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Developmental Insights into Osteoarthritis Increase the Applicability of New Animal Models

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Abstract

Osteoarthritis (OA) is an increasingly common degenerative joint condition, estimated to affect more than 100 million people worldwide and more than 40% of people over 70 years of age. There is currently no pharmacological cure. The genetic contribution to osteoarthritis is estimated at between 39 and 60% in knee and hip OA respectively and a number of Genome Wide association studies have identified a number of alleles and loci that confer increased susceptibility to OA. In the last few years a number of key studies have demonstrated that genes known to play a role in the normal skeletal developmental processes of endochondral ossification, chondrocyte hypertrophy and joint formation are reactivated during osteoarthritis progression. Significantly, in some cases modification of these developmental genes can modulate osteoarthritis severity.

The high levels of genetic contribution make osteoarthritis an ideal target for both forward genetic screening efforts focused on identifying novel genes involved in chondrocyte differentiation and hypertrophy, which are likely to be involved in osteoarthritis progression and reverse genetics focused on modulation of genes involved in developmental skeletogenesis in later life. The functional study of genes associated with aging can be problematic in part because of the limited availability of animal models and the high costs associated with keeping aged animals. Therefore, the developmental aspect opens up the prospect of using chemical screens, such as those commonly performed in the zebrafish and frogs, to identify potential pharmaceutical modifiers of disease phenotypes. Here, we explore the current models for OA and compare genes, including mechanosensitive genes, involved in developmental joint formation and osteoarthritis.

Keywords

Osteoarthritis, Development, Chondrocyte hypertrophy, Endochondral ossification, Genetics, Joint morphogenesis, Animal models

A Brief Introduction to Osteoarthritis

Osteoarthritis is the most common form of arthritis, and represents a major cause of disabilities in the western world [1]. Current treatment for OA includes pain management and in more severe cases surgical replacement of the joint, known as total joint replacement (TJR) is performed [2]. However, a pharmaceutical cure to reverse the damage caused by OA has not yet been found.

Osteoarthritis is a disease of the whole joint, involving changes to many of the joint tissues. These include significant loss of the articular cartilage, which normally covers the bone surfaces, through breakdown of the structural proteins of the cartilage matrix [3]. This loss of cartilage is twinned with changes to the underlying bone, primarily thickening of the subchondral bone and the formation of osteophytes, which can protrude into the joint causing stiffness [4] and an inflammatory response mediated principally by proinflammatory cytokines [5].

Much is known about the environmental factors that increase OA susceptibility, principally age, sports injury, obesity and diet [6,7]. However, in recent years there has been a shift in attention from studies focused primarily on these environmental effects on cartilage destruction, towards the genetic factors regulating disease onset. It has been estimated from a number of twin studies that the genetic contribution to OA lies between 39 and 60% in hip and knee OA, respectively [8,9]. One aspect that makes large scale genetic epidemiology studies of OA somewhat difficult is the use of an unambiguous disease phenotype, as the disease is highly variable ranging from symptom-free radiographic disease to painful end stage OA requiring joint replacement surgery (reviewed in [10]). The largest and most extensive Genome Wide Association Study (GWAS) published to date was the arcOGEN study, which included 7400 OA cases and identified a number of loci [11]. Since then many other association studies have been published (with varying power) which have identified further risk alleles in a number of other genes [12-14]. Genes involved in early stages of disease pathogenesis are likely to present the best targets for development of new therapies; therefore their identification is of paramount importance.

It has long been appreciated that one of the most damaging aspects of OA is the body's own vigorous attempt to repair the damage to articular cartilage [15]. Cartilage repair recapitulates a number of developmental pathways, including those involved in the normal embryonic replacement of cartilage with bone during endochondral ossification [16,17]. Of note, there is an increasing amount of evidence that aberrant reactivation of embryonic skeletal processes might play a contributory factor in disease progression. In this review we show recent evidence that reactivation of pathways relevant to skeletal development and joint morphogenesis contribute to OA risk, and discuss how this might open up new avenues for therapeutic intervention. Given that animal models of OA are of great value in addressing these issues, we also summarize the advantages and limitations of the different models.

