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CHONDROCYTE EXPANSION IS ASSOCIATED WITH LOSS OF PRIMARY CILIA AND DISRUPTED HEDGEHOG SIGNALLING

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Abstract

Tissue engineering-based therapies targeting cartilage diseases, such as osteoarthritis, require *in vitro* expansion of articular chondrocytes. A major obstacle for these therapies is the dedifferentiation and loss of phenotype accompanying chondrocyte expansion. Recent studies suggest that manipulation of hedgehog signalling may be used to promote chondrocyte re-differentiation. Hedgehog signalling requires the primary cilium, a microtubule-based signalling compartment, the integrity of which is linked to the cytoskeleton. We tested the hypothesis that chondrocyte dedifferentiation caused alterations in cilia expression that influence hedgehog responsiveness.

In vitro chondrocyte expansion to passage 5 (P5) was associated with increased actin stress fibre formation, dedifferentiation and progressive loss of primary cilia, compared to primary (P0) cells. P5 chondrocytes exhibited ~50 % fewer cilia with a reduced median length. Cilia loss was associated with disruption of ligand-induced hedgehog signalling, such that P5 chondrocytes did not significantly regulate the expression of hedgehog target genes (*GLI1* and *PTCH1)*. This phenomenon could be recapitulated by applying 24 h cyclic tensile strain, which reduced cilia prevalence and length in P0 cells. LiCl treatment rescued cilia loss in P5 cells, partially restoring hedgehog signalling, so that *GLI1* expression was significantly increased by Indian hedgehog.

This study demonstrated that monolayer expansion disrupted primary cilia structure and hedgehog signalling in association with chondrocyte dedifferentiation. This excluded the possibility to use hedgehog ligands to stimulate re-differentiation without first restoring cilia expression. Furthermore, primary cilia loss during chondrocyte expansion would likely impact other cilia pathways important for cartilage health and tissue engineering, including transforming growth factor (TGF), Wnt and mechanosignalling.

Keywords: Chondrocyte, dedifferentiation, re-differentiation, primary cilia, cilium, hedgehog, lithium chloride.

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Introduction

Articular cartilage has a limited capacity for repair, mostly due to its complex anisotropic structure and avascularity. Consequently, trauma to the articular surface often results in development of osteoarthritis (OA), a degenerative cartilage disease. Current surgical treatments utilise tissue engineering approaches to repair cartilage lesions and prevent further cartilage degradation. Autologous chondrocyte implantation (ACI), perhaps considered the most successful treatment for focal lesions, requires the removal and expansion of patient chondrocytes (Rai *et al.*, 2017). In a second surgery, these autologous cells are re-implanted, in combination with a membrane or matrix scaffold, to repair a cartilage defect. However,

upon monolayer expansion, articular chondrocytes undergo dedifferentiation, such that expression of the chondrogenic matrix molecules, aggrecan (*ACAN*) and collagen type II (*COL2*) are reduced (Holtzer *et al.*, 1960; Benya *et al.*, 1978). Consequently, defects repaired in this way often heal as fibrocartilage, which is mechanically insufficient and cannot support long term recovery, merely delaying the need for total knee arthroplasty. Thus, strategies targeting either the prevention of dedifferentiation or the re-differentiation of expanded cells aim to improve the quality of the tissue generated by these approaches.

The phenotypic changes associated with dedifferentiation in monolayer culture are widely attributed to alterations to the cytoskeleton, which occur with expansion, specifically an increase in actin stress fibre formation (Benya, 1988; Parreno *et al.*, 2017). Indeed, depolymerisation of the actin cytoskeleton in expanded cells results in alterations in cell shape and increased expression of cartilage matrix molecules (Benya, 1988; Parreno *et al.*, 2017). The disruption of the actin filaments will influence a plethora of systems and cellular processes, such as proliferation, migration and mechanotransduction. Moreover, several studies have shown that changes to the actin filament network influence primary cilia expression and length (Kim *et al.*, 2010; Sharma *et al.*, 2011).

The primary cilium is a microtubule-based signalling compartment present in most cell types, including chondrocytes (Haycraft and Serra 2008). The cilium comprises a basal body, which arises from the cell's "mother" centriole and nucleates the formation of a '9 + 0' microtubular scaffold, which is sheathed in a specialised membrane to form the ciliary axoneme. Protein entry into this compartment is tightly regulated at the base of the cilium, thus, a unique signalling compartment is formed whereby proteins can be shuttled into and out of the axoneme by a process called intraflagellar transport (IFT, for review see Veland *et al.*, 2009). Cilia integrity is essential for the function of a rapidly expanding list of pathways that include growth factor signalling (Schneider *et al.*, 2005; Zhu *et al.*, 2009; Christensen *et al.*, 2012), mechanotransduction (Praetorius and Spring 2001; Wann *et al.*, 2012) and inflammation (Wann and Knight, 2012; Wann *et al.*, 2014). Earlier reports also show the effects of extended culture and cellular senescence on primary cilia with conflicting results. Breslin *et al.* (2014) report an increased frequency and length of cilia in senescent fibroblasts, while Bishop *et al.* (2010) show that senescence is associated with cilia loss in epithelial cells. However, both studies show the downregulation of hedgehog signalling components during chondrocyte expansion. Therefore, in this study, we examined the expression of cilia in the context of chondrocyte expansion.

Growth factors, such as insulin growth factor-I (IGF-I), bone morphogenic protein-2 (BMP-2), transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF) and hedgehog (Hh) are employed to improve the quality of tissue engineered cartilage (Zimber *et al.*, 1995; Lohmann *et al.*, 2000; Kellner *et al.*, 2002; Ko *et al.*, 2012). These factors influence cartilage growth and metabolism through their effects on chondrocyte proliferation and differentiation and, intriguingly, are all linked to primary cilia (Huangfu *et al.*, 2003; Schneider *et al.*, 2005; Zhu *et al.*, 2009; Christensen *et al.*, 2012).

Hedgehog signalling is perhaps the most well-characterised of the cilia-mediated signalling pathways and it has been shown to both increase cartilage matrix production in tissue engineered constructs (Kellner *et al.*, 2002) and promote chondrocyte re-differentiation (Lin *et al.*, 2014). In the absence of hedgehog ligands, full-length Gli transcription factors (Gli2 and Gli3) traffic through the ciliary compartment and undergo protein kinase A (PKA)-dependent phosphorylation, resulting in proteolysis and production of the transcriptional repressor Gli3R (Sasaki *et al.*, 1999; Huangfu *et al.*, 2003). In the presence of hedgehog ligands, the transmembrane protein smoothened (smo) traffics into the cilium (Corbit *et al.*, 2005). PKA activity is inhibited by the removal of the G-protein coupled receptor (GPCR) Gpr161 from the cilium and consequently full-length Gli activators are formed (Mukhopadhyay *et al.*, 2013; Nager *et al.*, 2017). Activated Gli proteins enter the nucleus and promote the transcription of hedgehog target genes, such as the negative hedgehog regulator protein patched homolog 1 (*PTCH1*) and the transcriptional activator *GLI1*, which further potentiates hedgehog response (Kim *et al.*, 2009).

