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Cell cycle deregulation and mosaic loss of *Ext1* drive peripheral chondrosarcomagenesis in the mouse and reveal an intrinsic cilia deficiency

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Abstract

Peripheral chondrosarcoma (PCS) develops as malignant transformation of an osteochondroma, a benign cartilaginous outgrowth at the bone surface. Its invasive, lobular growth despite low-grade histology suggests a loss of chondrocyte polarity. The known genetics of osteochondromagenesis include mosaic loss of *EXT1* or *EXT2* in both hereditary and non-hereditary cases. The most frequent genetic aberrations in human PCS also include disruptions of *CDKN2A* or *TP53*. In order to test the sufficiency of either of these to drive progression of an osteochondroma to PCS, we added conditional loss of *Trp53* or *Ink4a/Arf* in an *Ext1*-driven mouse model of osteochondromagenesis. Each additional tumour suppressor silencing efficiently drove the development of growths that mimic human PCS. As in humans, lobules developed from both *Ext1*-null and *Ext1*-functional clones within osteochondromas. Assessment of their orientation revealed an absence of primary cilia in the majority of mouse PCS chondrocytes, which was corroborated in human PCSs. Loss of primary cilia may be responsible for the lost polarity phenotype ascribed to PCS. Cilia deficiency blocks proliferation in physal chondrocytes, but cell cycle deregulation is sufficient to rescue chondrocyte proliferation following deciliation. This provides a basis of selective pressure for the frequent cell cycle regulator silencing observed in peripheral chondrosarcomagenesis. Mosaic loss of *Ext1* combined with loss of cell cycle regulators promotes peripheral chondrosarcomagenesis in the mouse and reveals deficient ciliogenesis in both the model and the human disease, explaining biological behaviour including lobular and invasive growth.

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Statement of Author Contributions

KBJ and JVMGB conceived of the study. CEa, JFZ, HJ, and KBJ collected and analysed data. CEa, KBJ, and JVMGB interpreted data. KBJ wrote the manuscript and all authors edited it and had final approval of the submitted version.

Keywords

chondrosarcoma; osteochondroma; primary cilium; tumour suppressor; mouse genetic model

Introduction

Chondrosarcoma is the second most common type of primary bone sarcoma [1]. Generally recalcitrant to chemotherapy and radiation, wide surgical resection remains the only effective modality for treatment. Surgical resection of localized chondrosarcoma is challenged by the invasive growth pattern and large size of many tumours.

Chondrosarcomas occur either at the bone surface, termed peripheral chondrosarcoma (PCS), or centrally within the bone. PCS differs from central chondrosarcoma beyond its unique location with respect to the bone, including molecular dissimilarities [2]. Most PCSs arise specifically from an osteochondroma (OC), an otherwise benign outgrowth of physal chondrocytes followed by a bony stalk [3]. In nearly half of cases, PCS arises in patients with a heritable predisposition to form many osteochondromas, called multiple osteochondromas (MO) or multiple hereditary exostoses (MHE or HME) [4]. This makes PCS one of exceedingly few sarcoma types that arise from a precursor lesion and in the context of a heritable syndrome.

MO is characterized genetically by inheritance of a dysfunctional germ line allele of exostosin 1 or 2 (*EXT1* or *EXT2*) and phenotypically by the development of many OCs arising from the skeletal surface during growth [5]. Experiments in mice [6, 7], and with human specimens [8, 9] have established the role of loss of heterozygosity in the development of individual OCs. However, the cartilaginous caps of OCs are not clonal neoplasms, instead consistently including mixed populations of chondrocytes, some bearing homozygous loss of either *EXT* gene and some yet bearing the germ line wild-type allele [6]. A recent analysis of human PCSs showed that they were frequently dominated by chondrocytes that retain at least one functional copy of both *EXT* genes [10]. Few of the PCS cases included in this prior study arose in MO patients, but the *EXT*-mosaic character of OC chondrocytes has also been demonstrated in sporadic/solitary OCs arising in individuals without hereditary MO. This prior analysis therefore suggested that PCS was developing from outgrowth of the “passenger” *EXT*-functional chondrocytes within the original OC.