Table 1: Comparative genetics of endochondral ossification/osteoarthritis.

Gene or pathway	Role in Endochondral Ossification	Implication in OA	Selected references
Col10a1 (type X collagen)	Secreted by hypertrophic chondrocytes	Increase in type X collagen as chondrocytes lose their 'articular cartilage identity' and become hypertrophic and calcified	[40,41]
PTHrP	Secreted by cells in the perichondrium, acts to keep chondrocytes proliferating and to prevent differentiation	Increased production of PTHrP by synovial fibroblasts in human OA, although decreased levels of PTH1R (its receptor) in rabbit OA	[42 and reviewed in 43]
Runx2	Stimulates chondrocyte hypertrophy and osteoblast differentiation	Upregulated in cartilage during early stages of OA	[44-46]
Hif2a	Transcriptionally activates *Runx2*	Highly expressed in OA cartilage, overexpression leads to cartilage destruction	[17,47]
Mmp-13 (Collagenase 3)	Required in the growth plate to prevent premature hypertrophy	Increased expression in OA cartilage leading to catabolism of Type II cartilage	[48,49]
Sox9	Required for expression of a number of cartilage specific genes, including type II collagen	Levels are decreased in OA. Reintroduction of *Sox9* leads to increased synthesis of cartilage matrix	[50,51]
Gdf5	Required for correct cartilage development and joint placement	SNPs in *Gdf5* associated with disease onset in multiple cohorts	[52,53]
Frzb	Expressed during chondrocyte condensation and promotes chondrocyte maturation	SNPs in *Frzb* associated with OA in some cohorts but not in others	[54-57]
Hedgehog signalling	Indian Hedgehog is produced by prehypertrophic chondrocytes and regulates both hypertrophic differentiation and chondrocyte proliferation	Increased hedgehog (Hh) signalling levels lead to increased OA severity, modulation of Hh signalling can reduce OA severity	[18,58]
Bmp and TGFbeta	Antagonistic interactions in the growth plate in the control of chondrocyte proliferation and hypertrophy	Implicated in osteophyte formation	[59,60]
Sulf2	Endochondral ossification, mutations lead to vertebral fusions changes and growth plate dynamics	Increased expression in osteoarthritic cartilage	[12,61]
COL11A1	Important for normal differentiation and spatial organization of growth plate chondrocytes	Reduced expression in OA cartilage. Heterozygotes show higher levels of MMP13 and reduced tensile stiffness in articular cartilage. COL11A1 significantly associated with OA.	[14,62,63]
Chondroitin sulfatases	Designate early stages of stem/progenitor cell differentiation	CHST11 identified as an OA susceptibility gene via GWAS	[11,64]
Syndecan 4	Expressed during chondrocyte differentiation. Its deficiency inhibits chondrocyte proliferation	Mediates matrix degradation by controlling ADAMTS-5 through direct interaction with the protease and through regulating MAPK-dependent synthesis of MMP-3.	[64]
Erg	Transcription factor induced by *Gdf5*. Involved in maintaining chondrocyte differentiation and inhibiting maturation to hypertrophy	Conditional *Erg* mouse mutants at the joint are more susceptible to OA defects in aging and following surgically-induced OA	[66-68]

Endochondral ossification and osteoarthritis

Recently, a number of groups have identified a set of genes with a central role in OA progression and pathogenesis (summarised in Table 1), and these genes share well-characterised roles in developmental endochondral ossification; spanning a number of types, from signalling proteins like Gdf5, hormones such as PTHrP, transcription factors such as Sox9, ERG and structural matrix proteins such as Type X collagen, amongst others. Together these reports suggest the involvement of a number of important and well known signalling pathways such as the Hedgehog, Wnt, BMP and TGFbeta pathways in influencing OA susceptibility and disease progression. Many of the genes (summarised in Table 1 and in Figure 1) appear to exert their effects in the disease by promoting the transition from a quiescent state to the re-entry of the chondrocyte into the cell cycle and differentiation to a hypertrophic state, similar to their role in the control of developmental endochondral ossification. A number of these genes appear to play a role in the 'early' stages of OA, raising the tantalising prospect that amongst the proteins controlling the entry to chondrocyte hypertrophy may be pharmaceutical drug targets that could be manipulated to limit OA progression. Supporting this idea, modulation of some of these 'developmental' genes has been demonstrated to decrease the severity of OA *in vivo* [18]. Indeed many new OA drugs currently in preclinical studies or in early stages of human trials modulate the developmental pathways discussed above; reviewed in [2].