Within this study, we evaluated the effects of chondrocyte expansion on primary cilia and determined the consequences for ciliary function, with a focus on hedgehog signalling. We showed that expansion of articular chondrocytes to passage 5 (P5) resulted in a reduction in cilia prevalence and length, with increasing passage. We showed that this was associated with reduced responsiveness of these cells to exogenous Indian hedgehog. Then, we examined the potential of drug targeting primary cilia, cytochalasin D (cyto D) and lithium chloride (LiCl), to restore hedgehog signalling in P5 cells. Our results highlighted the importance of maintaining ciliation in order to ensure the success of hedgehog-dependent re-differentiation strategies. Moreover, the role of primary cilia in a range of growth factor signalling pathways suggested that maintaining cilia could also improve existing tissue engineering techniques.

Materials and methods

Chondrocyte isolation and culture

Primary bovine articular chondrocytes were isolated from full-depth slices of articular cartilage removed from the proximal surface of the metacarpal

phalangeal joint. Cells were isolated by enzymatic digestion: cartilage was first incubated for 1 h at 37 °C with 35 U/mL protease (P5417, Sigma-Aldrich, Poole, UK), followed by 16 h at 37 °C in 100 U/mL collagenase (C7657, Sigma-Aldrich). Next, the cartilage digest was filtered and cells collected by centrifugation. Chondrocytes were cultured at 37 °C and 5 % CO_2 in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % (v/v) foetal calf serum, 1.9 mM L-glutamine, 96 U/mL penicillin, 96 mg/mL streptomycin, 19 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer and 0.74 mM L-ascorbic acid (all from Sigma-Aldrich).

Primary chondrocytes were cultured until confluence, when they were either used for experiments as P0 cells or passaged for up to five passages to generate P5 cells. For experiments, chondrocytes were seeded onto serum-coated glass coverslips, plastic tissue culture plates or collagen type I-coated Flexcell® membranes (Dunn Labortechnik GMbH, Asbach, Germany) and cultured until confluence.

Application of cyclic tensile strain

To induce physiologically relevant primary cilia disassembly, chondrocytes were subjected to uniform, equibiaxial cyclic tensile strain (CTS) using the Flexcell® FX4000-T system (Dunn Labortechnik GMbH) with circular loading posts with a diameter of 25 mm. Cells were subjected to 10 % strain for 24 h at 0.33 Hz. For unstrained controls, chondrocytes were cultured in an identical manner, but without the application of strain. At the end of the strain period cells were immediately processed for subsequent analysis.

Immunocytochemistry, confocal and super resolution microscopy

For immunocytochemistry, samples were fixed for 10 min in 4 % paraformaldehyde. For basal body staining, fixation was followed by 5 min in ice-cold methanol. Then, samples were permeabilised with 0.5 % Triton X-100 and blocked with 5 % donkey serum. Labelling with primary antibodies was performed overnight at 4 °C. The primary antibodies used were α-tubulin (ab4074, Abcam, Cambridge, UK), acetylated α-tubulin (T7451, Sigma-Aldrich), ADP-ribosylation factor-like protein 13B (ARL13B) (17711-1-AP, Protein Tech, Manchester, UK), Ki-67 (9129S, Cell Signalling Technology, Danvers, MA, USA) and γ-tubulin (sc-7396, Santa Cruz Biotechnology, Dallas, TX, USA), while actin was labelled with Alexa Fluor 633-conjugated phalloidin (Life Technologies, Paisley, UK). Following repeated washing, samples were incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies) for 1 h at room temperature. Nuclei were detected with 1 μg/mL DAPI. Coverslips were mounted with ProLong Diamond reagent (Life Technologies).

Confocal microscopy and super resolution structured illumination microscopy (SIM) were both performed using a Zeiss 710 ELYRA PS.1 microscope (Carl Zeiss, Oberkochen, Germany) with a 63×/1.4 NA objective, yielding images with an x-y pixel size of 0.1 μm and 0.033 μm, respectively. Confocal z-stacks were generated throughout the entire cellular profile using a z-step size of 0.5 μm. SIM z-stacks were produced through individual cilia and processed using Zeiss Zen Black software (Carl Zeiss). In both cases, z-stacks were reconstructed and an x-y maximum intensity projection was used for the measurement of cilia length with ImageJ software (Image Processing and Analysis in Java; U.S. National Institutes of Health, Bethesda, MD, USA). Semi-automated analysis of SIM staining intensity along the entire length of cilia axonemes was performed using an ImageJ plugin, specially developed and validated in association with Dr Novak (Institute of Bioengineering, Queen Mary University of London, London, UK). For the quantification of cell proliferation, nuclear intensity of Ki-67 was quantified from confocal z-stacks using Image J and the number of positive nuclei quantified above a set threshold. The proportion of Ki-67 positive cells was expressed relative to total cell number and quantified using DAPI staining.

Protein isolation and western blotting

For protein isolation, chondrocytes were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Roche, Basel, Switzerland). Following homogenisation, the lysate was centrifuged for 15 min at 13,000 rpm and the supernatant was collected for subsequent analysis. Protein concentration was determined by bicinchoninic acid assay. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 20 μg of total protein and 4-20 % gradient gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred to nitrocellulose membranes, which were subsequently blocked with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE, USA) and incubated with primary antibodies overnight at 4 °C. Additional primary antibodies used were Gli3 (sc-6155, Santa Cruz Biotechnology) and β-actin (ab8226, Abcam). Next, membranes were washed, incubated for 1 h at room temperature with appropriate infrared secondary antibodies (Li-Cor) and visualised using the Li-Cor Odyssey imaging system (Li-Cor). Analysis of band intensity was performed using Li-Cor Image Studio™ Lite.

RNA isolation, cDNA synthesis and quantitative real time PCR

RNA isolation was performed using an RNeasy Kit (Qiagen, Germantown, MD, USA) and converted to cDNA using the QuantiTect reverse transcription kit (Qiagen), according to the manufacturer's instructions. Quantitative real time PCR was

Table 1. Primers used for real time PCR.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
18S	CGGCTACCACATCCAAGGAA	GGGCCTCGAAAGAGTCCTGT
ACAN	GAGTTTGTCAACAACAATGCC	TGGTAATTACATGGGACATCG
*COL2*A	ACGTCCAGATGACCTTCCTG	GGATGAGCAGAGCCTTCTTG
GLI1	TTCCTCAGTGGAACCCAAG	CCTGCATTTCCAGTCATTC
PTCH1	ATGTCTCGCACATCAACTGG	TCGTGGTAAAGGAAAGCACC

performed using QuantStudio 7 (Applied Biosystems, Foster City, CA, USA) and analysed using the relative standard curve method (Larionov *et al.*, 2005). Reactions were performed in 5 µL volumes, containing 1 µL cDNA (diluted 1 : 2), 2.5 µL Kapa SYBR Fast Universal 2× qPCR Master Mix (Kapa Biosystems, London, UK), containing SYBR green and ROX reference dye, and 500 nM optimised primer pairs (Table 1). Samples and standards were run in triplicate and gene expression was normalised to 18S ribosomal RNA endogenous control.

Statistical analysis

Statistical analyses were performed using Graph Pad Prism 7.01 (GraphPad, La Jolla, CA, USA). When data sets adhered to normal distribution, two sample *t*-test or ANOVA with Sidaks test for multiple comparisons were used. For non-parametric data sets, Mann-Whitney tests were used. For contingency data, chi-squared analysis was performed. *N* referred to the number of donors and *n* referred to the number of technical replicates analysed per experimental group. Data were presented as mean ± SEM, except for cilia length, for which median values were described with error bars depicting 10-90 percentiles, due to the non-Gaussian distributions. Details of specific statistical tests and *n* values can be found in the figure legends.

