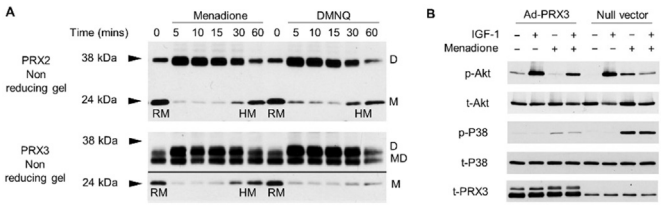

Figure 1. Effect of oxidative stress on PRX hyperoxidation and chondrocyte cell signaling. (A) Chondrocytes were treated with menadione (25 μM) or DMNQ (25 μM) for the indicated times and PRX2 and PRX3 redox status was evaluated by immunoblotting for PRX2 and PRX3 under non-reducing conditions. D = dimer; MD = mixed disulfide; RM = reduced monomer; HM = hyperoxidized monomer. (B) Chondrocytes were transduced with an adenoviral vector encoding PRX3 or null empty vector and stimulated with IGF-1 (50 ng/ml), menadione (25 μM), or both for 60 minutes and phosphorylation was analyzed by immunoblotting. Immunoblots shown are representative of three independent experiments.

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AGRIN INHIBITS CANONICAL WNT SIGNALLING THROUGH LRP4

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Purpose: Osteoarthritis (OA) is a leading cause of disability for which there is no cure. The identification of molecules supporting cartilage homeostasis and regeneration is therefore a major pursuit in musculoskeletal medicine. Agrin is a heparan sulfate proteoglycan which, through binding to low-density lipoprotein receptor-related protein 4 (LRP4), is required for neuromuscular synapse formation. We recently identified Agrin as an essential molecule in articular cartilage homeostasis, uniquely requiring both α-dystroglycan and LRP4 to support SOX9 expression and chondrocyte differentiation *in vitro* and *in vivo*. Despite identifying α-dystroglycan and LRP4 as necessary for the chondrogenic effects of Agrin in chondrocytes, the signalling pathway(s) by which this occurs remains unclear. LRP4 signalling is known to negatively regulate WNT signalling. Therefore, we tested whether Agrin is required for the WNT-inhibitory properties of LRP4 and whether this is the mechanism by which Agrin signals in chondrocytes.
Methods: Mammalian Agrin or GFP was overexpressed in bovine primary chondrocytes and cultured in micromass for 5 days in the presence or absence of 100ng/ml WNT3a. Chondrogenic potential was assessed by qPCR for SOX9 mRNA expression. Agrin or GFP stably expressing COS7 cells were transfected with the SUPER8TOPFLASH reporter plasmid in combination with Vehicle or LRP4 plasmids. COS7 monolayer cultures were treated with vehicle or increasing doses of WNT3a (50, 100, 200ng/ml). WNT signalling activation was determined by luciferase expression.
Results: LRP4 overexpression was sufficient to induce SOX9 upregulation in chondrocytes, however this effect was abolished following Agrin knockdown demonstrating the requirement of Agrin for the chondrogenic effect of LRP4 signalling. In addition, downregulation of SOX9 mRNA expression induced by WNT3a treatment was rescued by Agrin overexpression in micromass cultures. Using the WNT reporter assay, activation of canonical WNT signalling was inhibited in COS7 cells transduced with human Agrin compared to GFP-transduced control COS7 cells; and this effect was exacerbated by overexpression of LRP4.
Conclusions: Agrin inhibits canonical WNT signalling through LRP4 and enhance chondrocyte differentiation. However, it remains unclear how Agrin-LRP4 signalling achieves its specificity in promoting chondrogenesis compared to other LRP4 ligands.

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PHOSPHOPROTEOMICS ANALYSIS OF SIGNALING CHANGES IN HUMAN CHONDROCYTES FOLLOWING TREATMENT WITH IL-1, IGF-1 AND DEXAMETHASONE