This re-employment of developmental transcription factors and return to the 'fetal gene program' is a mechanism common to number of pathological states of the heart [19,20] and the brain [21].

Traditional models of OA: *In vitro* models, stem cells, tissue engineering and *in vivo* models

Currently the majority of research focused on OA is performed using either *in vitro* models (such as primary cultures of normal and osteoarthritic chondrocytes), or using various rodent models, predominantly in mouse, rat or guinea pig.

Since chondrocytes constitute the main cell type in a normal adult joint, cell cultures of chondrocytes are commonly used. These can either be monolayer cultures [22,23], 3D cultures [24], or cultures on artificial substrates [25]. They can be derived either from chondrocyte cells lines or cultured from stem cells such as mesenchymal stem cells (MSCs), which are often taken from patients carrying specific risk alleles to test the effects on chondrogenesis [26]. More recently, induced pluripotent stem cells have been used [27]. Additionally, explants from laboratory animals, such as mouse ribcage explants or calvaria have been used to study the effects of known OA-inducing stimuli (e.g. levels of mechanical stress, inflammation) *in vitro* [28]. Culture systems and cartilage tissue engineering are reviewed in detail elsewhere [29] and will not be covered further here.

Many animal models traditionally used to study OA have focused on destruction of the cartilage in weight bearing joints. This is usually achieved either through surgery by disrupting the medial meniscus, by destabilising the whole joint by anterior cruciate ligament transection, or by destruction of cartilage through chemical means, such as injection of collagenases or papain [30]. The extent of OA progression is then characterised by histomorphological studies, applying a set of defined criteria, which has been set out for each animal species by OARSI (Osteoarthritis Research Society International) to allow closer correlation between the results obtained from animal studies to human clinical characteristics [31-34].