Results

Expansion of articular chondrocytes resulted in dedifferentiation and loss of phenotype

Freshly-isolated bovine articular chondrocytes (P0) were cultured for 7 d in a 2D monolayer until confluence, then passaged again until confluence (2-3 d), for up to 5 passages (P5). Serial passages resulted in classic changes indicative of de-differentiation: very little actin stress fibre formation was observed in P0 chondrocytes, while P5 chondrocytes exhibited an increase in cell size and actin stress fibre formation and cells appeared more fibroblastic (Fig. 1**A**). Moreover, the expression of *Sox9*, the chondrocyte master transcriptional regulator required for their differentiation, was downregulated at P5 compared to P0 (Fig. 1**B**,**C**). This was accompanied by a significant reduction in the expression of the genes encoding the chondrocyte matrix proteins aggrecan (*ACAN*) and collagen type II (*COL2*A) (Fig. 1**D**,**E**, respectively), thus confirming that dedifferentiation occurred in this system.

Dedifferentiation of articular chondrocytes was associated with loss of primary cilia

Primary cilia expression and structure are intrinsically linked to changes in the cytoskeleton (Pitaval *et al.*, 2010; McMurray *et al.*, 2013). However, there are conflicting reports as to the effect of extended cell culture upon cilia prevalence and length in other cell types (Bishop *et al.*, 2010; Breslin *et al.*, 2014). Therefore, primary cilia expression was examined in P0 and P5 articular chondrocytes. P0 chondrocytes exhibited primary cilia in 59 % of cells, with a median length of 2.42 µm (Fig. 2**A**,**B**,**C**). Cilia length and prevalence were significantly reduced in P5 cells, such that only 31 % of cells exhibited a cilium with a median length of 2.03 µm (Fig. 2**A**,**B**,**C**). This loss of cilia occurred progressively within each successive passage (data not shown); however, cilia loss did not occur as the result of an increased proliferation, as no significant difference in the proportion of Ki-67 (proliferation marker)-positive cells was observed at P0 and P5 (Fig. 2**D**).

Tubulin acetylation promotes microtubule stability and is suggested to regulate cilia length, thus ciliary microtubules exhibit significant acetylation (Nakakura *et al.*, 2015; Portran *et al.*, 2017). However, despite the differences in cilium length, the level of whole cell tubulin acetylation between P0 and P5 chondrocytes, immunolabelled with α-tubulin and acetylated α-tubulin, did not appear different (Fig. 2**E**). This observation was supported by western blot analyses showing that the total level of tubulin acetylation was not significantly different between P0 and P5 (Fig. 2**F**,**G**).

Dedifferentiated chondrocytes exhibited a reduced response to exogenous hedgehog ligand

The primary cilium is essential for hedgehog signalling, which promotes chondrocyte re-differentiation (Lin *et al.*, 2014). Therefore, we examined the responsiveness of P0 and P5 chondrocytes to the addition of exogenous hedgehog ligand. Cells were treated for 24 h with 1 µg/mL recombinant Indian hedgehog (r-Ihh) and the pathway activation was examined by real time PCR. In control cells, the expression of the hedgehog markers *GLI1* and *PTCH1* was not significantly different in P5 cells relative to P0 (Fig. 3**A**,**B**), despite lower expression of the 83 kDa Gli3 repressor (Gli3R, Fig. 3**C**) protein in P5 cells (Fig. 3**D**). In P0 cultures, the expression of *GLI1* and *PTCH1* was significantly increased by 20.97 and 6.85-fold, respectively, in response to r-Ihh (Fig. 3**A**,**B**). By

contrast, the expression of these genes was not significantly altered in P5 chondrocytes, consistent with the observed reduction in prevalence of cilia (Fig. 3**D**). The level of Gli3R was reduced relative to the untreated control in both P0 and P5 chondrocytes, following r-Ihh treatment (Fig. 3**D**). These data indicated that hedgehog pathway activation was disrupted in P5 chondrocytes, where ciliation was reduced, as a result of serial passage.

Previous studies report that the addition of hedgehog proteins to de-differentiated chondrocytes can trigger re-differentiation of these cells and induce the expression of *ACAN* and *COL2*A (Lin *et al.*, 2014). However, in P5 cells, we found that the expression of these genes was not significantly different from the control. In contrast, *ACAN* and *COL2A* expression were increased in response to r-Ihh in P0 cells (data not shown).

Loss of hedgehog signalling was associated with changes in Arl13b cilia localisation

The small GTPase, Arl13b, is required for the dynamic translocation of hedgehog pathway components into and out of the cilium in response to ligand (Larkins *et al.*, 2011). Previously, we have shown that a loss of hedgehog signalling associated with the deregulation of ciliary length is accompanied by changes in the ciliary distribution of Arl13b (Thompson *et al.*, 2016; Thorpe *et al.*, 2017). Therefore, we examined ciliary Arl13b content in P0 and P5 cells using SIM (Fig. 3**E**). Analysis of Arl13b intensity along the length of the cilium revealed that in P0 cells the localisation of Arl13b was approximately constant along the majority of the axoneme, but reduced sharply at the tip. By contrast in P5 cells, Arl13b localisation was much less even and reduced along the length of the cilium, such that intensity values in the central portion of the cilium were significantly lower in P5 cells compared to P0 (Fig. 3**F**). This resulted in an overall reduction in the total intensity of Arl13b within the cilium (Fig. 3**F**) in addition to the loss of cilia at P5 (Fig. 2**A**-**D**).

Mechanically-induced reduction of primary cilia length and prevalence in P0 chondrocytes inhibited ligand-induced hedgehog signalling

Previously, we have reported that mechanical loading inhibits basal hedgehog signalling through the modulation of primary cilia length (Thompson *et al.*, 2014). To further examine the effect that decreased cilia length and prevalence had upon ligand-induced hedgehog signalling, P0 chondrocytes were subjected to 10 % CTS to reduce ciliation in combination with hedgehog stimulation. Primary cilia length was

