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Endochondral growth defect and deployment of transient chondrocyte behaviours underlie osteoarthritis onset in a natural murine model

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Abstract

Objective

To explore whether aberrant transient chondrocyte behaviours occur in STR/Ort mouse joints (spontaneously osteoarthritis) and whether this is attributable to an endochondral growth defect.

Methods

STR/Ort mouse knee joints at advancing osteoarthritis stages and age-matched CBA (control) joints were examined, by affymetrix microarray profiling, multiplex PCR analysis and immunohistochemical labelling of endochondral markers including sclerostin and MEPE. The endochondral phenotype of STR/Ort mice was analysed by histology, microCT and *ex vivo* organ culture. A novel protocol for quantifying bony bridges across the murine epiphysis (growth-plate fusion) using synchrotron x-ray computed microtomography was developed and applied.

Results

Meta-analysis of transcriptional profiles revealed significant elevation in functions linked with endochondral ossification in STR/Ort mice (in comparison to CBA; $P<0.05$). Consistent with this, immunolabelling revealed increased MMP13 and collagen type-X expression in STR/Ort joints and multiplex RT-qPCR showed differential expression of known mineralisation regulators suggesting an inherent chondrocyte defect. Support for an endochondral defect was provided by accelerated growth, increased zone of growth-plate proliferative chondrocytes ($P<0.05$) and widespread collagen type-X/MMP13 labelling beyond the expected hypertrophic zone distribution. We found osteoarthritis development involved concomitant focal suppression of sclerostin/MEPE in STR/Ort mice. Our novel

synchrotron radiation microtomography method revealed increased numbers ($P<0.001$) and mean areal growth plate bridge densities ($P<0.01$) in young and aged STR/Ort compared to age-matched CBA mice.

Conclusion

Our data collectively supports an inherent endochondral defect that is linked to growth dynamics and subject to regulation by the MEPE/sclerostin axis that may represent an underpinning mechanism in pathological ossification in osteoarthritis.

Key Words: osteoarthritis, growth plate, mineralisation, chondrocyte, STR/Ort

Osteoarthritis (OA) is a degenerative joint disease and a world-wide healthcare burden. Characterised by articular cartilage loss, subchondral bone thickening and osteophyte formation, the OA joint afflicts much pain and disability. Its underpinning molecular mechanisms are, nevertheless, not fully understood; indeed it is even still a matter of debate as to which is the precipitating pathology. As such, there is an ever growing need for an effective disease modifying treatment. Canine hip dysplasia is a hereditary predisposition to the development of degenerative OA and this is more common in certain breeds, in particular those larger breeds which tend to grow more rapidly (1). Whilst no direct link has been made between growth dynamics and osteoarthritis, recent murine and human studies have prompted speculation that the articular cartilage chondrocytes may undergo a switching from their inherently stable phenotype to a more transient one characteristic of the chondrocytes in the growth plate (2-9).

The epiphyseal growth plates are responsible for long bone development (endochondral ossification) and growth. This is secured by growth plate chondrocytes undergoing differentiation, maturation, hypertrophy and death, resulting in mineralisation of the cartilage matrix (10-13). Transience of growth plate cartilage chondrocytes is thus a crucial attribute. However, this could hardly contrast more than with the inherent stability of articular cartilage chondrocytes, in which these dynamic events must be restricted to assure life-long articular integrity and joint function. Interlinks between these apparently discordant phenotypes are not fully understood and whether switching in these behaviours may contribute to the structural demise of articular cartilage in OA joints is not yet established (13-15). However, based on the common embryology of cartilage and bone, along with the recent evidence supporting uncommon origins for growth plate and articular cartilage chondrocytes, it is not surprising that this hypothesis has been controversial (16-18). Regardless, an exploration of

the mechanisms controlling changes that chondrocytes undergo during their transition through the various stages of endochondral ossification may help to decipher those which underpin pathological ossification in OA.

The STR/Ort mouse is a well-established, natural OA model, resembling the human condition. Mice develop articular cartilage lesions on the medial tibia plateau, with subchondral bone thickening and expected degenerative changes in other joint tissues from ~18 weeks of age, coincident with attainment of skeletal maturity (19-22). CBA mice, the closest available parental strain show, in contrast, very low spontaneous OA susceptibility (21, 23). We therefore aimed to establish whether an aberrant deployment of the transient chondrocyte phenotype is observed in STR/Ort mouse joints and whether this can be attributed to modified growth dynamics underpinned by an inherent endochondral growth defect.

Methods

Animals. Male STR/Ort (bred in-house), and CBA (Charles River, UK) mice were used in all experiments. All procedures complied with Animals (Scientific Procedures) Act 1986 and local ethics committee.

Meta-analysis of microarray data. Gene ontology classification was performed using DAVID (http://david.abcc.ncifcrf.gov/) on Affymetrix mouse gene microarray profiling of articular cartilage performed by us previously (22, 24).

RNA extraction. RNA was extracted from knee joint articular cartilage from STR/Ort and CBA joints (n=3 joints/sample for each strain at each age) at ages 8-10, 18-20 and 40+ weeks of age, as described (22).

Multiplex RT-qPCR. A GeXP multiplex RT-qPCR assay was designed for gene targets; *Ank*, *Dmp1*, *Enpp1*, *Mepe*, *Opn (Spp1)*, *Phex*, and *Sost* (Suppl. Table 3). Target-specific reverse transcription was performed as previously described using 50ng of total RNA (25, 26).

