JAWS coordinates chondrogenesis and synovial joint positioning

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Properly positioned synovial joints are crucial to coordinated skeletal movement. Despite their importance for skeletal development and function, the molecular mechanisms that underlie joint positioning are not well understood. We show that mice carrying an insertional mutation in a previously uncharacterized gene, which we have named JAWS (joints abnormal with splitting), die perinatally with striking skeletal defects, including ectopic interphalangeal joints. These ectopic joints develop along the longitudinal axis and persist at birth, suggesting that JAWS is uniquely required for the orientation and consequent positioning of interphalangeal joints within the endochondral skeleton. Jaws mutant mice also exhibit severe chondrodysplasia characterized by delayed and disorganized maturation of growth plate chondrocytes, together with impaired chondroitin sulfation and abnormal metabolism of the chondroitin sulfate proteoglycan aggrecan. Our findings identify JAWS as a key regulator of chondrogenesis and synovial joint positioning required for the restriction of joint formation to discrete stereotyped locations in the embryonic skeleton.

KEY WORDS: Chondrogenesis, Synovial joints, Interzone, Gdf5, Chondroitin sulfate, Extracellular matrix, Mouse embryo

INTRODUCTION

Synovial joints segment the developing cartilage template into individual skeletal elements while providing flexibility and structural stability between these elements. Histologically, the earliest sign of synovial joint formation is the appearance of the interzone (Mitrovic, 1977), which occurs at approximately embryonic day (E) 12.5-13.5 in mouse digits. Several signaling pathways are implicated in the molecular specification of the joint interzone, including those downstream of the secreted proteins growth differentiation factor 5 (Gdf5) and Wnt9a (Hartmann and Tabin, 2001; Pacifici et al., 2005; Khan et al., 2007). By contrast, the molecular mechanisms that control spatial positioning of the joint interzone are not well understood. Here we identify a previously uncharacterized protein, which we have named JAWS (joints abnormal with splitting), as a novel and essential coordinator of cartilage formation and synovial joint positioning.

MATERIALS AND METHODS

Animals

The KST245 mouse embryonic stem cell line, containing an insertion of the pGT1Tmpls gene trap vector in the Jaws/Impad1 locus, was isolated and characterized as described (Mitchell et al., 2001). Jaws F1 heterozygotes were backcrossed to C57BL/6 mice for six generations before intercrossing. Genotyping was performed by X-gal staining of yolk sacs and/or tail biopsies, or by RT-PCR using primers flanking the insertion site (sequences available upon request).

Expression studies

Jaws transcripts were detected by northern blotting of Trizol-extracted total RNA, probed with a cDNA fragment spanning nucleotides 726-1158 of the transcripts were detected by northern blotting of Trizol-extracted total biopsies, or by RT-PCR using primers flanking the insertion site (sequences characterized as described (Mitchell et al., 2001). The KST245 mouse embryonic stem cell line, containing an insertion of the JAWS protein.

lysates (60 μg), a polyclonal antiserum was raised against the peptide epitope N-CRESNLHESKGKTREGADD-C corresponding to residues 83-102 of the JAWS protein.

Histology, in situ hybridization and immunohistochemistry

Alcian Blue and Alizarin Red staining of skeletal preparations and histological sections, X-gal staining of embryos, and in situ hybridization were performed as described (Nagy, 2003). To enhance contrast against the Hoechst counterstain, the radioactive ISH images were photographed in bright-field and then pseudo-colored red using the ‘Colorize’ option within the ‘Hue/Saturation’ command in Adobe Photoshop 7.0. Immunohistochemistry on paraffin sections was carried out after antigen retrieval, using Alexa Fluor-conjugated secondary antibodies and the following primary antibodies: mouse aggrecan 12/21/1-C-6 and link protein 9/30/8-A-4 (Developmental Studies Hybridoma Bank), mouse β-catenin (BD Biosciences), mouse chondroitin sulfate CS-56 (Sigma-Aldrich), rabbit collagen II (Abcam) and rat CD44S (Chemicon). Apoptotic cells were labeled using the In Situ Cell Death Detection kit (Roche).

Fluorophore-assisted carbohydrate electrophoresis (FACE)

The composition and amount of chondroitin sulfate and heparan sulfate were analyzed as described (Plaas et al., 2001; Gao et al., 2004). Values for total CS, HS and HA were normalized to wet tissue weight. FACE data were analyzed by a two-tailed Student’s t-test, and values were considered statistically significant at P<0.05.