Osteochondromagenesis has long been considered a process of misorientation [11]. Chondrocytes at the periphery of the physis begin to grow in a peripheral—rather than a longitudinal—direction, yielding the cartilage-capped outgrowth. While OC chondrocytes somewhat recapitulate the architecture of a growth plate, they are not as strictly organized. When the primary cilium first offered a means of determining the polarity or orientation of an individual chondrocyte, cilia staining was employed to assess polarity of OC chondrocytes in humans [12]. The primary cilia of human OC chondrocytes were found to align with neither the OC growth direction nor the linear growth direction of the host bone, in strict contrast to the highly aligned chondrocyte cilia of the normal physis.

As an often low-grade, matrix-producing tumour, PCS rarely grows in a truly infiltrative pattern. Instead, lobules of cartilage push inexorably in anatomically wayward directions. Frequently pushing their way back even into bones [13], lobules of PCS grow as if their cells retain no sense of direction. This observation begs interrogation of the orienting capacity of PCS chondrocytes.

With the goal of understanding the biology of PCS on a more fundamental level, we endeavoured to model the neoplasm genetically in the mouse. Genetic profiling of human PCS specimens has highlighted *p16* (also termed *CDKN2A* and harbouring the overlapping *INK4A* and *ARF* coding regions) as a frequently lost locus in addition to the *EXT* genes themselves [2]. Moreover, overexpression of TP53, suggestive of inactivation by missense mutation, was noted in 10 percent of low-grade and 71 percent of high grade PCSs [2]. These results implicate cell cycle deregulation in this particular form of oncogenesis. With this information in mind, we directed our modelling efforts toward genetic disruption of the cell cycle in mouse OC chondrocytes. Knowing that cartilaginous cap size is the most reliable method for diagnosis of transformation of an osteochondroma [14], we planned to use this as well as corroborative histological and immunohistochemical analyses to test any model that developed.

Materials and Methods

Mice

All mouse work was performed with the approval of the institutional animal care and use committee and in accordance with international legal and ethical standards. The *Col2rtTACre* [15], *Ext1^{e2fl}* [6], *Ink4a/Arf^{fl}* [16], and *Trp53^{fl}* [17] mouse lines have been described previously. Doxycycline was administered at 4mg/mL concentration in 5 percent sucrose water during the second week of life.

Imaging

Radiographs were obtained using a Kodak Carestream 4000 Pro Fx (Carestream Health, Inc., Rochester, NY, USA). Photomicrographs were obtained with an Olympus BX43 microscope and DP26 camera (Olympus America, Center Valley, PA, USA). Fluorescently labeled primary cilia were imaged using a confocal laser scanning microscope (LSM 710; Zeiss, Jena, Germany) and a plan apochromat $\times 63/1.40$ oil or a C-Apo $\times 40/1.2$ water immersion objective lens (both from Zeiss).

Histology

Tissues were harvested post-mortem, fixed in 10% buffered formalin overnight, decalcified for 2 weeks at 4°C in 14 percent ethylenediaminetetraacetic acid (EDTA) (pH7.4), and embedded in paraffin wax following serial dehydration in ethanol. Sections of 8µm thickness were deparaffinised and stained with haematoxylin and eosin (Fisher Scientific, Pittsburgh, PA, USA). For immunohistochemistry against heparan sulphate, 8µm sections were pre-treated with 10,000 units/mL hyaluronidase I (Sigma, St. Louis, MO, USA) for 30 min at 37°C, quenched with 3% H₂O₂ in PBS for 30 min, blocked in 7% goat serum in PBS for 60 min, and incubated overnight at 4°C with mouse anti-10E4 antibody (Seikagaku,

Tokyo, Japan), diluted 1:100 in blocking solution. Goat biotinylated secondary antibody against mouse IgM (Vector Laboratories, Burlingame, CA, USA) at a dilution of 1:250 was applied for 60 min. For parathyroid hormone-like hormone (PTH_{LH}) immunohistochemistry, trypsin antigen retrieval was performed, followed by an overnight incubation at 4°C with anti-PTHLP primary antibody (Ab-2, Oncogene cat nr PC09-100UG), 1:75 diluted in PBS/5% BSA. For visualization, the DAKO envision + system-HRP anti rabbit kit, K4011, was used. For BCL2 immunohistochemistry, following heated antigen retrieval, we incubated slides overnight at 4°C in a 1:500 dilution of anti-BCL2 primary antibody (clone mw-26, Santa Cruz Biotechnology) then IgG-horse radish peroxidase goat anti-rabbit secondary (1:5000 dilution, sc-2004, Santa Cruz Biotechnology). For all immunohistochemical stains other than PTH_{LH}, detection was performed with the Vectastain elite ABC kit (Vector) following the manufacturer's instruction. Slides were counterstained with haematoxylin. For immunofluorescence to detect primary cilia and for cilia analysis, 20µm sections were stained with primary monoclonal antibodies against acetylated alpha-tubulin (clone 6-11b-1, 1:1000; Sigma-Aldrich, Steinheim, Germany), as previously described [12]. For proliferation index analysis, 4µm sections were stained with a monoclonal antibody against Ki67 (clone MIB1, 1:100; Dako, Glostrup, Germany), as previously described [12]. Ki67 positive nuclei were counted per 100 tumour cells in areas containing the largest number of positive cells.