Immunohistochemistry. Immunohistochemistry was performed on 6μm coronal sections using anti-sclerostin (1/100; R&D systems), anti-MMP13 (1/200; Abcam), anti-Col10a1 (1/500; Prof. Boot-Handford, University of Manchester) or anti-MEPE antibodies (1/200; Prof. Rowe, KUMC).

Articular cartilage and growth plate zone analysis. Multiple toluidine blue stained coronal sections (n>6) from the joints of 4 individual mice (at each age) were used to measure the width of: (i) joint compartments (ii) growth plate zones based on established cell morphology (27).

Joint imaging by micro-computed tomography (μCT). Joints were scanned using a laboratory source for 5 micron voxel size and at a synchrotron for 1 micron voxel size. The laboratory scans were performed with an 1172 x-ray microtomograph (Skyscan, Belgium) to evaluate the cortical and trabecular bone geometry. The synchrotron radiation microtomography was performed at Diamond Light Source on the Diamond-Manchester Branchline I13-2 with projections being reconstructed and a procedure developed to characterise each individual bridge and map their location on the tibial joint surface (Supplementary methods) (28-30).

Metatarsal organ cultures. Metatarsal bones (E15) were cultured for up to 7 days (31, 32). The total length of the bone through the centre of the mineralising zone, and the length of the central mineralisation zone were determined using Image J analysis software.

Sclerostin ELISA. Serum sclerostin levels in CBA and STR/Ort mice at 8-10 weeks, 18-20 weeks, and 40+ weeks (n=4 for each strain at each age) were measured using a mouse/rat sclerostin ELISA kit (R&D systems).

Statistics. Data were analysed by one-way analysis of variance (ANOVA), the Student's *t*-test, or a suitable non-parametric test using GraphPad prism 6 and following normality checks (La Jolla, USA). All data are expressed as the mean ± SEM.

Results

Retention of calcified cartilage thickness despite articular cartilage loss and subchondral bone thickening in STR/Ort mice. We first sought to determine temporospatial patterns of changing joint architecture in the STR/Ort mice. Consistent with previous findings (33) we show that young STR/Ort mice have thicker medial tibia articular cartilage than age-matched CBA controls ($P<0.001$). As STR/Ort mice age, this becomes thinner with concomitant thickening of subchondral bone, neither of which occur in CBA mice ($P<0.001$) (Fig. 1A&B). Despite this, there is no change in calcified cartilage thickness (Fig. 1B) and further analysis discloses similar changes in the medial femur (Suppl. Fig. 1). Lateral condyles in STR/Ort mice were unaffected by such age-related structural modifications. The lateral tibia also exhibited greater articular cartilage thickness, maybe in compensation to altered mechanical loads (Suppl. Fig. 1).

Articular cartilage in STR/Ort mice deploys transient chondrocyte behaviours prior to OA onset. We next examined whether this predisposition to age-related subchondral bone thickening and loss of articular cartilage in STR/Ort OA was linked to the expression of markers of the transient chondrocyte phenotype. Initially, we used the DAVID functional annotation clustering tool to identify biological functions enriched within differentially expressed genes in articular cartilage from OA affected mice (STR/Ort >18weeks) compared

to unaffected mice (CBA 8-40 weeks, STR/Ort at 8-10 weeks) (22). Within the major gene ontology classifications, there was significant up-regulation of endochondral bone growth ($P<0.01$) (Table 1, Suppl. Table 1). No gene ontology classifications associated with skeletal development and ossification were found to be significantly down-regulated.

These endochondral ossification gene ontologies in STR/Ort OA articular cartilage led us to examine protein expression of well-established chondrocyte hypertrophy markers in OA development. Immunohistochemistry revealed positive MMP13 labelling in both superficial and deep articular chondrocytes in the joints of STR/Ort mice prior to OA onset (Fig. 1C&D). In accord with previous findings, an expected pattern of collagen type X expression was observed in the unaffected condyles of STR/Ort mouse joints, with immunolabelling restricted to hypertrophic chondrocytes (Fig. 1F). Intriguingly, collagen type X immunolabelling was observed throughout the medial condylar articular cartilage matrix in STR/Ort mice aged 8-10weeks, before histologically detectable OA (Fig. 1E). Multiplex gene analysis of genes associated with matrix mineralization confirmed significant elevation in *Enpp1* and *Ank* mRNA isolated from 8-10 week-old STR/Ort mice articular cartilage, compared to CBA mice ($P<0.001$, Fig. 1G&H). Interestingly, these increases were diminished upon OA onset ($P<0.001$, at 18-20weeks, Fig. 1G&H). These findings suggest that OA-prone regions of the STR/Ort mouse cartilage exhibit an inherent instability defect in articular chondrocytes.

STR/Ort mice exhibit accelerated growth, dysfunctional growth plate morphology and matrix mineralisation. To more fully define this inherent endochondral chondrocyte defect, we examined growth trajectories of 1-8 week old STR/Ort and CBA mice. We found that STR/Ort mice weigh less ($P<0.05$) until 4-5 weeks of age, corresponding with the attainment of sexual maturity, when they overtake the CBA mice (Fig. 2A). Consistent with this, analysis of embryonic longitudinal growth and mineralisation *in vitro* revealed aberrant rates

of growth and endochondral ossification in STR/Ort mouse metatarsals (31, 32) (Fig. 2C-F), involving both a slowing of metatarsal growth (Fig. 2E) and marked reductions in the mineralised portion of the element (Fig. 2F).