One problem with surgical models, while undoubtedly of value, is that by focusing mainly on cartilage destruction they may not give much insight into subtle changes occurring during the early stages of OA and often do not fully recapitulate the transcriptional changes which are observed between human normal and osteoarthritic
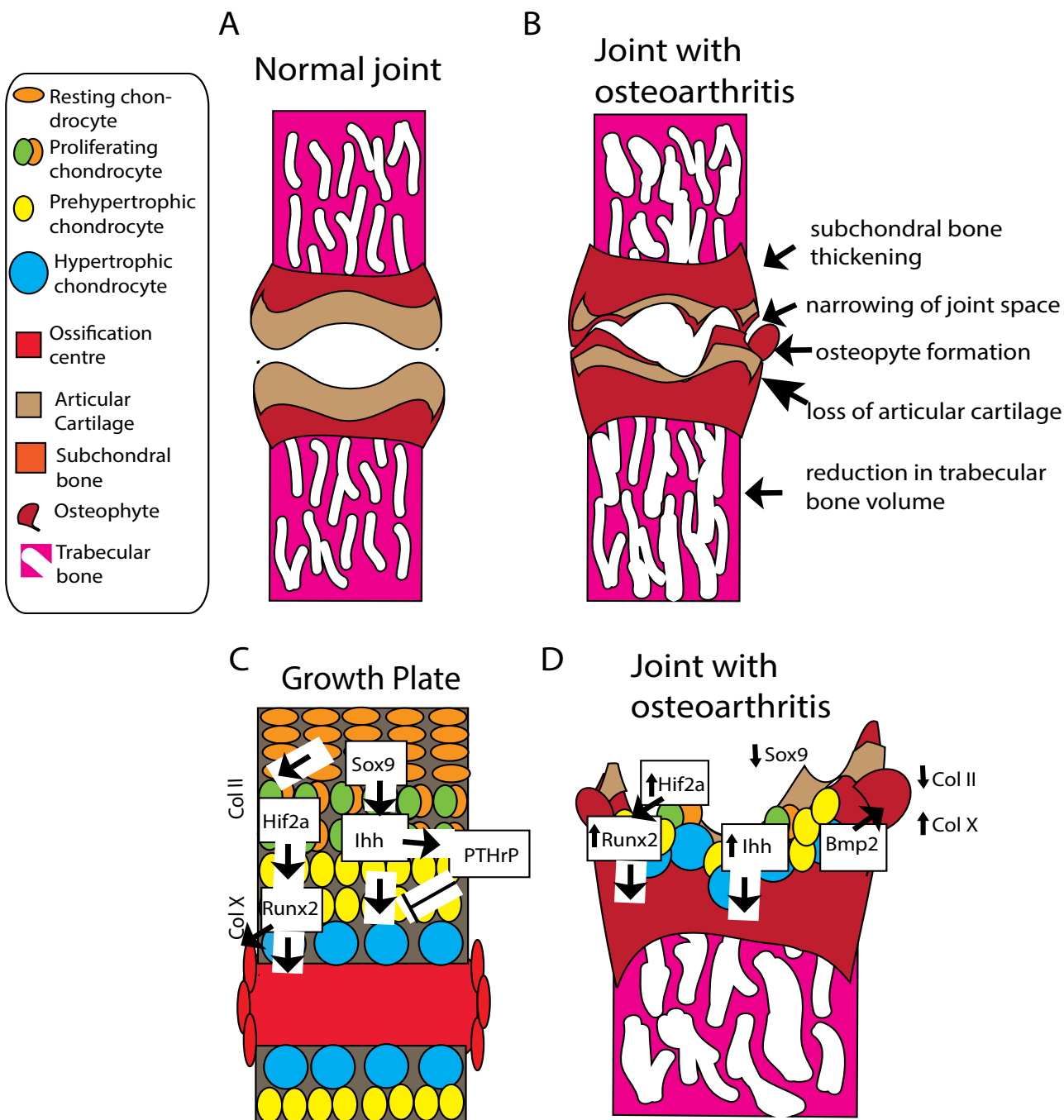
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Figure 1: Osteoarthritis phenotype vs. endochondral ossification –changes and markers common to both processes.

A: Normal joints feature intact articular cartilage covering subchondral and trabecular bone; B: Osteoarthritic joint features include thinned articular cartilage, exposed subchondral bone, osteophytes formation and trabecular thinning; C: Schematic of a cartilage growth plate undergoing endochondral ossification from the resting chondrocytes to the ossification centre. Cell types/stages are referenced in the key. Molecular regulators and markers of stages of endochondral ossification. Activation arrowheads and inhibitory arrows represent diffusion of signals to site of action; D: Molecular changes related to osteoarthritis. Large arrows represent diffusion of signals to site of action. Small arrows represent up and down regulation of expression in osteoarthritis.

cartilage [4]. They, therefore, risk missing some of the changes occurring in the earliest and potentially most treatable stages of OA.