Clinical

Tissue blocks from 5 pathologically confirmed human osteochondromas and 5 peripheral chondrosarcomas were sectioned and stained using the same protocols as for mouse tissues. All samples were handled according to the Dutch code of proper secondary use of human material as accorded by the Dutch society of pathology (www.federa.org). The samples were handled in a coded (pseudonymised) fashion according to the procedures as accorded by the LUMC ethical board.

Tissue Culture

Chondrocytes were harvested in sterile fashion from the anterior rib cage of 5 day old mice (5 pooled from each genotype: wildtype, *Ext1^{e2fl/e2fl};Trp53^{fl/fl}* and *Ext1^{e2fl/e2fl};Ink4a/Arf^{fl/fl}*), washed in 4°C Hank's buffered salt solution (HBSS, Invitrogen, Life Technologies, Grand Island, NY, USA), cleared of soft-tissues in 2mg/mL protease (Sigma), then 3mg/mL collagenase D (Roche, Indianapolis, IN, USA), minced and dissociated using the Cartilage Dissociation System 4 (CHI Scientific, Inc., Maynard, MA, USA), filtered through a 40µm cell strainer (BD Bioscience, San Jose, CA, USA), and cultured in Ham's F12 Gluta Max (Invitrogen) supplemented with sodium pyruvate, MEM non-essential amino acid, 100ug/ml L-Ascorbic acid (Sigma), penicillin-streptomycin and 10 percent foetal bovine serum. Serum-free media with or without 2.5µM TATCre was applied for 2 hours, after which complete media was added. RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA, USA), then reverse transcribed with the Superscript First-Strand synthesis system (Invitrogen). Polymerase chain reaction in 35 cycles of 30 seconds 56°C annealing and 1 minute 72°C elongation for collagen I (F: CCC TGG TAT GAC TGG CTT; R: GAC CAC GAA TCC CTT CCT), collagen II (F: GCC CGT CAG GAA GTA CC; R: ACC AGC ATC TCC TTT CTG T), aggrecan (F: TTC CAT CTG GAG GAG AGG G; R: ATC TAC TCC

TGA AGC AGA TGT C), and control *Gapdh* (F: ACC ACA GTC CAT GCC ATC AC; R: CAC CAC CCT GTT GCT GTA GCC). For growth in chloral hydrate (Fagron, St. Paul, MN, USA) cells were plated at 5×10^4 /mL density in 96-well format. CellTiter-Glo luminescent cell viability assay was performed per manufacturer's instructions (Promega, Madison, WI, USA) and luminescence measured with an Envision 2104 multilabel reader (PerkinElmer, Akron, OH, USA). Each experiment with TATCre application and/or chloral hydrate exposure had at least 3 biological replicates and was also independently repeated twice with another 3 replicates each time for separate confirmation. The data shown in each graph are from one run (3 replicates).

Results

Loss of tumour suppressive cell cycle regulators enables transformation of osteochondromas