Consistent with this accelerated growth phenotype, STR/Ort mice also have shorter tibiae at 3 weeks of age ($P<0.05$), which is seemingly revised as tibia length at 6 weeks of age is not significantly different (Fig. 2B). This is further endorsed by comparisons of 8-week-old mice when the STR/Ort tibia is longer than in age matched CBA mice ($P<0.01$, Fig. 2B). MicroCT analysis revealed significantly enhanced cortical and trabecular parameters in 6 week old STR/Ort tibia, with higher % differences in BV/TV (12%; $P<0.01$), cortical thickness (23%; $P<0.05$), cortical area (23%; $P<0.001$), polar moment of inertia (46%; $P<0.05$), trabecular pattern factor (27%; $P<0.05$) and SMI (13%, $P<0.05$) compared to age-matched CBA mice (Suppl. Table 2). In comparison, there were no significant differences at 3 weeks of age (Suppl. Table 2).

Growth plate zone analysis revealed a significantly enlarged proliferating zone of chondrocytes in both 3- and 6-week-old STR/Ort mice ($P<0.001$ and $P<0.05$, respectively; Fig. 2G). This was not apparent in 8-week-old STR/Ort mice when compared with age-matched CBA (Fig. 2G). Despite the lack of any differences in the size of hypertrophic chondrocyte zones, immunolabelling for collagen type X disclosed the expected localisation in CBA mouse growth plates, limited exclusively to the hypertrophic zone and underlying metaphyseal bone at both 3- and 6-weeks of age (Fig. 2H&I). In contrast, STR/Ort mice at both ages showed considerably greater and more widely dispersed collagen type X expression which extended additionally into the proliferative chondrocyte zone (Fig. 2J&K). This disrupted distribution of growth plate zone markers was also evident for MMP13 in the growth plates of STR/Ort mice (Fig. 2L-O).

STR/Ort mouse OA is linked to modifications in the MEPE/sclerostin axis. Molecular mechanisms controlling endochondral ossification may help identify those involved in OA. We have previously shown MEPE, a member of the SIBLING family, to be a negative regulator of growth plate chondrocyte matrix mineralisation (31). Examination of MEPE expression by multiplex analysis found significantly higher *Mepe* mRNA in STR/Ort mouse articular cartilage than CBA mice ($P<0.001$) (Fig. 3A). Immunolabelling for MEPE showed differential expression across the tibia of the STR/Ort mouse joints, with a distinct lack of positive MEPE protein labelling in the medial (affected) aspects both prior to and during OA progression (Fig. 3C). This is in contrast to the lateral (unaffected) aspect of the STR/Ort joints and throughout all aspects of CBA joints where labelling for MEPE is observed throughout the depth of the articular cartilage tissue (Fig. 3C&D).

Assessment of the MEPE regulator, *Phex,* revealing elevated mRNA in 8-10 week STR/Ort articular cartilage compared to age-matched CBA mice ($P<0.001$, Fig. 3B). In STR/Ort mice, this expression significantly decreased with OA onset ($P<0.001$, Fig. 4B). Identical age-related expression patterns were found for *Dmp1* mRNA, another SIBLING family member ($P<0.001$, Fig. 3E). Analysis of mRNA levels of osteopontin (*Opn*), a SIBLING with shared roles in biomineralisation, revealed significantly higher levels in aged STR/Ort compared to young STR/Ort mice ($P<0.05$, Fig. 3F) and age-matched CBA mice, resembling patterns of *Mepe* expression ($P<0.05$, Fig. 3F). Taken together, this suggests a regulatory role for the SIBLING family of proteins in OA development in these mice.

We next sought to examine the temporal expression of another important regulator of MEPE expression, the Wnt signalling inhibitor sclerostin (*Sost*) (34). Our analyses showed greater *Sost* mRNA levels in articular cartilage of 8-10 week STR/Ort than in age-matched CBA mice ($P<0.05$, Fig. 4A), which significantly decreased with OA onset ($P<0.01$, Fig. 4A). Despite this, no differences were observed in circulating serum sclerostin concentrations in

these mice at any age, supportive of solely local effects (Fig. 4B). Consistent with this, sclerostin immunolabelling disclosed a clear enrichment in cells at the osteochondral interface in unaffected regions of STR/Ort mouse joints (arrows, Fig. 4C). In contrast, STR/Ort mice with OA showed suppression in positive sclerostin labelling in regions of subchondral bone-thickening underlying those with compromised articular cartilage integrity (Fig. 4D).

Premature growth plate closure in STR/Ort mice linked to OA development. To directly test whether longitudinal growth, growth plate fusion and osteoarthritis exhibit inter-relationships in STR/Ort mice we developed a novel protocol for quantifying bony bridges formed across the entire murine tibia epiphysis during growth plate fusion (Suppl. Methods, Fig. 5A-C). Applying this novel method to examine growth plate closure in STR/Ort and CBA mice at 8 and 40+ weeks of age revealed a dramatically (10-fold) greater total number of bridges in 8-week-old STR/Ort (137 ± 10) than in CBA mice (14 ± 10; Fig. 5D, E&H; $P<0.001$; Suppl. Fig. 2). This enriched growth plate bridging was apparent in all aspects of STR/Ort mouse tibiae ($P<0.05$; Fig. 5H). Although still evident in aged STR/Ort mice (40+ weeks), this enriched bone bridging was much more discrete (STR/Ort: 295 ± 72, CBA: 266 ± 53; Fig. 5F, G&I; Suppl. Fig. 2). Mean areal bridge densities were also greater in STR/Ort mice at both ages ($P<0.01$, Fig. 5J). These exciting data reveal an accelerated cartilage-bone transition in the growth plate which, together with our aforementioned data, support an inherent endochondral defect in both the articular and growth plate cartilage in STR/Ort mice.