In addition to surgical models, an increasing number of genetically modified mouse models are established, in which genes identified from *in vitro*, human genetic, or other animal research models are knocked out in a targeted fashion, then analysed for joint phenotypes and OA. These include such mouse knockouts as Type II and Type IX collagen [35,36], COMP (Tsp5) [37], CCN3/Nov [38] and Ephrin B2 [39]. While these rodent mutants allow OA onset to be studied, it remains difficult to follow disease progression at a cellular level, as dynamic imaging of the skeleton at cellular resolution is impossible. As such imaging tends to focus on radiography and MRI in live animals twinned with post mortem histology. Therefore, ideally new models will combine the ability to identify novel genes with functional
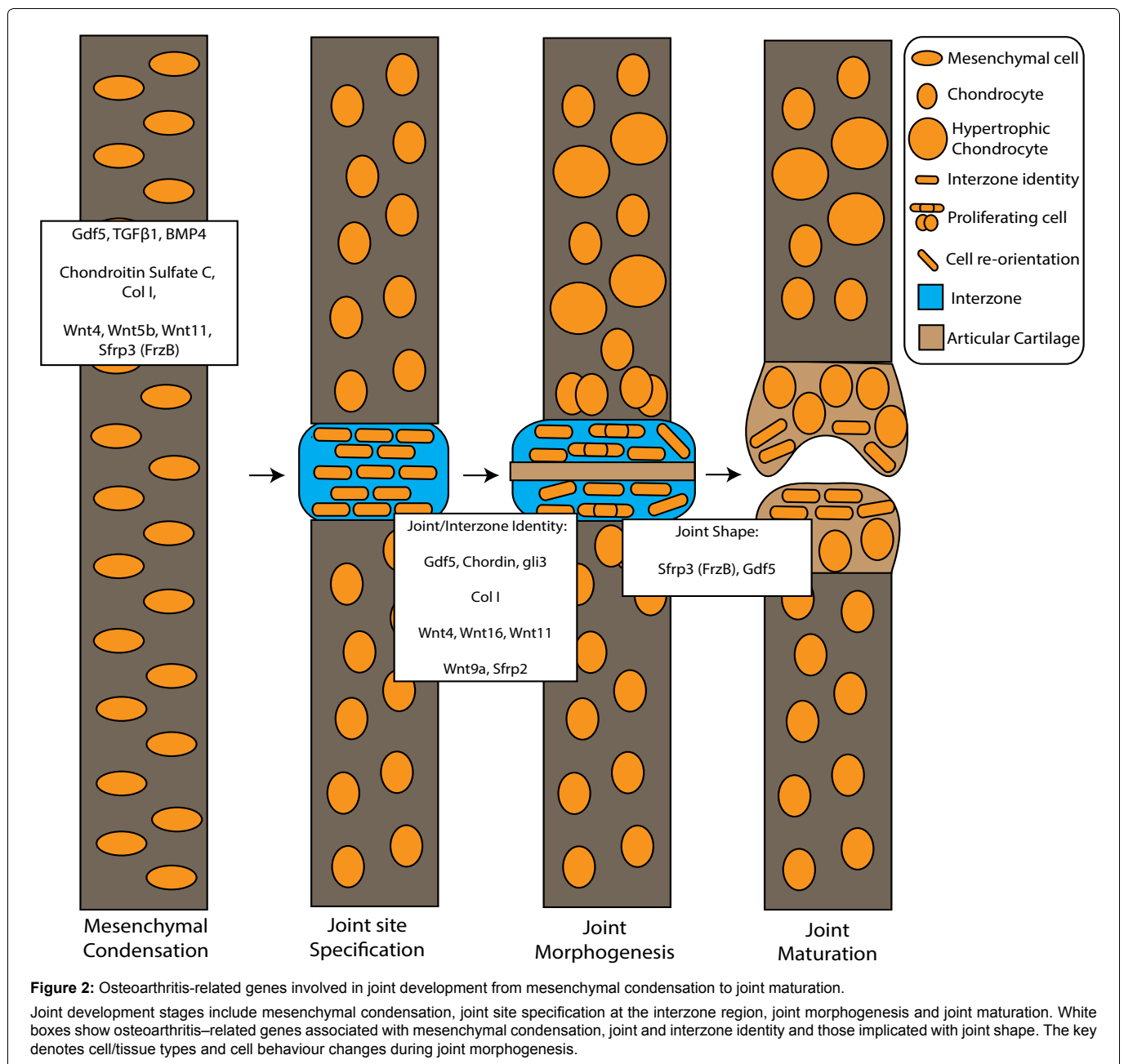
genomics and biochemical data and on the mechanisms by which these genes exert their effects and ideally the ability to use dynamic imaging. The developmental angle to the disease raises the tantalising prospect of using additional models to complement those currently used for OA research such as zebrafish, medaka and xenopus.