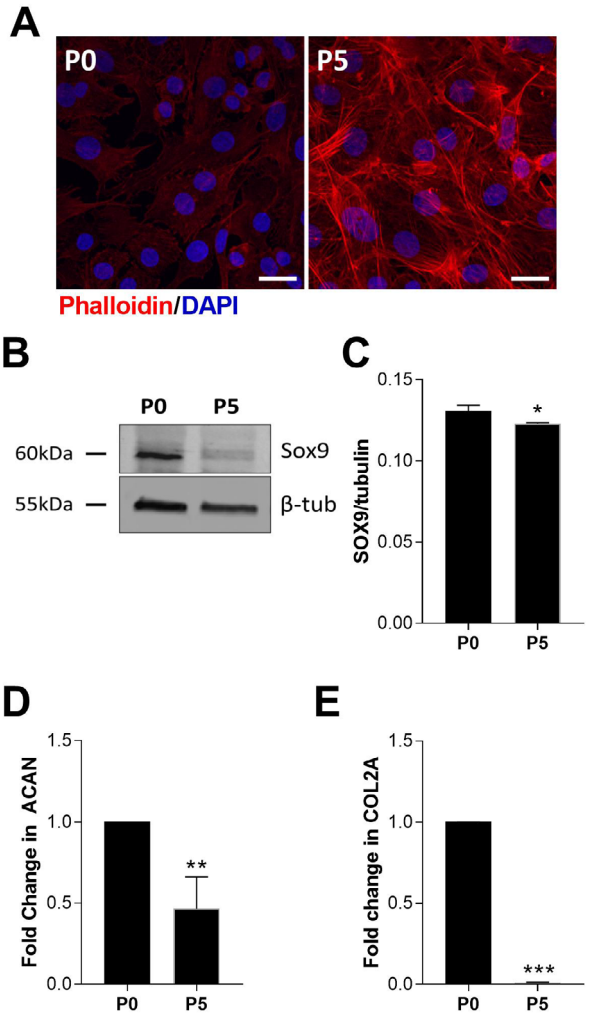

Fig. 1. Serial passage was associated with chondrocyte dedifferentiation. Primary bovine articular chondrocytes cultured in 2D monolayer at P0 and P5. (**A**) Representative images of actin stress fibres (phalloidin, red) counterstained with DAPI (blue). Scale bar = 20 μm. (**B**) Representative western blot of Sox9 protein expression in P0 and P5 cells; β-tubulin (β-tub) was used as a loading control. (**C**) Quantification of band intensity of Sox9 western blotting expressed relative to tubulin ($N = 3$, $n = 3$, two sample *t*-test: * $p < 0.05$). Real-time PCR of (**D**) *ACAN* and (**E**) *COL2A* expression; data were expressed as a fold change relative to P0 ($N = 6$, $n \geq 15$, paired *t*-test: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

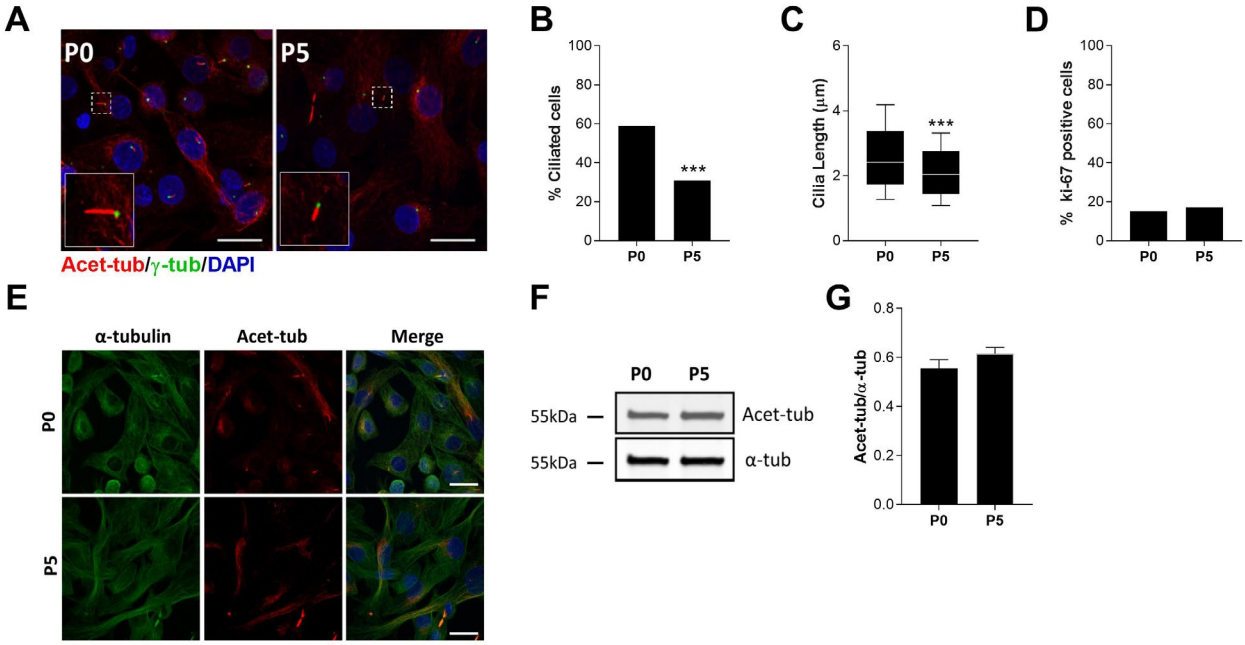

Fig. 2. Chondrocyte dedifferentiation was associated with primary cilia loss, without changes in proliferation or tubulin acetylation. Primary bovine articular chondrocytes cultured in 2D monolayer at P0 and P5. (**A**) Immunocytochemistry of primary cilia. Ciliary axonemes were labelled with acetylated α-tubulin (acet-tub, red) and basal bodies with γ-tubulin (γ-tub, green), nuclei were counter stained with DAPI (blue). Scale bar = 20 μm. Inserts showed individual cilia highlighted in the main image by the dashed white box. Quantification of (**B**) cilia prevalence ($N = 6$, $n \geq 529$ cells, chi-squared test: *** $p < 0.001$), (**C**) cilia length ($N = 6$, $n > 135$ cilia, Mann-Whitney test: *** $p < 0.001$) and (**D**) Ki-67 prevalence ($N = 3$, $n \geq 187$ cells, chi-squared test). (**E**) Immunocytochemistry of α-tubulin (green) and acetylated α-tubulin (acet-tub, red), nuclei were counter stained with DAPI (blue). Scale bar = 20 μm. (**F**) Representative western blot of whole cell α-tubulin (α-tub) and acetylated α-tubulin (acet-tub) protein expression in P0 and P5 chondrocytes. (**G**). Quantification of band intensity of acetylated α-tubulin (acet-tub) expressed relative to α-tubulin (α-tub) ($N = 3$, $n = 3$, two sample *t*-test).

reduced from 3.31 μm to 2.77 μm in loaded CTS cultures (Fig. 4**A**,**B**), while cilia prevalence was reduced from 70 % to 59 % (Fig. 4**C**). The expression of *GLI1* and *PTCH1* was significantly upregulated in response to r-Ihh in the unloaded No CTS control group (Fig. 4**D**,**E**). By contrast, in the mechanically-loaded CTS group the expression of these genes was no longer significantly upregulated by r-Ihh (Fig. 4**D**,**E**). In P5 cells, which already had reduced cilia expression, mechanical loading did not further influence ciliation or hedgehog response (Fig. 4**A-C**,**F**,**G**).

Actin depolymerisation increased cilia prevalence and length in P5 chondrocytes, but could not restore hedgehog signalling

Given the increased stress fibre formation in P5 chondrocytes (Fig. 1**A**), we examined the effects of actin depolymerisation on primary cilia. Cytochalasin D (cyto D, 10 μm) had little effect on the minimal actin stress fibre formation at P0 (Fig. 5**A**). Similarly, cyto D produced minimal changes in the proportion of ciliated cells in P0 cultures and had no significant effect on cilia length (Fig. 5**B**,**C**). By contrast, the enhanced formation of actin stress fibres in P5 cultures was disrupted by addition of cyto D (Fig. 5**A**). This was associated with a significant increase in both cilia prevalence and length, such that, with a value of 77.5 %, the level of ciliation exceeded that in P0 cultures, where only 53.8 % of the cells were ciliated (Fig. 5**B**). Moreover, median cilia length was significantly increased to 4.27 μm (Fig. 5**C**). These data suggested that the loss of primary cilia, as the result of serial passage, was likely due to changes in actin organisation.