Discussion

Our data reveal changes in the articular cartilage of STR/Ort mouse knee joints consistent with an aberrant deployment of endochondral processes. This is associated with inherent longitudinal growth modifications, disrupted growth plate morphology, premature growth

plate fusion, and aberrant bone formation and matrix mineralisation prior to OA onset. These data indicate, at least in the spontaneous human-like OA in STR/Ort mice, that growth-related endochondral ossification abnormalities may forecast mechanisms of OA development in articular cartilage.

There is certainly some tantalising previously published data exploring expression of endochondral ossification markers which agrees with this notion (14). Collagen type X is a marker of chondrocyte hypertrophy, usually found in the growth plate and unique to the calcified cartilage in normal joints (35). Expression of collagen type X mRNA transcripts, as examined by *in situ* hybridization, have however been observed throughout articular cartilage in both young (9 week) and older (41+ weeks) STR/Ort mice (36). Here we provide evidence for the first time for associated collagen type X protein expression in these mice. In agreement with our studies, an additional marker of chondrocyte hypertrophy, MMP13 has also been detected in the calcified cartilage chondrocytes of STR/Ort mice at both young and old ages, at levels greater than in age-matched CBA (37). Similarly, higher expression levels of several other MMPs (MMP2, MMP3, MMP7, MMP9, MT1-MMP) were observed in the tibial articular chondrocytes of the STR/Ort mouse (37). Indeed, many of these MMPs were also shown to be significantly increased in our previous microarray study, further highlighting their likely role as key players in cartilage degradation in OA (22). The STR/Ort mouse growth plate has remained relatively unexamined with, to our knowledge, only one paper describing phenotypic changes associated with ageing. Chambers et al describe collagen type X mRNA expression localized to hypertrophic chondrocytes, as expected, in both young CBA and STR/Ort mice. However in the aged mice, there was no expression of collagen type X mRNA observed despite the preservation of collagen type II mRNA throughout the depth of the thinned growth plate cartilage (36). Our results now reveal aberrant expression of collagen type X and MMP13 additionally in the growth plate of young

STR/Ort mice. STR/Ort mice also display an increased zone of proliferative chondrocytes, based on well-established cell morphological features (27). As such, these results may seem counterintuitive and just highlight that there is clearly an inherent endochondral defect in the STR/Ort mice which may also precipitate OA pathogenesis.

Molecular mechanisms controlling endochondral ossification may help identify those involved in OA. Effective control of the Wnt signalling pathway is certainly proving critical in regulating both the extent of OA joint pathology (38) and growth plate chondrocyte behaviour, and our data here corroborates this.

Genetic and microarray analyses have been performed in STR/Ort mice in order to better elucidate their OA aetiology (39-42). Jaeger and colleagues identified a QTL associated with articular cartilage degeneration on chromosome 8 of the STR/Ort mouse (39). This however was not corroborated in a more recent QTL analysis in which STR/Ort mice were backcrossed to the C57BL/6N strain (43). This QTL was therefore suggested to be a recessive trait, as originally implicated by Walton and colleagues, among the polygenetic factors in STR/Ort OA (19, 43). Instead the authors identify a QTL for the OA phenotype that is mapped to chromosome 4 (43). Chromosome 8 was however revisited and fine mapping of the OA-QTL in a more recent study revealed Wnt related genes associated with altered chondrogenesis, including *dickkopf 4* (*Dkk4*), *secreted frizzled related protein 1* (*Sfrp1*) and *fibroblast growth factor 1* (*Fgfr1*) (38, 42). Whilst a number of genes, including Wnt related genes, have been implicated in OA by association studies in human populations, there is a distinct lack of functional data to support a causative link between these associated genes and OA.

Pasold et al., attempted to find such link and identified 23 polymorphic changes in the *Sfrp1* gene in STR/Ort in comparison to C57BL/6 mice (42). Further immunohistochemical studies

revealed the expression of sFRP1 was reduced in articular chondrocytes of young STR/Ort mice, and this was confirmed by *in vitro* analysis of STR/Ort mesenchymal stem cells (42). Our meta-analysis of previous STR/Ort microarray data herein did not reveal any significant changes in the gene expression of *Dkk4, Sfrp1* or *Fgfr1*. Instead we provide evidence for the role of the Wnt inhibitor sclerostin, in OA development in STR/Ort mice. This is consistent with other studies which have shown expression of sclerostin in articular cartilage of various species including mice and sheep (44, 45). Whilst we observed no differences in serum sclerostin levels in STR/Ort mice, in humans an inverse relationship with radiographic knee OA severity has been observed, therefore implicating sclerostin as a potential biochemical marker (46).

This does not mean that the role of sclerostin in OA is uncontroversial. Recent studies examining OA in aged sclerostin-deficient mice and in rats treated with sclerostin neutralising antibodies following surgical induction (45), concluded that genetic ablation of sclerostin does not alter spontaneous age-dependent murine OA development, nor does pharmacological inhibition of sclerostin in a surgical model of OA (45). This therefore highlights the growing need for further investigations into the precise role of sclerostin in this debilitating disease. This may come from examining the role of its downstream pathways; here we were interested in identifying the role of one such novel pathway involving the SIBLING protein MEPE (34). MEPE has been shown to be a critical regulator of osteoblast and chondrocyte matrix mineralisation (12, 31, 47, 48). The similarity in differential patterns of MEPE and sclerostin expression we observe in STR/Ort mice implies a novel mechanism by which sclerostin may function in OA. Alterations in this pathway support the case for abnormal Wnt/β-catenin signalling, as has been reported in many OA studies including in the STR/Ort mouse (42).