Protein extraction and aggrecan immunoblotting

Successive nondissociative extractions (0.15 M NaCl, then 0.15 M NaCl + 0.5% CHAPS) of E14.5 limb tissue were followed by one dissociative extraction (4 M guanidinium HCl + 0.5% CHAPS) to solubilize matrix-associated proteins. Loadings for SDS-PAGE were normalized to wet tissue weight, and immunoblots were processed with polyclonal CDAGWL antibodies directed against the N-terminal G1 domain of aggrecan. Blots were exposed to film simultaneously and developed identically.

RESULTS AND DISCUSSION

JAWS is required for the development of the endochondral skeleton

Jaws (also known as Impad1 – Mouse Genome Informatics) was identified in an insertional mutagenesis screen for genes encoding secreted and transmembrane proteins essential for mammalian development (Mitchell et al., 2001). The Jaws/Impad1 locus encodes a predicted protein of ~39 kDa with an N-terminal hydrophobic domain and a consensus inositol monophosphatase


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catalytic motif. Insertion of the gene-trap vector into the third of four introns fuses the first 213 amino acids of the JAWS protein in-frame with a βgeo reporter, resulting in less than 0.2% expression of wild-type (WT) transcripts and negligible expression of the endogenous protein (see Fig. S1 in the supplementary material). Together with the observation that βgeo fusion proteins generated by this ‘secretory trap’ accumulate in cytoplasmic inclusion bodies (Skarnes et al., 1995; Mitchell et al., 2001), these data suggest that the JAWS insertion creates a null or strongly hypomorphic allele.

JAWS mRNA and protein expression is widespread during embryogenesis and early postnatal life (Fig. 1A; see Fig. S1 in the supplementary material). In the developing skeleton, X-gal staining demonstrated β-galactosidase activity throughout the cartilage growth plate and in primary ossification centers, with prominent staining in chondrogenic condensations, perichondrium and articular chondrocytes (Fig. 1A; data not shown).

Mice carrying one copy of the JAWS insertional mutation (JAWS+/–) appeared phenotypically indistinguishable from wild-type littermates. By contrast, mice homozygous for the insertion (JAWS–/–) died perinatally with cleft secondary palate (see Fig. S2 in the supplementary material). JAWS–/– neonates exhibited severe dwarfism with stunted limbs, hypoplastic ribs and rounder shortened craniofacies (Fig. 1B,C). The cranial vault and clavicles, which form without a cartilage intermediate, were largely unaffected (Fig. 1C; data not shown). These results reveal that the skeletal defects in JAWS–/– embryos were restricted to elements formed through endochondral ossification. Defects in chondrogenesis were apparent by E12.5 in JAWS–/– embryos (see Fig. S3 in the supplementary material), despite robust expression of the chondrogenic markers Sox9, Sox5 and Col2a1 (see Fig. S4 in the supplementary material) (Akiyama et al., 2002). This latter observation suggests that JAWS is dispensable for the formation of prechondrogenic condensations from limb bud mesenchyme.

In the embryonic growth plate, chondrocytes align in a pseudocolumnar arrangement along the longitudinal axis according to their differentiation status, which is reflected in the demarcation of four histologically and molecularly distinct chondrocytic zones: resting, proliferative, prehypertrophic and hypertrophic (Kronenberg, 2003). Histologically, JAWS–/– chondrocytes lacked the pseudocolumnar organization of their wild-type counterparts (Fig. 1D). Mutant growth plates were hypocellular, with discontinuities in Alcian Blue staining revealing regions devoid of extracellular matrix (ECM). After E15.5, ectopic chondrocytes were regularly seen impinging on the primary ossification center in JAWS–/– embryos (Fig. 1D, arrow).

JAWS deficiency delays chondrocyte maturation and disrupts long-range Ihh signaling in the growth plate