In order to determine the impact of losing cell cycle regulation in the chondrocytes in the cap of an OC, we bred mice bearing an *Ext1* allele with a trans-floxed second exon (*Ext1^{e2fl}*) [6] to mice bearing standard floxed alleles of either tumour related protein 53 (*Trp53^{fl/fl}*) [17] or the cyclin-dependent kinase inhibitor 2a locus, which harbours the *Ink4a* and *Arf* genes (*Ink4a/Arf^{fl/fl}*) [16], then to mice bearing a transgene expressing Cre-recombinase under the control of the collagen IIa1 promoter and the reverse Tet transactivator (*Col2rtTA-Cre*) [15]. Double homozygous mice bearing the Cre-recombinase, *Col2rtTA-Cre;Ext1^{e2fl/e2fl};Trp53^{fl/fl}* and *Col2rtTA-Cre;Ext1^{e2fl/e2fl};Ink4a/Arf^{fl/fl}*, received doxycycline during the second week of life through the drinking water, along with controls lacking the Cre-recombinase or lacking the conditional cell cycle regulator alleles (Fig. 1A). Cohorts of these mice were followed until the age of 6 and 9 months. Beyond 9 months, added morbidity and a few osteosarcomas in the *Trp53*-conditional group made regimented assessment difficult, and older cohorts were not collected. Radiographic imaging demonstrated the development of larger and more involved surfaces masses in the regions where simple OCs were expected in the control mice lacking the additional loss of the tumour suppressors (Fig. 1B–C).

In order to determine whether the OCs had transformed, we assessed three parameters used to diagnose PCS in humans: cartilage cap thickness, lobular growth, and cellular morphology [14]. Either *Ink4a/Arf* or *Trp53* conditional disruption led to a statistically significant thickening of the cartilaginous cap (Fig. 2A), the development of lobules of cartilage growing beyond the cap (Fig. 2B), and increased cellularity, in which nuclei were slightly enlarged and demonstrated coarse chromatin (Fig. 2C). We also evaluated mouse tumours in each group by immunohistochemistry against PTHLH and BCL2 (Fig. S1). Similar to humans, there was some variability in the staining for these markers in both mouse OCs and PCSs, but significantly more prevalent positive staining for each marker in the growths arising in mice that also had *Ink4a/Arf* or *Trp53* conditional disruption.

Lobular growth can derive from either *Ext1*-null or *Ext1*-wildtype chondrocytes

This distinct character of the inverting and deleting loxP flanked alleles in our model enabled the possibility for either *Ext1*-functional or *Ext1*-null OC chondrocytes to transform from the additional tumour suppressor silencing (Fig. 1A). In order to determine whether the

PCS lobules growing from OCs in our MO model retained at least one functional copy of *Ext1*, we stained for heparan sulphate with the 10E4 antibody, a proven surrogate for functional *Ext1* in the model [6].

Cartilage lobules in each combination allele model varied in their retention of 10E4 staining. Some lobules showed normal heparan sulphate (Fig. 3B) and others no staining (Fig. 3C). Critically, though, the homogeneous staining in each lobule contrasted sharply to the typical, mosaic pattern of 10E4 staining in non-transformed OCs [6], which demonstrate a mixture of retained and absent 10E4 staining within the same cartilaginous cap (Fig. 3A). This suggests that either cell population included in the cartilage caps of OCs can grow into a PCS.

Peripheral chondrosarcomas exhibit dysfunctional ciliogenesis

In order to assess chondrocyte orientation in the PCS mice, we stained sagittal histologic sections from 9 month old mice in the control and PCS groups with an anti-acetylated- α -tubulin antibody. Matching the previously reported human data, the primary cilia of mouse physal chondrocytes were well-aligned, but those of mouse OCs much less so (Fig. 4A).

To our surprise, a large number of chondrocytes in lobular growth areas in the PCS mice lacked primary cilia altogether (Fig. 4A). The prevalence of cells lacking a primary cilium was increased in both *Ink4a/Arf*- and *Trp53*-associated mouse PCS models at 9 months, compared to *Ext1*-driven osteochondroma controls at the same age (Fig. 4B).

To determine whether defective ciliogenesis also occurs in human PCS, we assessed cilia counts in 5 human PCS specimens and 5 human OCs, all from patients with MO. We found a significant loss of ciliated chondrocytes in human PCSs as well, compared to human OCs (Fig. 4C).

Deficient ciliogenesis in PCS cannot be explained by proliferation alone

To further investigate likely potential causes of deficient ciliogenesis in PCS, we assessed the proliferative index in the mouse and human PCSs. Very rapid proliferation is one potential cause of apparently deficient ciliogenesis in many cancer types. In contrast to highly proliferative physal chondrocytes (which notably retain primary cilia), the Ki67 proliferative indices from mouse and human PCS samples were low relative to the prevalence of deciliation (Fig. 5), suggesting that proliferation alone does not explain the lack of cilia.