In endochondral growth, the Wnt pathway is known to play an intricate and yet critical role with cartilage specific β-catenin deficient mice lacking typical growth plate zones and exhibiting delayed endochondral ossification (49). It has been shown that appropriate control of Wnt signalling in the growth plate is essential in regulating proliferation, alignment, differentiation, hypertrophy and replacement of calcified matrix with bone (38). During chondrogenesis, Wnt signalling is thought to influence the cell-cell and cell-extracellular matrix interactions upon which this fundamental process depends. Various Wnt factors, including Wnt3a, Wnt6, Wnt7a and many more, have been implicated as inhibitors of chondrogenesis, whilst a similar number, including Wnt5a and Wnt5b, have stimulatory roles (38). Similar ambiguity applies to the role of Wnt signalling in chondrocyte hypertrophic differentiation (38). In particular, it has been revealed that Wnt signalling regulates the parathyroid related peptide (PTHrP), Indian hedgehog (ihh) and transforming growth factor (TGF)-β feedback loop (49). Chondrocytes undergoing hypertrophy secrete Ihh which acts upon the proliferating chondrocytes to maintain their proliferative state and to restrict hypertrophy. Ihh also stimulates TGF-β production which in turn upregulates PTHrP. This acts on the pre-hypertrophic chondrocytes to prevent their further differentiation and thus Ihh production (50). This pathway has also been implicated in OA with increased Ihh expression reported in OA cartilage and selective Ihh deletion protecting against surgical OA progression (51, 52). Whilst this pathway was not examined here, it would be interesting to examine whether sclerostin/MEPE regulates the PTHrP/Ihh pathway in the STR/Ort mouse and whether this contributes to their OA pathology. Our data do however strengthen the intimacy of the relationship between molecular dysregulation of the Wnt/β-catenin pathway and endochondral growth defects.

Whether the results presented here are a unique feature of OA in the STR/Ort mouse or of OA in general is an interesting consideration and one that should be deliberated upon, as OA is being more widely accepted as a clinicopathological syndrome with multiple aetiologies. An elegant and comprehensive microarray study by Bateman and colleagues (53) in which gene expression profiling was performed in cartilage from wild-type mice with surgically induced OA (destabilisation of the medial meniscus, DMM) at 1, 2, and 6 weeks post-surgery, details the full list of differentially expressed genes between DMM-operated and sham-operated mice. Bateman et al., found that the marker of hypertrophy MMP13, was unchanged in DMM-operated mice in comparison to sham-operated mice at all stages post-surgery (53). This contrasts with our data in STR/Ort mice where elevated MMP13 expression levels were found prior to and during OA. In accord with our data, Bateman et al., found *Col10a1* to be significantly increased in DMM-operated mice in comparison to sham-operated mice at 1 and 2 weeks post-surgery and the matrix mineralisation regulators *Enpp1* and *Ank* to be increased at all time points post-surgery (53). The differential expression of these matrix and mineralisation markers at early time points post-surgery suggests their involvement in the initial OA processes in the DMM model. This is in accord with our data which shows similar changes prior to OA development in STR/Ort mice. Together this suggests a point of integration with these endochondral pathways at which the different OA subtypes, surgical (DMM) and natural (STR/Ort), may converge.

In this manuscript we highlight the MEPE/sclerostin pathway as a potential pathway for future investigation in OA research. Our data shows differential expression of MEPE and sclerostin in the STR/Ort mouse, along with the MEPE regulator PHEX and other members of the SIBLING family of proteins, DMP1 and osteopontin. In the DMM model, none of these genes of interest were changed (53). This therefore identifies this subset of genes as selective to the STR/Ort mouse OA and our identification of this molecular phenotype will

not only aid understanding into this diverse human condition, but also suggests that we may be able to identify specific gene signatures within particular at-risk human patients.

Our reports of an inherent endochondral defect in STR/Ort mice is further strengthened by our data acquired using synchrotron x-ray computed microtomography that shows premature growth plate closure in STR/Ort mice. This novel method for 3D quantification of bony bridging will no doubt advance understanding of growth plate closure mechanisms, and our unique data revealing the complex internal topographies of the growth plate cartilage layer in CBA and STR/Ort mice (Fig. 5B&D) may also yield more insights into the micro-mechanical environment of the cells in the growth plate (54).

This method has revealed that OA-prone STR/Ort and healthy CBA mice both display overt bone bridges prior to growth cessation. More specifically, spatial localisation of these bridges has shown greater clustering in STR/Ort mice, suggesting that their formation is driven by local factors, likely altered mechanical loading. The idea that STR/Ort mouse OA is driven by loading has certainly been supported by previous reports suggesting an association with medial patellar dislocation (55) and by those showing accelerated OA following mechanical joint loading (33). There have also been direct links made between growth plate function and mechanical loads, but whether this extends to the regulation of growth plate closure has yet to be explored (56). Regardless, it remains clear that these studies have, for the first time, shown that early growth plate closure is indicative of modified growth trajectory in STR/Ort mice and that growth ceases sooner in the lifetime of these OA-prone mice than in the closely-related parental CBA strain which shows healthy joint ageing. These data may also be interpreted as evidence that the OA in STR/Ort mice is secondary to a chondrodysplasia. Data indicating internal tibia rotation of the tibia of STR/Ort mice may offer support for such a contribution (57). However, the linked deployment of transient chondrocyte behaviours in the articular cartilage prior to overt OA development that we observe suggests, instead, that

this reflects an inherent chondrocyte defect. Nonetheless, the likelihood that such relationships with modified growth trajectories are also prevalent in human OA is yet to be defined and longitudinal studies examining associations between growth plate dynamics and OA development in human patients would be particularly informative in our understanding of OA in general.