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Purpose: The combination of insulin-like growth factor 1 (IGF-1) and dexamethasone (Dex) treatment has been shown to reduce aggrecan and collagen degradation and promote sGAG synthesis in human and bovine cartilage explants treated with IL-1 (one of the pro-inflammatory cytokines reported to be upregulated in the synovial fluid after traumatic joint injuries). However, the underlying cellular signaling mechanisms are not well understood. Since phosphorylation is an important post-translational modification in mediating cellular response to stimuli, we employed an untargeted LC/MS/MS approach to study the phosphorylation changes in chondrocytes following treatment with IL-1, IGF-1, and Dex to achieve a better understanding of how human chondrocytes respond to these stimuli.
Methods: Chondrocytes were isolated postmortem from the ankle and knee cartilage (Collins Grade 1) from the same donor (56-year–old female), equilibrated for 3 days, and subjected to 8 treatment conditions: untreated control, IL-1 (10ng/mL), Dex (100nM), IGF-1 (300ng/mL), IL-1+Dex, IL-1+IGF-1, IL-1+Dex+IGF-1, and Dex+IGF. After 30 minutes of treatment, cells were lysed in 8M urea and cell lysates were reduced, alkylated and trypsin digested into peptides. Each peptide sample was labelled with isobaric tags using the 8-plex iTRAQ reagent protocol (Sciex), and the labelled peptides from the different conditions were combined. Two immunoprecipitation (IP) steps were carried out to enrich for phosphopeptides (one IP using phosphotyrosine (pY) antibodies and a second IP using an antibody specific to the pSer/pThr-Pro motif). The products from each IP were further enriched for phosphopeptides using an IMAC (immobilized metal ion affinity chromatography) column. Phosphopeptides were resolved by reverse phase HPLC coupled to tandem mass spectrometry. The identification and relative quantification of the proteins was carried out with Discoverer software. In separate tests, cartilage explants (3mm x 1mm) harvested from the femoropatellar groove of 1–2 weeks old calves were used for treatments with kinase inhibitors.
Results: In the phosphoproteomics analysis of pY peptides, 229 and 167 peptides were identified and quantified in ankle and knee chondrocyte samples, respectively. Among these pY peptides, 74 peptides were found in both ankle and knee chondrocyte samples. Interestingly, we found an upregulation in the phosphorylation levels (more than 2x relative to untreated control) of key components of the MAPK pathway (including ERK1, ERK2, ERK5, JNK1, JNK2, P38α, and P38γ) following IL-1 treatment (Fig 1). Treatment with IGF and Dex did not affect the IL-1 stimulated phosphorylation changes of these proteins, except for JNK1 and JNK2, which had slight downregulation in phosphorylation with the addition of Dex. In addition to MAPK protein peptides, IL-1 treatment also upregulated the phosphorylation (more than 2x) of 68 peptides with the pS/pT-P motif, indicating that a large number of substrates of the MAPK pathways are being modulated in response to IL-1 treatment. Inhibition of the MAPK pathways using small molecule inhibitors revealed that the JNK inhibitor, SP600125, reduced GAG loss, rescued sGAG synthesis, and improved viability of IL-1 treated bovine cartilage explants (Fig 2).
Conclusions: We harnessed an innovative phosphoproteomics approach to understand the changes in phosphorylation caused by treatment of human chondrocytes with IL-1, Dex and IGF-1 (individually and in combinations) that result in reprogramming of these cells. Using this approach, we found key regulatory kinases, including p38, JNK1/2, ERK1/2, ERK5, and substrates of the MAPK pathways that were highly upregulated in phosphorylation as a result of cytokine treatment. We further found that inhibition of JNK1/2 pathway has the anti-catabolic effect of reducing GAG loss and anabolic effect of promoting sGAG synthesis in cartilage explants treated with IL-1.

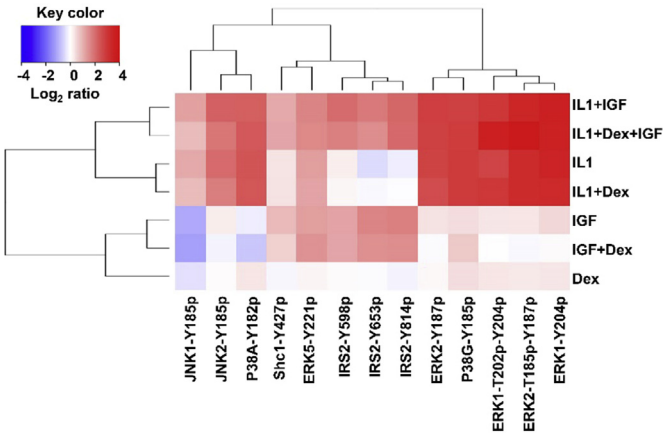

Figure 1. Heatmap of averaged $\log_2$ ratio of phosphotyrosine peptides that were highly upregulated by IL-1 or IGF-1 treatment.