The uncovering of a 'developmental' aspect of OA should facilitate use of traditional developmental models such as mice and zebrafish, which are ideally suited for functional genetic studies. These include phenotype-driven forward genetic screens to identify novel genes involved in OA, and offer the advantage of an unbiased approach to identifying new genes through randomly generated mutagenesis approaches. ENU-based (N-ethyl-N-nitroso urea) random mutagenesis forward genetic screens in mouse are increasingly being used [40]. Through one such screen a new allele

of *Gdf5* was identified, the mutant displaying joint malformation and early onset of OA [69]. Finding back a gene already implicated in OA pathogenesis provides a proof of principal that forward genetic screens can identify OA susceptibility genes. Forward genetic screens have been used successfully for 20 years in zebrafish [70,71]. They have only more recently been applied to skeletal research, but are already beginning to identify genes involved in skeletogenesis [72-76]. The zebrafish mutant's *dackel* (*dak*) and *pinscher* (*pic*), have identified genes involved in the human bone disease Hereditary Multiple Exostoses (HME) [77], and further insight into osteogenesis imperfecta has come from zebrafish [78]. Furthermore, ageing zebrafish has been shown to develop skeletal phenotypes that strongly resemble human osteoarthritis [79]. Therefore, while zebrafish form fewer synovial joints (they only have synovial joints in the craniofacial skeleton), they have the potential to complement existing small animal models in understanding the genetic link between skeletogenesis and osteoarthritis.

While forward genetic screening provides the potential of identification of new genes related to disease, reverse genetics is a useful tool for functional studies of known genes, for example those identified from GWAs studies that require further functional analyses. Site-specific genome editing has become easier and suitable over the years. Traditional technology of Cre/loxp highly used in mice

has enriched the knowledge on bone formation and also OA with the generation of conditional knockout animal models [80] but also transgenic lines for lineage tracing in mice and live zebrafish [81-85]. Lineage tracing experiments have contributed to the identification of different populations of osteoprogenitor cells in hypertrophic cartilage and origins of heterotopic ossifications, helping to understand the "developmental" aspect of OA and reasons why cartilage elements might respond differentially to treatments [86]. New technologies of genome editing have emerged, ZFN (Zinc Finger Nucleases) and TALEN (Transcription Activator-Like Effector Nuclease) [87] have been used elsewhere for functional tests in a variety of model organisms, for example frame shift alleles for *kif6* were generated in zebrafish through TALEN and confirmed its role in development of spinal scoliosis [88]. More recently the advent of Crispr (Clustered Regularly- Interspaced Short Palindromic Repeats) has revolutionized the field providing a powerful facility over other methods to edit the genome. The system has been used successfully in a diversity of organisms including mice, rat, zebrafish, other vertebrates and cell lines (stable, iPSCs and primary) to either generate knockouts, tissue specific gene disruption, deletion of regulatory regions or correction of single base pair mutations [89-93]. The technology has yet to be extensively used for the study of bone formation and related diseases, however Crispr/Cas9 has already contributed to attribute function to upstream regions of Mmp13 (Matrix metalloproteinase 13) that

Figure 2: Osteoarthritis-related genes involved in joint development from mesenchymal condensation to joint maturation.
Joint development stages include mesenchymal condensation, joint site specification at the interzone region, joint morphogenesis and joint maturation. White boxes show osteoarthritis–related genes associated with mesenchymal condensation, joint and interzone identity and those implicated with joint shape. The key denotes cell/tissue types and cell behaviour changes during joint morphogenesis.

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when deleted result in complete loss of basal transcript activity [94]. Crispr has also been used to generate stable rat chondrocyte Aggrecan knockout cell lines [95], and to study Lrp5 during cell migration and shaping of the craniofacial skeleton [96]. The possibility of correction of the causative mutations may in future be applied to treatment of a spectrum of diseases including OA. Furthermore, multi target Crispr/cas9 combined with faster platforms such as zebrafish has the potential to validate candidate genes previously identified through GWAS and elucidate the importance of groups of altered genes for the cause of complex diseases as OA.

The generation of an increasing number of transgenic lines marking chondrocytes, osteoblasts and osteoclasts via promoters such as *osterix* [97-99], *osteocalcin* and *runx2* [100,101], or *col2a1 and col10a1* [102,103] along with those reporting on signalling pathway activity [104] will further benefit fish skeletal research. The increasing use of zebrafish and *Xenopus* for skeletal research will likely include screening of chemical libraries [105,106]. This could be through classical screening techniques on the many fish skeletal transgenic lines. Additionally, *ex vivo* approaches such as screening for skeletal compounds on scales removed from transgenic zebrafish expressing an *sp7 (osterix)* luciferase transgene have already identified a number of novel osteogenic compounds [107].