Despite their predictable disease development with characteristics resembling those seen in human OA, including osteophytes, subchondral bone sclerosis, and synovial hyperplasia (19, 20, 58) the insights into the aetiology of OA provided by our data from this STR/Ort mouse model are limited. For instance, the aetiology of this OA is not yet known, despite extensive genetic and microarray analyses (39-41). As such, there are potential factors which may confound interpretation of our data, and it is vital to highlight that our findings define only the distinct pathophysiological mechanisms important in this subset of OA. Concurrent with this, the molecular phenotype we describe is unmodified in CBA mice suggesting that it is disease and indeed STR/Ort OA specific as opposed to any result of ageing. It is still however important to consider the molecular phenotype of this particular OA as it is becoming more widely accepted that generalisation of the OA disease is somewhat distracting and flawed in our pursuit of a disease modifying treatment (59).

In conclusion, our findings show that aberrant deployment of transient chondrocyte behaviours, consistent with re-initiation of endochondral processes, occurs in joints of spontaneously OA STR/Ort mice. Articular cartilage transcriptional profiles, labelling for endochondral markers and age-related growth plate dynamics, both in vivo and in vitro, support that OA in STR/Ort mice is allied to an inherent endochondral growth defect that is subject to regulation by the MEPE/sclerostin axis. Further investigation will determine whether this is an underpinning mechanism in some or all forms of pathological ossification in OA.

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Figure Legends

Figure 1. Measurements of uncalcified cartilage (white), calcified cartilage (grey) and subchondral bone (black) in the medial tibia of **(A)** CBA **(B)** STR/Ort mice at 8-10 weeks, 18-20 weeks, and 40+ weeks (10 measurements in >6 sections/mouse, n=4). Results are presented as % of the thickness of each zone measured from articular surface to subchondral bone. Immunolabelling of STR/Ort mice joints prior to OA onset for MMP13 in the **(C)** medial tibia **(D)** lateral tibia, and collagen type X in the **(E)** medial tibia **(F)** lateral tibia. Images are representative of 3 individual mice. GeXP multiplex RT-qPCR analysis of **(G)** *Enpp1* and **(H)** *Ank* mRNA in CBA and STR/Ort articular cartilage at 8-10weeks, 18-20weeks and 40+ weeks of age (n=3 joints/sample).

Figure 2. (A) Weights (g) of CBA and STR/Ort mice from 1-8 weeks of age (n>5). **(B)** Tibia length (mm) analysis in STR/Ort and age-matched CBA mice at 3-, 6- and 8-weeks of age (n=6). Measurements of digital images of E15 STR/Ort **(C)** and CBA **(D)** metatarsal bones in culture for 7 days. The growth rate **(E)** and **(F)** the percentage change in mineralisation zone length of metatarsal bones (n>8) **(G)** Growth plate zone lengths in 3-, 6-, and 8-week old STR/Ort and CBA mice (PZ – proliferative zone, HZ - hypertrophic zone) (10 measurements in >6 sections/mouse, n=4). Data are represented as mean ± SEM. *P<0.05, ***P<0.001. Immunohistochemical analysis of **(H-K)** Collagen type X in (H) 3 week CBA (I) 6 week CBA (J) 3 week STR/Ort (K) 6 week STR/Ort **(L-O)** MMP13 (L) 3 week CBA (M) 6 week CBA (N) 3 week STR/Ort (O) 6 week STR/Ort. Images are representative of 3 individual mice. Bar = 50μm. Data are represented as mean ± SEM. *P<0.05, **P<0.01, P<0.001***.

Figure 3. GeXP multiplex RT-qPCR analysis of **(A)** *Mepe* **(B)** *Phex* mRNA in CBA and STR/Ort articular cartilage at 8-10weeks, 18-20weeks and 40+ weeks (n=3 joints/sample).