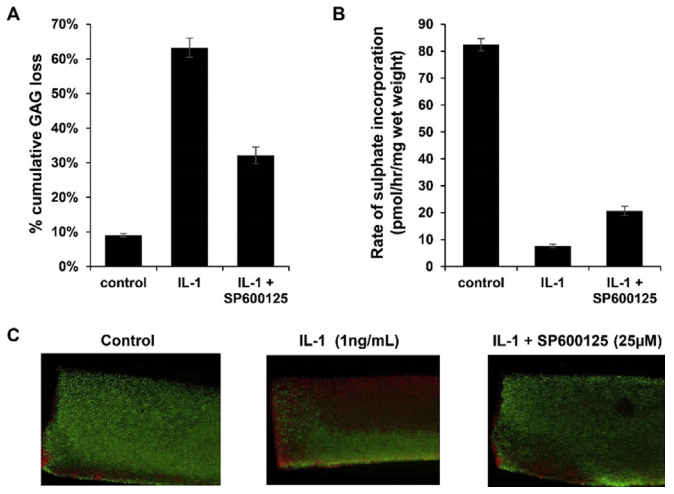

Fig 2A. Percent sGAG loss, from immature bovine explants treated with IL-1 +/- SP600125 (8 days experiment). 47 disks/condition from 8 animals were used, values are mean +/- SEM. B. Normalized sulfate incorporation rate measured during Day 6–8 of the same disks used in A. C. Bovine chondrocyte viability in cartilage disks in response to 8 day treatments. Cells were fluorescently labeled with fluorescein diacetate (green, viable) and propidium iodide (red, non-viable).

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INVESTIGATING THE INTERACTION BETWEEN RUNX2 AND PRB DURING IN VITRO CHONDROGENESIS AND OSTEOGENESIS OF HUMAN MESENCHYMAL STROMAL CELLS

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Purpose: The ability of mesenchymal stromal cells (MSCs) to differentiate towards chondrocytes and osteoblasts suggests great potential in the field of regenerative medicine for treating musculoskeletal disorders. However, their successful clinical application is hindered by the lack of specific markers and the inability to predict and control their fate *in vitro* and *in vivo*. In this study we wanted to investigate the effect of the modulation of two transcription factors, Runx2 and pRb, during *in vitro* chondrogenesis and osteogenesis of MSCs, with the hypothesis that the ratio between these transcription factors could be predictive of a stable induction fate.

Methods: First generation human adenovirus (hAd) serotype 5 dE/E3 carrying the Runx2 or shRNA-Runx2 gene was propagated and amplified in AD-293 cells. A second generation hAd serotype 5 carrying the Rb1 gene was generated. Adenoviral vectors were purified over successive caesium chloride gradients and used to transduce human bone marrow-derived MSCs (hBM-MSCs) by lanthofection. To study the effect of overexpression/knockdown of the gene of interest, five different conditions were tested: Runx2 overexpression, Rb1 overexpression, Runx2 knockdown, Runx2+Rb1 overexpression and Rb1 overexpression+Runx2 knockdown. Untransduced and GFP-Ad transduced cells were used as controls. The presence of a fluorescent tag protein in each adenoviral vector allowed the efficiency of transduction to be determined by flow-cytometric analysis. hAd-transduced hBM-MSCs were plated for standard chondrogenesis and osteogenesis assays and untransduced cells were used as a control. Chondrogenic differentiation was assessed histologically by determining proteoglycan content in pellet cultures using Safranin O-Fast green staining. Osteogenic potency was assessed by quantification of alkaline phosphatase (ALP) activity and Alizarin red staining. RNA, isolated from pellet cultures and matrix layers at day 0, 14, 21 and 28, was reversed transcribed before quantitative RT-PCR to study relative mRNA expression of Runx2 and Rb1 in addition to a panel of genes known to have a role in chondrogenic and osteogenic differentiation.