Due to the increased ciliation in P5 cultures, as a result of cyto D treatment, we hypothesised that hedgehog responsiveness would be restored in these cells. Therefore, cultures were treated for 24 h with r-Ihh in the presence of cyto D and hedgehog signalling examined by real time PCR (Fig. 5**D**,**E**). In P0 cultures, consistent with our previous data, the expression of *GLI1* and *PTCH1* was significantly increased by r-Ihh treatment. However, cyto D treatment inhibited this response (Fig. 5**D**,**E**) without modulating cilia expression (Fig. 5**B**,**C**). In P5 cultures, *GLI1* and *PTCH1* expression were not significantly increased by r-Ihh treatment and cyto D treatment had no effect on this response (Fig. 5**D**,**E**). Thus, despite its effects on cilia at P5, cyto D was unable to restore hedgehog signalling in these expanded cells. These data suggested that even mild

disruption of actin may have a negative effect on cilia-mediated signalling, independent of changes in cilia structure or prevalence.

Pharmacological modulation of cilia length partially restored hedgehog signalling

Finally, we attempted to restore cilia in P5 cells without influencing stress fibre organisation, using the psychoactive drug lithium chloride (LiCl). LiCl is a potent regulator of cilia length promoting a dose-dependent cilia elongation in numerous cell types, including chondrocytes (Miyoshi *et al.*, 2009; Ou *et al.*, 2009; Thompson *et al.*, 2016). P0 and P5 cultures were treated with 25 mM LiCl for 24 h and the actin cytoskeleton examined by immunocytochemistry. Surprisingly, LiCl triggered some aggregation of actin structures in P0 cells, but had no significant effect on stress fibre formation in P5 cells (Fig. 5**A**). In P0 cultures, while LiCl did not significantly affect cilia prevalence (Fig. 5**B**), a dramatic increase in cilia length was observed, such that median cilia length was increased from 2.27 μm to 3.48 μm (Fig. 5**C**). In P5 cultures, LiCl resulted in a significant increase in primary cilia prevalence from 23.9 % to 52.8 % (Fig. 5**B**), which was accompanied by an increase in cilia length from 2.00 μm to 2.45 μm (Fig. 5**C**). Thus, LiCl restored cilia length and prevalence in P5 cells, such that these values were no longer significantly different to P0 cells (Fig. 5**B**,**C**).

Consistent with a previous report (Thompson *et al.*, 2016), the induction of *GLI1* and *PTCH1* in response to r-Ihh was inhibited by LiCl in P0 cells (Fig. 5**D**,**E**).

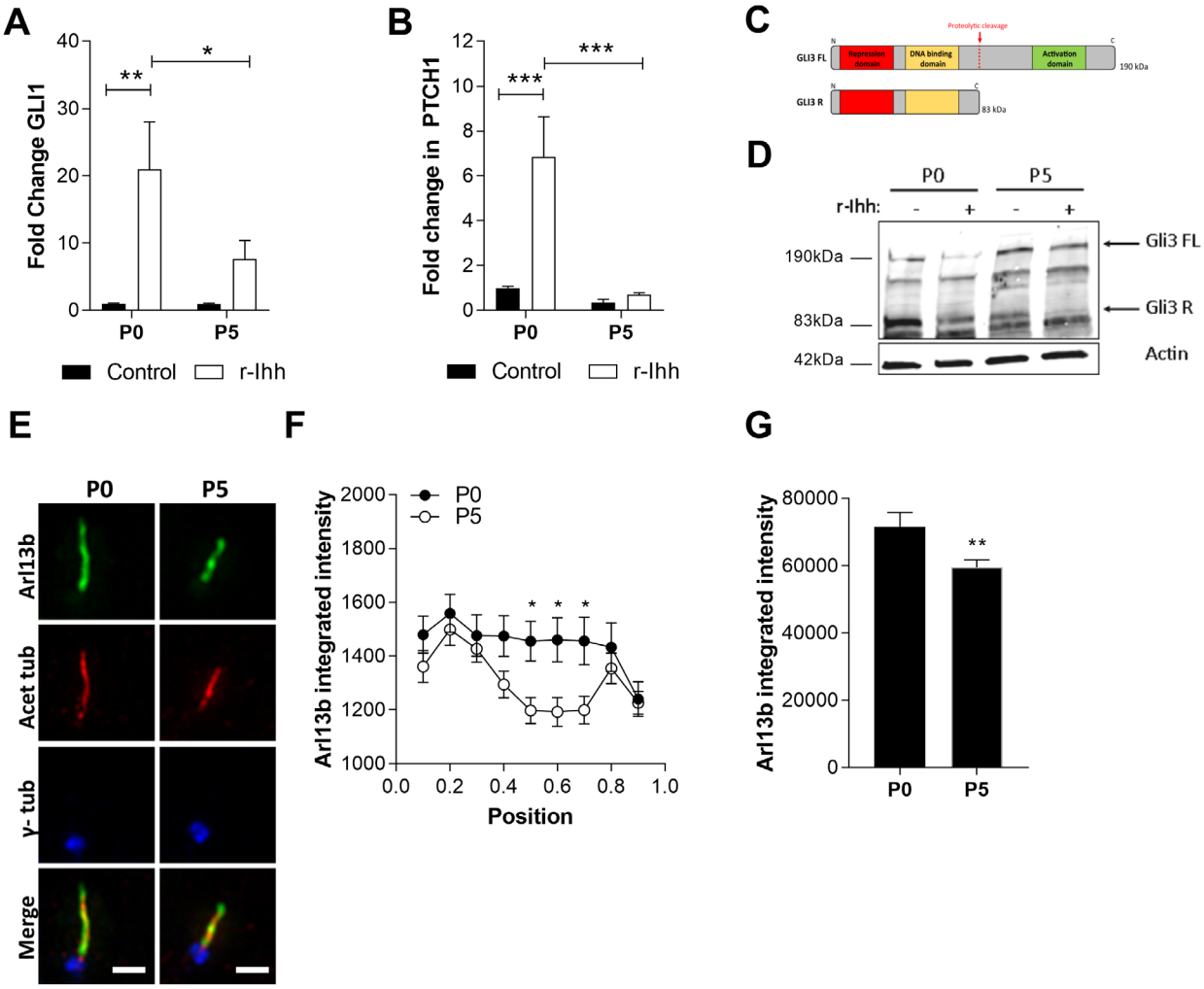

Fig. 3. Hedgehog pathway activation was disrupted in chondrocytes following serial passage, in association with changes in cilia expression and Arl13b cilia localisation. P0 and P5 chondrocytes cultured in 2D monolayer and treated for 24 h with r-Ihh. Real time PCR of (**A**) *GLI1* and (**B**) *PTCH1* expression ($N = 6$, $n \geq 16$, two-way ANOVA with Sidaks multiple comparison: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$). (**C**) Schematic of full-length and repressor forms of Gli3 (adapted from Alman, 2015). (**D**) Representative western blot of Gli3 expression in P0 and P5 cells, actin was used as a loading control. (**E**) Structured illumination microscopy of primary cilia from P0 and P5 cells labelled for Arl13b (green), acetylated α-tubulin (acet-tub; red) and γ-tubulin (γ-tub; blue). Scale bar = 1 μm. (**F**) Arl13b intensity along the length of the ciliary axoneme, where position 0.0 = cilium base and 1.0 = ciliary tip ($N = 3$, $n \geq 67$ cilia, two-way ANOVA with Sidaks multiple comparison: * $p < 0.05$). (**G**) Total Arl13b integrated intensity ($N = 3$, $n \geq 67$ cilia, Mann-Whitney test: * $p < 0.05$).