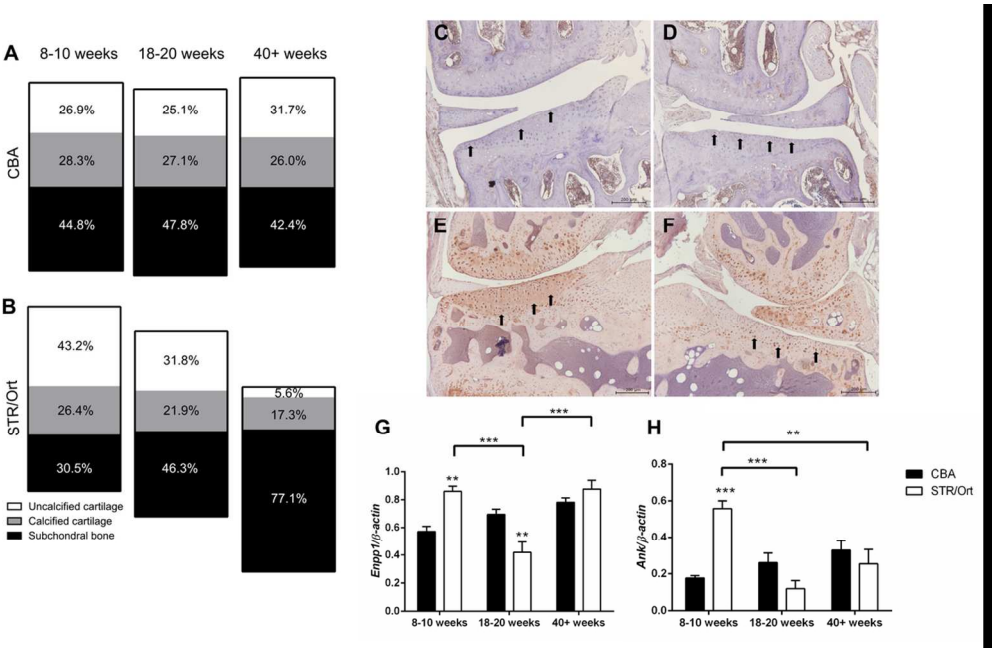
(C) Immunohistochemistry of MEPE in the lateral (non-affected) and medial (affected) tibia condyles in STR/Ort mice at stages prior to and at the onset of OA. **(D)** Immunohistochemistry of MEPE in the CBA tibia condyles. Images are representative of 3 individual mice. GeXP multiplex RT-qPCR analysis of **(E)** *Dmp1* **(F)** *Opn* mRNA in CBA and STR/Ort articular cartilage at 8-10weeks, 18-20weeks and 40+ weeks of age (n=3 joints/sample).

Figure 4. GeXP multiplex qPCR analysis of **(A)** *Sost* mRNA in CBA and STR/Ort articular cartilage at 8-10weeks, 18-20weeks and 40+ weeks of age (n=3 joints/sample). **(B)** Serum sclerostin in CBA and STR/Ort mice at the aforementioned ages (n=4). **(C)** Immunohistochemistry of sclerostin in the lateral (non-affected) and **(D)** medial (affected) tibia condyles in STR/Ort mice at stages prior to and at the onset of OA. Images are representative of 3 individual mice.

Figure 5. Development of a 3D quantification method for growth plate bridging. **(A)** 3D representation of the whole 40+ week STR/Ort joint. **(B)** 3D representation of the growth plate cartilage (yellow) underneath the tibial joint surface (grey, A) **(C)** 3D representation of bridges crossing the growth plate underneath the tibial joint (black crosses indicate bony bridges identified by an observer). Location and areal density of bridges across the growth plate projected on the tibial joint surface: **(D)** STR/Ort 8 weeks, **(E)** CBA 8 weeks, **(F)** STR/Ort 40+ weeks, **(G)** CBA 40+ weeks. Number of bridges per tibia at **(H)** 8 weeks **(I)** 40+ weeks (n=3). Lateral/medial and anterior/posterior segments were split in order to examine whether bridging is balanced during fusion. **(J)** Areal density, d. The areal density of bridges, d, is defined as the number of bridges per 256 μm x 256 μm window. Data are represented as mean of 3 animals ± S.E.M. $P<0.05$*, $P<0.01$**, $P<0.001$***.

Table 1: Gene ontology analysis of genes up-regulated in osteoarthritic articular cartilage in comparison to non-osteoarthritic articular cartilage. Genes showing >1.5 fold were analysed with DAVID software (n=491)

Term	%	P-value
Bone development	3.1	1.00E-05
Ossification	2.8	2.80E-05
Bone mineral formation	1.7	1.60E-04
Skeletal system development	4	3.40E-04
Cartilage development	1.7	6.10E-03



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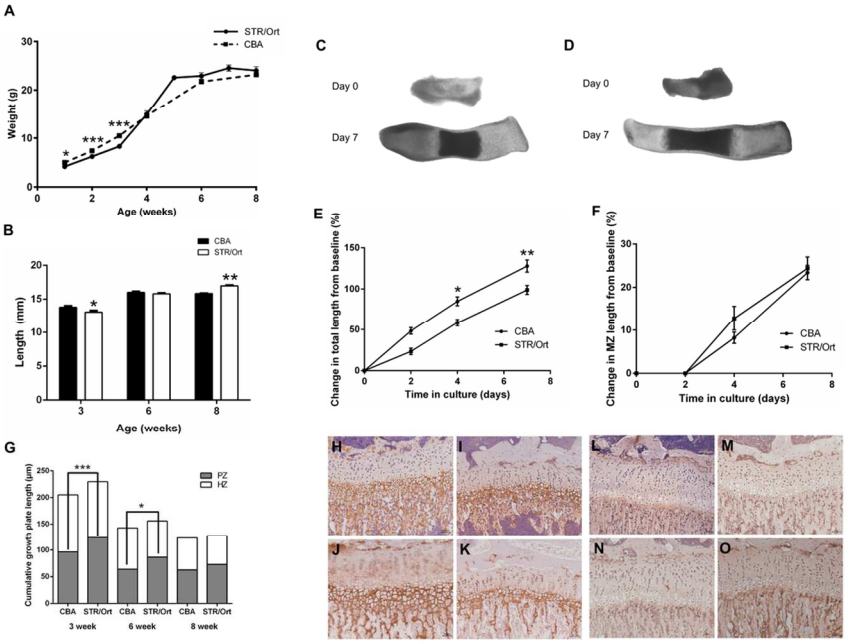