Joint shape, foetal movement and biomechanics

Loading, wear and tear and biomechanics have long been understood to play a role in OA pathogenesis, and it may be that there is a developmental aspect to the role of joint biomechanics in OA pathogenesis (see Figure 2 for genes in common between both joint development and OA). We now appreciate that the mechanical environment experienced during early development is important for normal skeletal development and correct joint morphogenesis. There are a number of conditions and syndromes for which abnormal or reduced movement are believed to be causal; including the relatively common developmental dysplasia of the hip (DDH sometimes also known as congenital dysplasia of the hip or CDH), which affects between 1.3 per 1000 births [108], arthrogryposis affecting 1:4000 births [109] and fetal akinesia deformation sequence (FADS) in which fetal movements are completely lacking, leading to joint contractures, craniofacial deformities and growth restriction, which affects 1:15000 births [110,111].

Genes and joint shape

From developmental studies in animal models, it is evident that mechanical forces from muscle activity are required for accurate joint formation [112,113]. The identification of mechanosensitive genes and the roles they play at the developing joint are starting to be elucidated from regulation of interzone cell differentiation, aiding cavitation and promoting chondrogenesis and morphogenesis [114-116]. The Wnt pathway has been implicated as mechanosensitive with 34 Wnt genes differentially expressed between control and muscle-less Splotch mutant mice in the developing humerus, [117]. Beta-catenin, which acts downstream of the canonical Wnt pathway, also showed reduced activation at the joint site in mice Splotch mutants and local loss of Wnt9a expression, [118]. Interestingly, the Wnt pathway has been implicated as a major player in OA progression [119]. As a result of muscle paralysis during early development many genes relevant to OA, such as Col2a1, BMP2, and PTHrP, show altered expression at the developing knee joint, [116]. Jaw movement constraint in ex utero mice resulted in reduction of Ihh and PTHrP expression [114]. Gdf5, which is normally down regulated prior to cavitation was reduced in cultured articular surface cells following the application of mechanical strain [115]. Immobilisation lead to elevation of Gdf5 in fused joints, reflecting the increase in numbers of chondrocytes at the joint site that express Gdf5 for chondrogenesis. Splotch mice, however, displayed an initial maintenance, and then decreased expression of Gdf5 at the interzone as a result of the absence of mechanical strain, [118]. The identification of genes that are misexpressed at the joint in animal models following reduced muscle activity can lead to further opportunities to identify the role that these mechanosensitive genes play in OA.

Locating regions that are under strain during development can help pin point those cells which are most likely to be responding to biomechanical cues to identify novel mechanosensitive genes and pathways. Finite Element (FE) modelling is an engineering technique that can computationally calculate and map the magnitude and location of strains acting on a 3d model [120]. FE analysis (FEA) has been exploited to model strains acting on skeletal elements during bone formation [121], the pattern of strains present during chick knee joint and zebrafish jaw joint morphogenesis [122,123]. Areas of high strain have been found to occur at regions that normally undergo cell behaviour changes such as proliferation or cell reorientation that impact on joint shape and that are affected in immobilised models [122,123]. FE modelling can be used to predict changes to strain patterns as a result of reduced muscle activity and can be used to identify mechanosensitive genes, such as *ColX* and *Ihh* involved in bone formation [124]. Further studies could help identify more mechanosensitive genes involved in joint morphogenesis in development that play a role during OA.

Future of OA research and potential for use of new models/ screens

As outlined above, recent genetic findings have identified a number of osteoarthritis susceptibility genes which also regulate Endochondral ossification and joint morphogenesis during skeletal morphogenesis; variation in these genes seems likely to be involved both in the onset of osteoarthritis and subsequent progression towards clinical outcomes. This developmental focus will allow a number of powerful models currently used to study developmental genetics and cell behaviour including chick, xenopus and zebrafish to be brought into osteoarthritis research, to complement existing research tools. These include forward genetic screens, reverse genetics with CRISPr and high-throughput *in vivo* chemical screens, to identify and functionally characterise new targets for pharmaceutical intervention and novel chemical modulators of pathways. This heralds an exciting period for OA research and hopefully new therapies for this disease will soon follow.

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