By contrast, in P5 cells, in the presence of LiCl, the expression of *GLI1* was significantly increased by the treatment with r-Ihh. The up-regulated *GLI1* reached levels similar to the ones in P0 cells in the absence of LiCl (Fig. 5**D**). However, there was no significant effect on *PTCH1* expression (Fig. 5**E**). These results suggested that LiCl induced a partial rescue of cilia hedgehog function in P5 cultures, associated with reversing the reduction in cilia prevalence and length induced by serial passages.

Discussion

This study examined the effects of serial passage and dedifferentiation on chondrocyte primary cilia. We demonstrated that dedifferentiation was accompanied by primary cilia loss, most likely due to changes in actin organisation. Primary cilia loss resulted in the disruption of ligand-dependent hedgehog signalling. We showed that a partial rescue of hedgehog signalling could be achieved in passaged cells through the restoration of primary cilia with LiCl.

Our findings were consistent with previous studies performed in other cell types showing that downregulation or inhibition of hedgehog signalling occurs as consequence of dedifferentiation and cellular senescence (Bishop *et al.*, 2010; Breslin *et al.*, 2014). However, the mechanisms responsible for this phenomenon are still objects of debate. The cilium is absolutely required for hedgehog signalling, as many of the components of this pathway traffic through the ciliary compartment and are modified here in order to regulate pathway activity (Corbit *et al.*, 2005; Haycraft *et al.*, 2005). Therefore, our findings suggested that it was the loss of this regulatory compartment that resulted in pathway inhibition. Furthermore, the reduced length of cilia observed in passaged cells was indicative of IFT dysfunction. IFT is required for

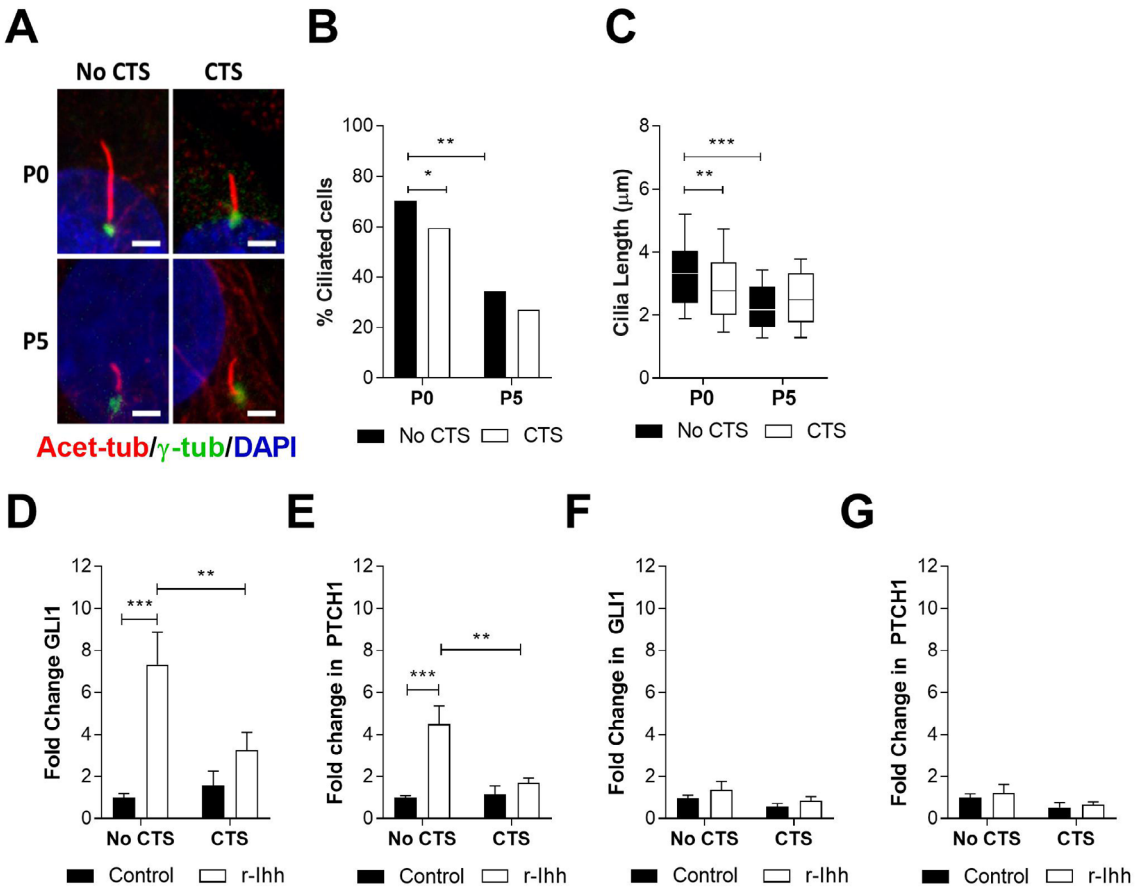

Fig. 4. Mechanical loading reduced primary cilia length and prevalence in P0 chondrocytes and inhibited ligand-induced hedgehog signalling, replicating the behaviour of P5 cells. P0 and P5 chondrocytes were subjected to 10 CTS, 0.33 Hz for 24 h in the presence of r-Ihh. (**A**) Immunocytochemistry of primary cilia. Ciliary axonemes were labelled with acetylated α-tubulin (red) and basal bodies with γ-tubulin (green), nuclei were counter stained with DAPI (blue). Scale bar = 2 μm. Quantification of (**B**) primary cilia prevalence ($N = 3$, $n \geq 290$ cells, chi-squared test: *** $p < 0.001$) and (**C**) primary cilia length ($N = 3$, $n \geq 165$ cilia, Mann- Whitney test: ** $p < 0.01$). Real time PCR of (**D**) *GLI1* and (**E**) *PTCH1* expression in P0 cells and (**E**) *GLI1* and (**F**) *PTCH1* in P5 cells. Data were normalised to GAPDH and expressed as a fold change relative to the NO CTS control ($N = 3$, $n \geq 8$, two-way ANOVA with Sidaks multiple comparison: ** $p < 0.01$ and *** $p < 0.001$).

cilia functionality in addition to cilia construction and maintenance; therefore, we would expect a reduced axoneme length to have direct consequences on signalling. Indeed, increasing numbers of studies are reporting small changes in the size of the ciliary compartment accompanied by more dramatic effects upon signalling (Tran *et al.*, 2008; Lancaster *et al.*, 2011; McMurray *et al.*, 2013; Thompson *et al.*, 2014; Sherpa *et al.*, 2016; Snouffer *et al.*, 2017; Spasic and Jacobs 2017).