To analyze growth plate maturation in molecular detail, we examined markers of chondrocyte differentiation by in situ hybridization at E14.5 (Fig. 2A). Expression of the proliferating chondrocyte marker Col2a1 was largely excluded from the central Col10a1-expressing hypertrophic zone in wild-type long bones, whereas in JAWS–/– embryos Col2a1 expression partially overlapped this Col10a1 domain. During hypertrophic differentiation, maturing chondrocytes abruptly switch from Col2a1 to Col10a1 expression; thus, this overlap in the two domains suggests that many JAWS–/– chondrocytes were in a delayed intermediate state of already expressing Col10a1 without yet having downregulated Col2a1 expression (St-Jacques et al., 1999). Similarly, wild-type expression of Ihh or Pthrp appeared in symmetrical domains of prehypertrophic chondrocytes flanking the central Col10a1 domain (Kronenberg, 2003); however, one continuous domain of expression overlapping that of Col10a1 was seen for each gene in JAWS–/– embryos. Altered expression of two downstream targets of Ihh signaling, Ptc1 and Pthrp, was also evident. Ptc1 expression was unchanged in the perichondrium and proliferating chondrocytes proximal to the Ihh source in JAWS–/– embryos; however, expression of Pthrp in resting and periarticular chondrocytes was markedly diminished, as was expression of Pthrp (Fig. 2A, arrows). This finding implies normal short-range, but disrupted long-range, Ihh signaling in the JAWS–/– growth plate, probably resulting from compromised ECM integrity.
Collectively, these data indicate a delay in chondrocyte maturation in Jaws–/– embryos. Terminal hypertrophic chondrocyte differentiation was also delayed in E15.5 Jaws–/– embryos. This delay was evident in expression of Col10a1 (see below). Collectively, these data indicate a delay in chondrocyte maturation in Jaws–/– embryos. Terminal hypertrophic chondrocyte differentiation was also delayed in E15.5 Jaws–/– embryos. Expression of Col10a1 expression and osteopontin (Spp1) (Fig. 2B). Delayed chondrocyte maturation was most conspicuous in the hindlimb, where the knee joint failed to form and where the tibia retained a homogeneous population of small, Col2a1-expressing chondrocytes at E18.5 (Fig. 3A; see Fig. S5 in the supplementary material). Finally, expression of the osteogenic markers Runx2 (Kronenberg, 2003) and Tcf1 (Glass, 2nd et al., 2005) was similar in wild-type and Jaws–/– long bones, excluding the tibia (Fig. 2A; data not shown), suggesting that subsequent osteogenesis, when it occurred, initiated normally.

**Ectopic joints form in the digits of Jaws–/– mice**

The most striking defect in Jaws–/– embryos was seen in the digits, where skeletal preparations revealed longitudinally oriented cavities that lacked Alcian Blue staining and were flanked by cartilage (Fig. 3A). These nonchondrogenic cavities, which were first apparent when the wild-type interzone formed at ~E13.5, extended from the presumptive metacarpophalangeal/metatarsophalangeal joint to the distalmost phalanx in all digits, appearing discontinuous in forelimbs while bisecting and spanning each hindlimb element. Two main possibilities could explain the occurrence of these cavities: either they result from aberrant cell death, or they represent ectopic joints. To distinguish these possibilities, we first examined the early joint marker Gdf5 by whole-mount in situ hybridization and found that Gdf5 expression extended longitudinally throughout these cavities (Fig. 3B; Fig. S6 in the supplementary material). Likewise, expression of all joint markers examined, including Gli3, Wnt9a, Sul1, Cutl1 (Cux1), Scl1, Hip1 and activated β-catenin (Guo et al., 2004; Hill et al., 2005; Pacifici et al., 2005; Mak et al., 2006; Zhao et al., 2006; Khan et al., 2007) overlapped the Gdf5 expression domain (Fig. 3C and data not shown), suggesting that cells within these cavities exhibited a joint-like fate. Histologically, these cells resembled wild-type joint progenitors in having a flattened, more mesenchymal appearance than surrounding chondrocytes (Fig. 3C; data not shown). TUNEL analysis demonstrated similar numbers of apoptotic cells in wild-type and Jaws–/– joints at all stages examined (E12.5–E17.5; see Fig. S7 in the supplementary material, and data not shown), further excluding cell death as a disproportionate contributor to the joint phenotype. Likewise, consistent cartilage staining in the digits of E12.5 Jaws–/– autopods prior to the appearance of the joint interzone suggested that ectopic joints were not a secondary consequence of diminished ECM formation (see Fig. S3 in the supplementary material; data not shown). Moreover, no changes in the expression of limb polarity markers were evident earlier in E11.5 or E12.5 Jaws–/– embryos (see Fig. S8 in the supplementary material).