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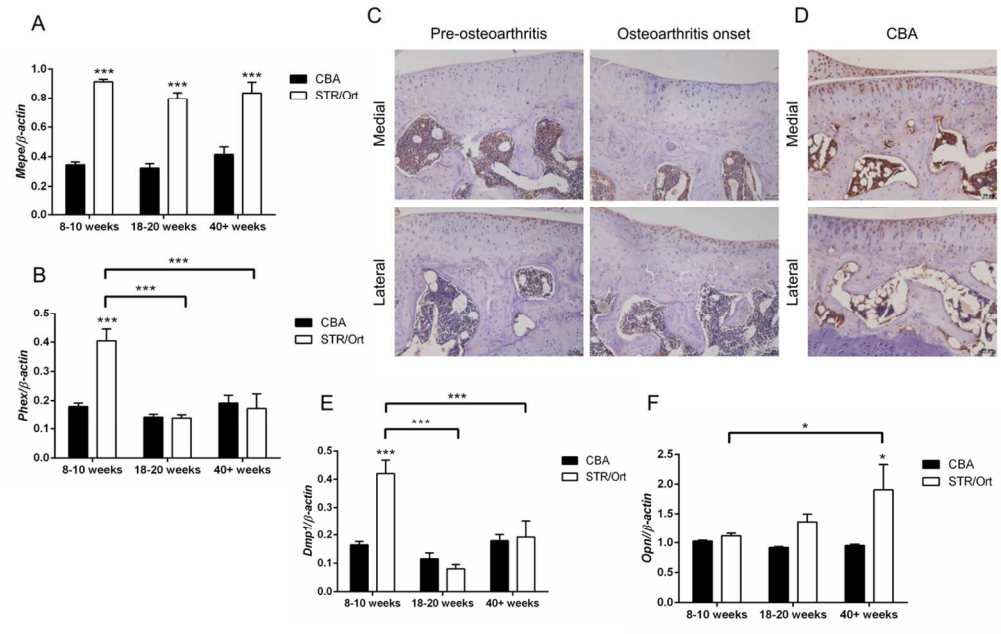

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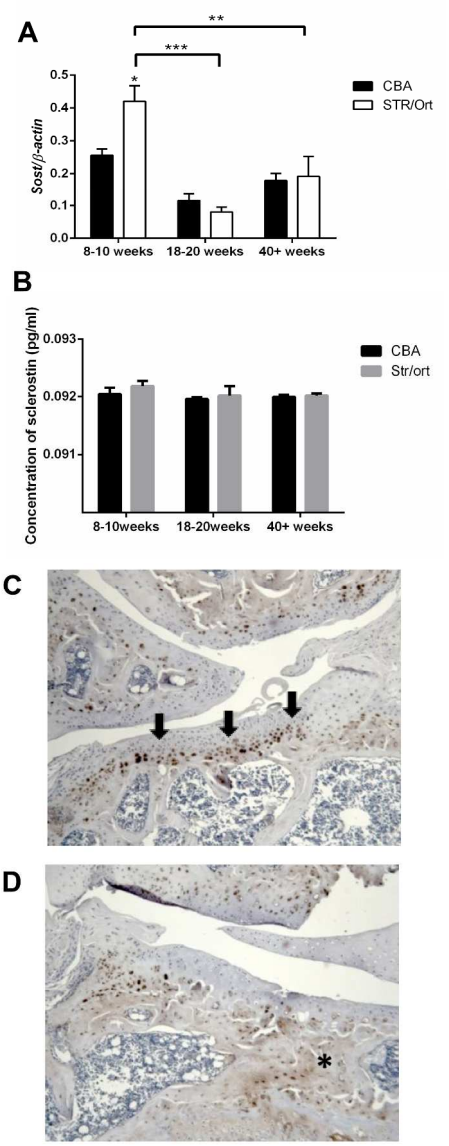

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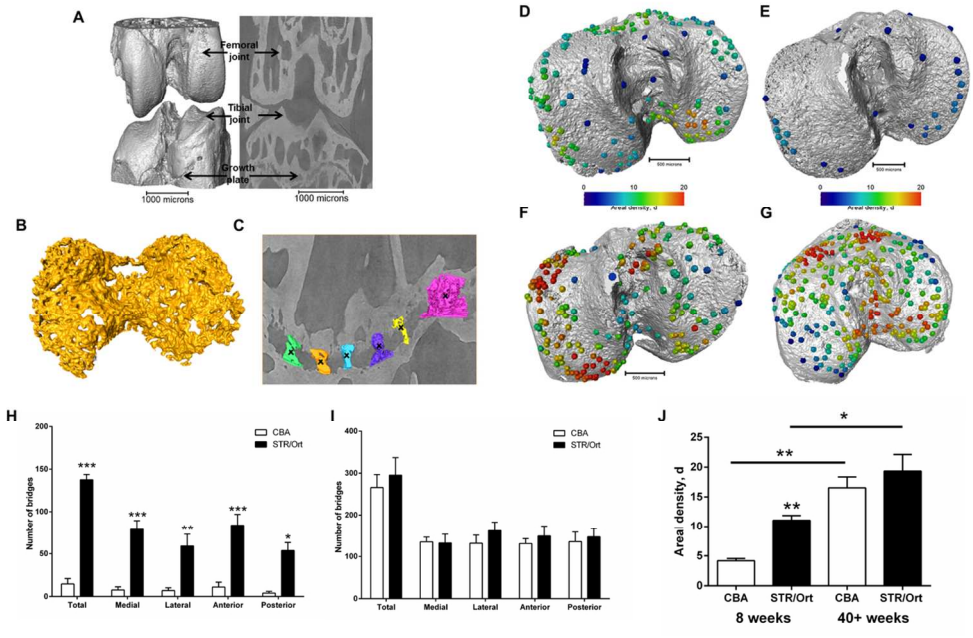

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