Previously, we have shown that alterations in the ciliary distribution of Arl13b occur as a result of ciliary lengthening and are associated with hedgehog inhibition (Thompson *et al.*, 2016). While this previous study reported a reduction in Arl13b localisation to the ciliary tip, in the current study

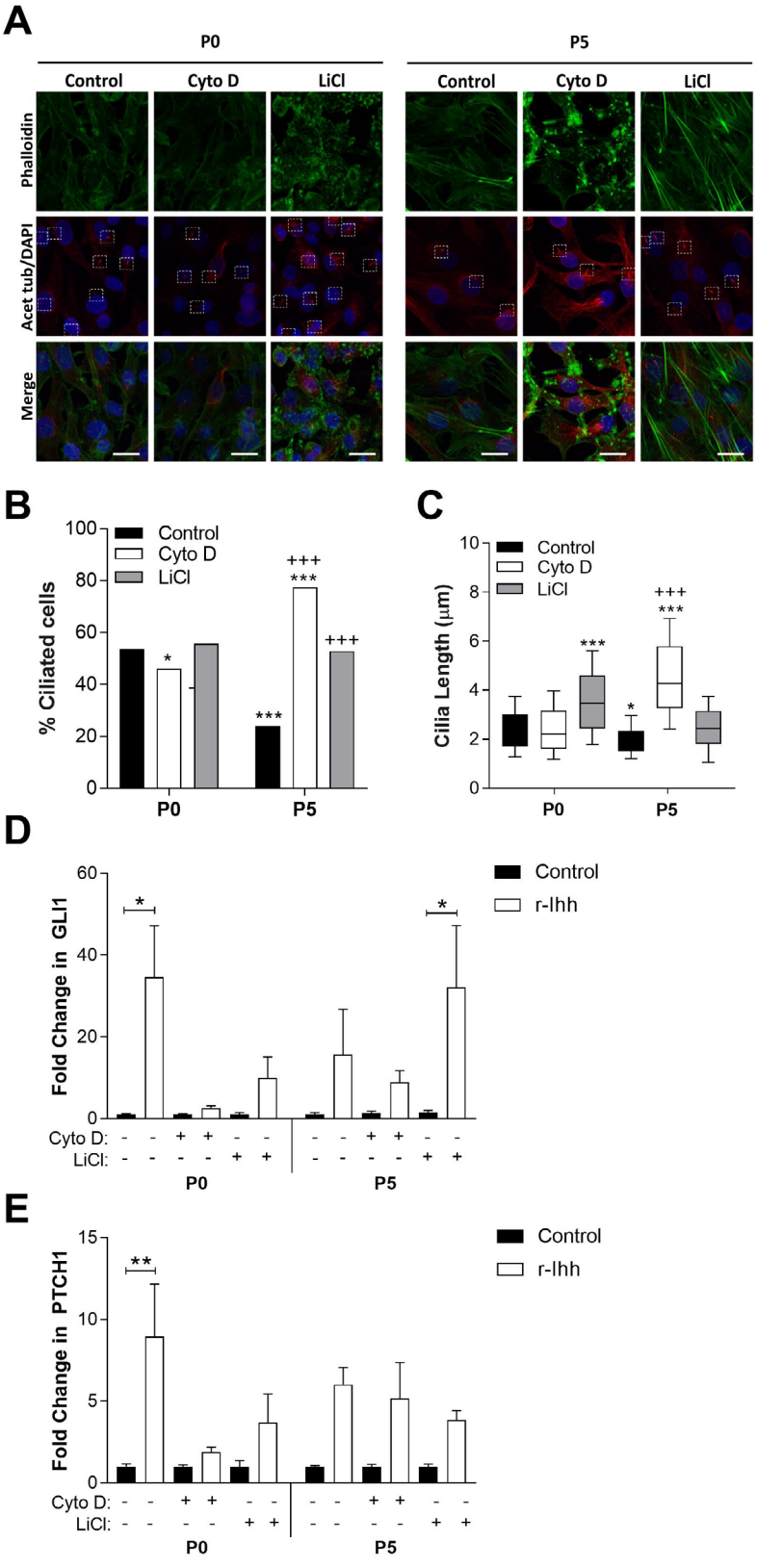

Fig. 5. LiCl increased primary cilia expression in P5 cells and partially restored hedgehog signalling. Primary bovine articular chondrocytes cultured in 2D monolayer at P0 and P5 then treated for 24 h with cytochalasin D (cyto D) and lithium chloride (LiCl) ± recombinant Indian hedgehog (r-Ihh). (**A**) Immunocytochemistry of actin stress fibres (phalloidin, green) and acetylated α-tubulin (acet-tub; red), nuclei were counter stained with DAPI (blue). Scale bar = 20μm. Individual cilia were highlighted by dashed white boxes. Quantification of (**B**) cilia prevalence ($N = 3$, $n \geq 132$ cells, chi-squared test: *** $p < 0.05$ and *** $p < 0.001$) and (**C**) cilia length ($N = 3$, $n \geq 50$ cilia, Mann-Whitney test: * $p < 0.05$ and *** $p < 0.001$ relative to P0 control, +++ $p < 0.001$ relative to P5 control). Real time PCR of (**D**) *GLI1* and (**E**) *PTCH1* expression, data were expressed as a fold change relative to the respective control for each condition ($N = 3$, $n \geq 6$, two-way ANOVA with Sidaks multiple comparison: * $p < 0.05$, ** $p < 0.01$).

we observed a reduction in Arl13b along the mid region of the axoneme, which accompanied ciliary shortening in P5 cultures (Fig. 3**D**,**E**, **F**). While this may also be indicative of alterations in trafficking, production of Gli3R was still observed in P5 cultures, albeit at a reduced level. Moreover, Gli3R expression was reduced following ligand treatment (Fig. 3**C**). The production of Gli3R is dependent on the trafficking of the full-length protein through the ciliary compartment, where it is modified by PKA and targeted for proteasomal cleavage (Sasaki *et al.*, 1999; Huangfu *et al.*, 2003). Thus, these data suggested that ciliary trafficking was intact in P5 cilia and supported the hypothesis that reduced Gli3R production was more likely a consequence of cilia loss.

Mechanical loading triggers primary cilia disassembly in a histone deacetylase 6 (HDAC6)-dependent manner (McGlashan *et al.*, 2010; Thompson *et al.*, 2014). We utilised this observation to reduce cilia length and prevalence in P0 cultures. Despite a comparatively small reduction in ciliation (Fig. 3**A**,**B**,**C**), ligand-dependent hedgehog signalling was inhibited by loading, providing further support for this hypothesis. However, the complete inhibition of this response suggested that there might be additional mechanisms at play. Primary cilia have been implicated in chondrocyte mechanosignalling and regulation of *ACAN* expression in response to load (McGlashan *et al.*, 2007; Wann *et al.*, 2012). While the effects of cilia loss on mechanosignalling were not investigated in the current study, future studies should examine the influence of cilia restoration upon this pathway, as effective matrix production is a key factor for the successful engineering of cartilage tissue. Indeed, a recent study by Spasic and Jacobs (2017) reports that targeting cilia with LiCl can improve mechanosensitivity in bone cells through its effects on primary cilia.

Several actin regulatory molecules are known to influence cilia length (Kim *et al.*, 2010), while the presence of branched F-actin negatively regulates ciliogenesis (for review see Yan and Zhu, 2013). Thus, the reduction in cilia expression observed in this study was likely a consequence of increased stress fibre formation and alterations in actin cytoskeletal tension that accompany dedifferentiation [(Benya, 1988; Parreno *et al.*, 2017) ; Fig. 1]. In support of this hypothesis, we found that cyto D-induced depolymerisation of the actin cytoskeleton in P5 cells restored ciliation beyond P0 levels (Fig. 5**A**,**B**,**C**). Interestingly, in the current study, we observed a higher level of ciliation and increased cilia length on Flexcell® membranes relative to the stiffer glass coverslips (Fig. 2 and 4). This suggested that the mechanical environment modulated chondrocyte ciliation, possibly through associated changes in actin organisation. Such findings have implications for the expansion of chondrocytes and the design of 3D scaffold materials for cartilage tissue engineering.