Loss of *Trp53* or *Ink4a/Arf* enables growth through deficient ciliogenesis in chondrocytes

In order to test whether deficient ciliogenesis directly impacts proliferation in the absence of the pleiotropic effects of organism-wide cilia deficiency, we harvested and cultured chondrocytes from both *Ext1^{e2fl/e2fl};Trp53^{fl/fl}* and *Ext1^{e2fl/e2fl};Ink4a/Arf^{fl/fl}* mice on the fifth day of life. Chondrocytes in each group were exposed either to control conditions or the *in vitro* administration of TATCre, a protein version of Cre-recombinase that enters cells and recombines floxed alleles [18]. Exposure to TATCre had no appreciable impact on chondrocyte phenotypic differentiation compared to control conditions (Fig. S2A–B), but

achieved very high density of recombination in the treated cells (Fig. S2C–D). Cells from each group were exposed to 4mM chloral hydrate, a well-established chondrocyte deciliating concentration [19, 20]. Non-recombined chondrocytes demonstrated a significant drop in viability after application of 4mM chloral hydrate (Fig. 6A). In contrast, recombined chondrocytes with mosaic loss of *Ext1* and loss of either *Trp53* or *Ink4a/Arf* demonstrated a partial rescue of viability/proliferation following chloral hydrate (Fig. 6B). Since loss of *Trp53* produced a chloral hydrate-independent increase in viability, the relative rescue of the chloral hydrate driven loss of proliferation was subtle at 4 days, but more pronounced at 8 days. This suggests that loss of one of these tumour suppressors may rescue proliferation following loss of primary cilia.

Discussion

We report a mouse genetic model of peripheral chondrosarcomagenesis, driven by loss of *Trp53* or *Ink4a/Arf* in addition to mosaic loss of *Ext1*. These primarily low-grade neoplasms fit the diagnostic criteria used for human PCS. Assessment for loss of polarity in the mouse model led to the discovery of deficient ciliogenesis in the chondrocytes of PCS. This finding was confirmed in human cases. While deficient ciliogenesis may enable certain oncogenic properties in chondrocytes, we found that in isolation, loss of cilia abrogated proliferation of non-transformed, physeal chondrocytes. Cell cycle deregulation was sufficient to rescue chondrocyte proliferation following loss of cilia. This may explain why loss of cell cycle checkpoint genes is so common in human PCS, despite their low-grade appearance.

Loss of cilia may contribute to peripheral chondrosarcomagenesis. The function of the primary cilium in a chondrocyte has not been thoroughly investigated. The strict alignment of primary cilia along the axis of growth in proliferating physeal chondrocytes suggests a role in polarity. Primary cilia are at least a marker of polarity, the axis-alignment of which is demonstrably lost in OC chondrocytes [12]. Nonetheless, OC chondrocytes retain primary cilia in general and do not exhibit invasive growth patterns. If chondrocyte cilia function as orienting organelles, sensing and interpreting the surrounding environment, and not as markers of orientation alone, then the lobular and invasive growth patterns characteristic of PCS may relate to more drastic compromise of chondrocyte orientation achieved via loss of cilia altogether. For a generally low-grade neoplasm, such growth patterns are remarkable.

A deficiency in primary cilia has also been reported in central chondrosarcomas. In strict contrast to OCs, the precursors of PCS, the presumed precursors of central chondrosarcomas also lack primary cilia. These enchondromas are benign islands of cartilage within the metaphyseal bone. It has even been shown that mosaic loss of cilia in physeal chondrocytes is sufficient to drive the formation of enchondromas [20]. What was not previously clear is whether deficient ciliogenesis contributed to proliferation or simply misorientation in the induction of enchondromas. Complete abrogation of ciliogenesis in chondrocytes leads instead to poor physeal organization and stunted growth [21]. As enchondromas are rarely proliferative and the loss of cilia in the physis itself leads to stunted growth, we hypothesized that the impact of lost ciliogenesis was distinct from a specifically pro-growth program and more related to loss of orientation. This hypothesis was substantiated by our assessment of the negative impact of the deciliation agent, chloral hydrate, on proliferation

in non-transformed chondrocytes. Our data suggests that primary cilia deficiency creates a proliferative block to be overcome, rather than a proliferative advantage to a chondrocyte.