If the longitudinal cavities in Jaws–/– digits represent joints, then they should not only express joint markers but also exclude markers of neighboring cell types. Indeed, expression of the chondrogenic markers collagen 2, aggrecan and link protein was excluded from prospective Jaws–/– joints (Fig. 3D and data not shown) (Schwartz and Domowicz, 2002). Similarly, expression of Bmpr1b, which is normally restricted to articular surfaces flanking the wild-type joint interzone at E14.5 (Baur et al., 2000; Yi et al., 2000), was seen in an analogous pattern bordering the longitudinal domain of Gdf5 expression in Jaws–/– digits (Fig. 3D). Thus, joint-like structures in Jaws–/– digits do not express chondrogenic markers but do maintain their immediate proximity to Bmpr1b—expressing articular chondrocytes. In addition, these joint-like structures did not express markers of hypertrophic chondrocytes (Col10a1) or osteoblasts (Runx2), while expressing wild-type levels of the tendon and ligament marker Scx (Fig. 3E) (Schweitzer et al., 2001). Collectively, these molecular and histological data establish the
longitudinal cavities in Jaws−/− digits as ectopic joints. The persistence of ectopic joints in neonatal Jaws−/− mice defines JAWS as a crucial novel regulator of synovial joint orientation and positioning. Because misorientation of the interzone necessarily causes an ectopic joint, we use the terms ‘orientation’ and ‘positioning’ interchangeably here.

Cavitation is the late-stage process culminating in formation of the synovial cavity. Cavitation in E18.5 wild-type forelimb digits was demonstrated by expression of the hyaluronan (HA) receptor CD44 on articular surfaces (Pitsillides, 1999). By contrast, CD44 immunostaining was largely absent from ectopic Jaws−/− joints, despite its presence in more proximal joints (Fig. 3F; data not shown). This result indicates a requirement for JAWS in the timely progression to cavitation, leading to a functional synovial joint.

**Impaired chondroitin sulfation and aberrant aggrecan metabolism in Jaws−/− cartilage**

From a mechanistic perspective, several features of the Jaws−/− phenotype are consistent with defects in chondroitin sulfation pathways. In particular, misaligned chondrocytes, hypocellularity and ECM discontinuities characterize the growth plates of animals lacking chondroitin-4-sulfotransferase 1 (C4st1; Chst11 – Mouse Genome Informatics) or the chondroitin sulfate (CS)- and heparan sulfate (HS)-rich proteoglycan perlecan (Klüppel et al., 2005; Schwartz and Domowicz, 2002). Moreover, mutant mice lacking components of the CS synthesis or transport machinery are chondrodysplastic (Schwartz and Domowicz, 2002). For these reasons, we examined whether CS was spatially or quantitatively altered in Jaws−/− embryos. Immunostaining for CS demonstrated highest levels in the perichondrium and articular zones of E14.5 limbs (Fig. 4A; data not shown), with intense uniform extracellular staining in wild-type digits. By contrast, a faint punctate pattern was observed in Jaws−/− digits, much of which appeared to localize intracellularly (Fig. 4A, insets).

We then used fluorophore-assisted carbohydrate electrophoresis to analyze and quantify glycosaminoglycan content in E14.5 limbs. Consistent with our immunostaining data, significant decreases in the proportions of both chondroitin-4-sulfate (C4S) and chondroitin-6-sulfate (C6S) were evident in Jaws−/− limbs, with concomitant increases in unsulfated chondroitin (C0S) (Fig. 4B). Importantly, no change in heparan sulfation was observed (Fig. 4C). Moreover, quantities of total CS (including C0S), HS and HA normalized to...
Fig. 4. Impaired chondroitin sulfation and altered aggrecan metabolism in Jaws−/− limbs. (A) CS immunostaining in hindlimb digits (E14.5). Higher-magnification views (top) reveal sparse irregular staining in the Jaws−/− joint. (B, C) Fluorophore-assisted carbohydrate electrophoresis analysis of chondroitin sulfate (CS, B) and heparan sulfate (HS, C) in E14.5 limbs. The relative proportions and percentages of disaccharides generated with the glycosaminoglycan-specific lysases are shown. For HS, no disaccharides containing 2-sulfated uronic acid (UA) or N,6-sulfated GlcN were detected. UA-galNAc; C4S, UA-galNAc4S; C6S, UA-galNAc6S; GlcNAc, N-acetylgalactosamine; GlcN, ΔUA-glcNAc; GlcN5, ΔUA-glcNS; GlcN(6S). *P<0.025; ‡P<0.0001, Jaws−/− versus wild type percentage (n=5 pairs per genotype). (D) Comparable amounts (mean and s.d.) of total glycosaminoglycans in wild type and Jaws−/− hindlimbs, normalized to wet tissue weight. Similar results were obtained for forelimbs. n.d., not determined; HA, hyaluronan. (E) Aggrecan immunostaining in the proximal end of the humerus (E14.5). (F) Anti-CDAGWL immunoblot of total limb protein extracts, showing less full-length aggrecan (arrow, ~350 kDa) and fewer aggrecan cleavage fragments (bracket) in Jaws−/− cartilage. Successive nondissociative extractions (lanes 1 and 2; arrowhead) and disassociative extraction (lane 3). HL, hindlimbs; FL, forelimbs.