Restoration of primary cilia in P5 chondrocytes through actin depolymerisation could not restore hedgehog response (Fig. 5). Moreover, cyto D treatment inhibited hedgehog signalling in P0 chondrocytes (Fig. 5). Together these data suggested that a complex relationship existed between actin, cilia and hedgehog. In addition to the cilia-dependent regulation of hedgehog signalling (Bershteyn *et al.*, 2010), the actin cytoskeleton can regulate hedgehog signalling through cilia-independent mechanisms. The dual specificity tyrosine-phosphorylation-regulated kinase 1A (DYRKA1) inhibits endogenous hedgehog signalling by negatively regulating the actin stabilising actin-binding LIM (ABLIM) proteins (Schneider *et al.*, 2015). Phosphorylation of ABLIM1 reduces F-actin assembly and nuclear localisation of the megakaryoblastic leukemia 1 (MKL1) protein. This reduces activation of the Jumonji domain-containing histone demethylase 1A (JMJD1A, encoded by the *Kdm3a* gene), which, in turn, stimulates the transcriptional activity of Gli1 (Schneider *et al.*, 2015), possibly accounting for hedgehog disruption in P0 cells. However, *kdm3a* mutant mice exhibit phenotypes overlapping with mouse models of ciliopathies (Inagaki *et al.*, 2009) and a recent study from Yeyati *et al.* (2017) demonstrates that KDM3A (JMJD1A) plays a role in mammalian ciliogenesis through the regulation of actin dynamics. Thus, separating cilia-dependent and -independent effects of actin modulation on hedgehog signalling is complex.

In this study, we employed LiCl to modify ciliation in P5 cells. This drug is widely used to consistently promote cilia elongation across numerous cell types, in contrast to more targeted molecular approaches (Miyoshi *et al.*, 2009; Ou *et al.*, 2009, Thompson *et al.*, 2016). Stress fibre formation was still apparent in P5 cultures following LiCl treatment, which restored ciliation and cilia length to the levels of P0 cells. However, only a partial rescue of Hh signalling was achieved (Fig. 5**D**,**E**). The shedding of ciliary GPCRs, such as Gpr161, within ciliary ectosomes, is regulated by actin and can regulate hedgehog signalling (Nager *et al.*, 2017). Ciliary ectosomes are shed from the ciliary tip as a mean for removing activated signalling molecules from cilia, which is accompanied by significant loss of ciliary material and ciliary shortening (Nager *et al.*, 2017). Therefore, if cilia loss in P5 chondrocytes occurred in part as a result of ectosome shedding, then it is conceivable that while LiCl might have effectively restored the cilia structure, the previous loss of protein by ectosomes might prevent full recovery of hedgehog signalling.

Hedgehog ligands can promote cartilage matrix production and the re-differentiation of articular chondrocytes (Kellner *et al.*, 2002; Lin *et al.*, 2014). Therefore, restoration of this signalling pathway could be beneficial for the generation of tissue engineered constructs. Indeed, Kellner *et al.* (2002) reports increased matrix production in tissue engineered constructs in the presence of hedgehog. This response is enhanced when constructs are

cultured in low serum conditions (Kellner *et al.*, 2002), which are likely to increase cilia expression (Wheatley *et al.*, 1996). Thus, this study further supports our finding that cilia restoration in dedifferentiated cells could improve hedgehog responsiveness. Lin *et al.* (2014) report that, in mice, hedgehog ligands induce the expression of *ACAN*, *COL2* and *SOX9* in P5 chondrocytes and improve the integration of tissue engineered constructs into cartilage defects. However, in the current study, while r-Ihh increased *ACAN* and *COL2* expression in P0 cultures, it did not induce the expression of matrix genes in P5 cells. This was consistent with cells at P5 showing cilia loss (Fig. 2) and disruption of hedgehog signalling (Fig. 3**A**-**C** and data not shown). However, the partial rescue of hedgehog signalling achieved with LiCl at P5 was not sufficient to restore this downstream response in terms of cartilage matrix gene expression (data not shown).

Targeting cilia for tissue engineering procedures and the concept of ciliotherapy has been in existence for some time with several studies providing proof of concept data suggesting that targeting this organelle can have great therapeutic benefit. For example, the restoration of cilia in pancreatic cancer reduces tumour size and disease progression (Gradilone *et al.*, 2013). While in the kidney, lengthening of primary cilia with feneldopam can effectively increase serum nitric oxide and reduce blood pressure in a mouse model of polycystic kidney disease (Kathem *et al.*, 2014). Therefore, it is timely that this study assessed the implications of cell expansion on cilia structure-function, such that future tissue engineering strategies may consider targeting this organelle for effective cartilage production.

Conclusions

Maintaining chondrocyte phenotype is a necessary part of cartilage tissue engineering. In the present study, we showed for the first time, that chondrocyte expansion and dedifferentiation disrupted primary cilia expression and hedgehog signalling and that this was associated with actin reorganisation. This excluded the use of hedgehog ligands for re-differentiation of expanded chondrocytes. However, treatment with LiCl partially restored cilia expression and hedgehog responsiveness, thus future strategies for tissue engineering cartilage should consider targeting primary cilia in order to restore normal chondrocyte function and enhance cartilage production.

Acknowledgements

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Discussion with Reviewer

Farshid Guilak: What is the relationship between continuous biaxial tensile stretch of chondrocytes and expansion-induced dedifferentiation? Please discuss how these mechanisms might be further interrelated to influence the cilia.

Authors: Mechanotransduction is involved in the maintenance of the chondrocyte phenotype, such that the application of dynamic mechanical loading can promote the chondrogenic phenotype (Holmvall *et al.*, 1995; Wong *et al.*, 2003). Indeed, the culture of primary bovine chondrocytes on a continuously expanding surface can inhibit the dedifferentiation that accompanies serial expansion (Rosenzweig *et al.*, 2012). Mechanical stimuli regulate the maintenance of chondrocyte ciliary structure, which is suggested to function as a mean to modify ciliary responses to environmental cues (McGlashan *et al.*, 2008; McGlashan *et al.*, 2010; Thompson *et al.*, 2014). In the current study, we reported that cells undergoing serial expansion exhibited cilia loss with an accompanying defect in cilia-mediated hedgehog signalling, suggesting that environmental regulation of ciliary signalling was disrupted in passaged cells. In addition to hedgehog signalling, cilia themselves were also required for a range of pathways, including the mechanical regulation of gene expression and matrix production. Therefore, the loss of cilia that occured with dedifferentiation might also be influencing other pathways and aspects of cell function important for tissue health and homeostasis. The fact that both mechanical loading and hedgehog signalling promoted redifferentiation and were both dependent on primary cilia, means that cell expansion conditions, which result in loss of primary cilia, are likely to preclude the use of these approaches to

redifferentiate chondrocytes. Therefore, this study highlighted the importance for tissue engineering approaches of maintaining ciliation.

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Editor note: The scientific editor for this paper was Martin Stoddart.