Loss of cilia may provide selective pressure favouring growth of clones that have lost cell cycle regulatory genes. Weakening of cell cycle check point regulation by loss of tumour suppressor regulators *Cdkn2a* or *Trp53* rescued chondrocyte proliferation in the setting of deficient ciliogenesis and promoted tumour growth in the mice. Importantly, the loss of cell cycle check-point regulators associates with progression to high grade histology and aggressive clinical behaviour in many cancers. Most PCSs, however, remain low-grade. It may be that in PCS, the cellular stress that must be by-passed by the loss of cell cycle check-point regulators is not some defect in DNA repair or chromosomal fidelity as much as it is loss of primary cilium signalling and misorientation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Modelling peripheral chondrosarcoma in the mouse

(A) Schematic demonstrates the alternative allele design of *Ext1^{e2fl}*, which results in reversible inversion, and therefore only mosaic loss of *Ext1* following Cre-mediated recombination that consistently disrupts both alleles of either *Trp53^{fl}* or *Ink4a/Arf^{fl}*. This produces chondrocytes lacking the cell cycle regulator and either lacking or retaining *Ext1*. (B) Radiographs from control and experimental mice demonstrate osteochondroma (open arrow) formation in mice with mosaic loss of *Ext1* alone, but formation of larger, more dysmorphic lesions with the addition of cell cycle deregulation (black arrows). (C) Schematic depicting the prevalence and anatomic distribution of osteochondromas in control mice with only mosaic loss of *Ext1* and more aggressive cartilaginous surface lesions in the combination genotype groups.

Figure 2. Mouse tumours match criteria for human peripheral chondrosarcoma

(A) linear measurement of the thickness of cartilaginous caps of the skeletal surface lesions in mice with mosaic loss of *Ext1* in chondrocytes either alone or in combination with *Ink4a/Arf* or *Trp53* finds the latter two groups to be increased (n = 5 mice for each assessment, * indicates t-test p-value < 0.0062). (B) Photomicrographs demonstrate the thin cartilaginous cap of a 9 month control osteochondroma, as well as lobules of cartilage (black arrows) extending beyond the thickened cap in both experimental models. (C) Higher power photomicrographs demonstrate the small chondrocytes with tight chromatin in the 9 month physis, while the transformed chondrocytes of peripheral chondrosarcoma have larger cells, more open and coarse chromatin patterns, and even binucleated cells (black arrows). (Magnification bars in B and panel widths in C are each 50µm.)

Figure 3. Both *Ext1*-functional and *Ext1*-null chondrocytes can transform

Photomicrographs of immunohistochemistry using the 10E4 antibody against heparan sulphate demonstrate a mix of cells with retained or lost *Ext1* function in an osteochondroma (A), but lobules with clonal retention (B) or loss (C) in mouse PCSs (Magnification bars are each 20µm).

Figure 4. Peripheral chondrosarcomas exhibit deficient ciliogenesis

(A) Photomicrographs of immunofluorescence against acetylated alpha-tubulin in a mouse physis, an osteochondroma, and a peripheral chondrosarcoma lobule, each at 9 months, demonstrate misorientation of primary cilia in the osteochondroma compared to the axis of growth (double-headed arrows) and loss of cilia in the peripheral chondrosarcoma lobule. Charts present the percentage of chondrocytes with primary cilia in each group of mice (B) and in human (C) osteochondroma (OC) and peripheral chondrosarcoma (PCS) samples (bars denote mean for each group; magnification bars are each 10 μ m).

Figure 5. Peripheral chondrosarcomas maintain a relatively low proliferative index

Photomicrographs demonstrating immunohistochemistry against Ki67 to mark proliferating cells in human physis (A) and a mouse PCS nodule (B). (C) Chart presenting the fraction of cells in each specimen staining positively for Ki67. (Magnification bars are each 10 μ m.)

Figure 6. Cell cycle deregulation rescues the block in chondrocyte proliferation from loss of cilia
 (A) Chart presenting relative cell viability of mouse chondrocytes cultured in the presence of increasing concentrations of chloral hydrate. (B) Charts demonstrating that chloral hydrate-blocked proliferation is partially rescued in chondrocytes that have lost *Ext1* in mosaic fashion with either *Trp53* or *Ink4a/Arf*. (Each bar presents the mean of 3 replicates with the error bar denoting the standard deviation.)