Results: Both Runx2 overexpression and shRNA-Runx2 reduced proteoglycan content under chondro-permissive culture conditions as demonstrated by Safranin O-Fast green staining. Rb1 overexpression caused a reduction of proteoglycan content under chondro-permissive culture conditions but induced proteoglycan accumulation under basal culture conditions. This positive effect on proteoglycan content in basal conditions was masked by the Runx2 overexpression or knockdown in the double transduced cells. During osteogenic differentiation, the overexpression of Runx2 increased ALP activity only in osteogenic medium. The overexpression of Rb1 increased the ALP activity under both culture conditions, and this stimulatory effect was also seen in the cells doubly transduced with both Runx2 and Rb1. The knockdown of Runx2 reduced ALP activity and, interestingly, the overexpression of Rb1 could not rescue the activity in basal medium. Runx2 overexpression increased mineral deposition in basal medium but did not further enhance mineralisation in osteogenic medium. In contrast, Rb1 overexpression increased mineralisation in basal medium but decreased it in osteogenic medium. Knockdown of Runx2 markedly reduced mineralisation under both culture conditions and, as seen with ALP activity, the overexpression of Rb1 could not rescue the effect of Runx2 knockdown on mineral deposition. Overexpression of both Runx2 and Rb1 further enhanced mineralisation in both basal and osteogenic conditions.

Conclusions: The present study reports a crucial role of Runx2 not only during osteogenic differentiation but, more interestingly, during chondrogenic differentiation of hBM-MSCs *in vitro*. This, together with the ability of pRB to stimulate proteoglycan deposition and enhance ALP activity in the absence of chondrogenic or osteogenic clues, respectively, opens new opportunities for investigations in understanding the molecular mechanisms underlying the fate choice and differentiation process of MSCs.

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SMAD3 AND SMAD4 HAVE A MORE DOMINANT ROLE THAN SMAD2 IN CHONDROGENIC INDUCTION OF MESENCHYMAL STEM CELLS

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Purpose: To improve cartilage formation by mesenchymal stem cells (MSCs), the signaling mechanism governing chondrogenic differentiation requires better understanding. Transforming Growth Factor-β (TGFβ) is well-known to promote chondrogenesis of MSCs; however, the role of downstream mediators of TGFβ signaling in chondrogenic induction remains largely unknown. We previously showed that the type I TGFβ receptor TGFBR1 (or ALK5) is crucial for TGFβ-induced chondrogenesis. Upon activation by TGFβ, ALK5 intracellularly phosphorylates SMAD2 and SMAD3 proteins, which then form complexes with co-factor SMAD4 to regulate gene transcription. Strikingly, the individual contribution of these crucial signaling molecules is still elusive. Therefore, the aim of this study was to determine the specific role of SMAD2, SMAD3 and SMAD4 in TGFβ-induced chondrogenesis of human MSCs.

Methods: In human fetal bone marrow-derived MSCs (ScienCell), expression of SMAD2, SMAD3 or SMAD4 was knocked down using short hairpin RNA (shRNA) or overexpressed with an adenovirus. Efficiency of knockdown/overexpression was assessed at gene and protein level by RT-qPCR and Western blot. Activation of TGFβ signaling was determined by Western blot analysis of (C-terminal) phosphorylated SMAD proteins. Chondrogenesis was induced by pellet-culturing MSCs for 14 days in serum-free chondrogenic medium supplemented with TGFβ1, and, thereafter, evaluated by mRNA expression of chondrocyte-specific genes; *ACAN*, *COL2A1* and *SOX9*, and deposition of collagen type II and glycosaminoglycans (GAGs) using histology and the DMMB assay, respectively.

Results: We confirmed efficient knockdown and overexpression of SMAD2, SMAD3 and SMAD4 at gene and protein level. Moreover, TGFβ-activated phosphorylation of SMAD2 was highly increased upon SMAD2 overexpression and decreased upon SMAD2 knockdown and, surprisingly, SMAD4 knockdown. Modulating SMAD3 expression did not affect SMAD2 phosphorylation. Activation of the alternative SMAD1/5/9 pathway by TGFβ was strongly reduced when SMAD4 was knocked down, while it was enhanced when SMAD2 or SMAD3 were knocked down. Subsequently, we induced chondrogenic differentiation of MSCs and observed that knockdown and overexpression of SMAD2 mildly (5–20%) inhibited *ACAN*, *COL2A1* and *SOX9* expression. These chondrocyte-specific genes were inhibited for 25-35% by SMAD3 knockdown and 70–80% by SMAD3 overexpression. Whereas SMAD4 knockdown resulted in 95–100% reduction in expression of *ACAN*,