wet tissue weight were comparable in wild-type and Jaws−/− limbs (Fig. 4D). The expression of C4st1, Slc26a2 and Papss2 – key regulators of CS biosynthesis and/or transport – was also unchanged, implying that JAWS does not influence CS levels through transcriptional regulation of these genes (see Fig. S9 in the supplementary material). As described above, this specific diminution of chondroitin sulfation is consistent with the Jaws−/− growth plate defects and suggests a molecular mechanism by which JAWS coordinates chondrogenesis and joint positioning. As extracellular HS is important for shaping the Ihh morphogen gradient (Koziel et al., 2004), these data also argue that the disrupted long-range Ihh signaling in the Jaws−/− growth plate (Fig. 2A) does not result from a change in HS levels but from a generalized loss of ECM integrity.

Decreased chondroitin sulfation may affect chondrogenesis in part by disrupting the normal metabolism of CS-containing proteoglycans such as aggrecan. Aggrecan is the predominant CS proteoglycan of the cartilage matrix and is crucial to normal chondrogenesis and ECM integrity (Schwartz and Domowicz, 2002). The CS chains of aggrecan are essential for its efficient secretion and for binding and proteolytic cleavage by the aggrecanase Adamts4 (Kiani et al., 2001; Tortorella et al., 2000). Therefore, we assessed whether Jaws−/− embryos show impaired expression and/or turnover of aggrecan. Aggrecan immunostaining revealed mottled, variable expression throughout E14.5 Jaws−/− cartilage, in contrast to the robust consistent wild-type staining pattern (Fig. 4E and data not shown). Notably, mRNA and protein levels of two other abundant cartilage ECM proteins, collagen 2 and link protein, were unchanged, suggesting that abnormal aggrecan expression was not a secondary consequence of delayed chondrocyte maturation (see Fig. S10 in the supplementary material; data not shown). To confirm and extend this aggrecan result, an antibody (anti-CDAGWL) that recognizes intact aggrecan and several well-characterized cleavage products was used to analyze limb protein extracts (Arner, 2002). Under dissociative conditions, markedly reduced amounts of both full-length aggrecan (Fig. 4F, arrow) and C-terminally cleaved aggrecan fragments (Fig. 4F, bracket) were extracted from Jaws−/− relative to wild-type limbs. This reduction was greater in hindlimbs, where more severe chondrogenic and joint defects were noted (Fig. 3A; Fig. S5 in the supplementary material). As an internal control, successive nondissociative extractions yielded similar levels of a 65 kDa aggrecan fragment that is likely to result from transient expression and rapid cleavage in extracartilaginous tissue (Fig. 4F, arrowhead) (Arner, 2002). Collectively, these data suggest that impaired CS sulfation and abnormal aggrecan metabolism contribute to the chondrodysplasia and aberrant joint positioning in Jaws−/− embryos.

Although the precise mechanism by which JAWS influences CS sulfation and ECM integrity is unclear, CS sulfation has been reported to modulate cell adhesion and migration (Zou et al., 2004), consistent with the misalignment of both growth plate chondrocytes and the axis of joint formation in Jaws−/− embryos. Moreover, the particular importance of CS sulfation for chondrogenesis may explain why mutation of Jaws, a widely expressed gene, produces defects that are relatively specific to endochondral ossification.

Importantly, this genetic loss-of-function approach distinguishes our findings from previous demonstrations of ectopic joint formation caused by overexpressing Wnt9a or activated β-catenin in vivo or by blocking α5β1 integrin function in limb explant cultures (Hartmann...
and Tabin, 2001; Guo et al., 2004; Garciadiego-Cázares et al., 2004). Our findings also contrast with data obtained by inactivation of the hypoxia-inducible factor 1α (Hif1α) gene in mouse limb mesenchyme. Conditional deletion of Hif1α using Prxl-Cre produces longitudinal cavities that are superficially similar to those seen in Jaws−/− digits; however, unlike ectopic Jaws−/− joints, Hif1α-null cavities form prior to the wild-type joint interzone and do not express joint markers such as Wnt9a and BMP2 (Amarilio et al., 2007; Provot et al., 2007). Gdf5 is expressed primarily in diffuse transverse (rather than longitudinal) stripes in Hif1α-null digits, leading Provot and colleagues to conclude that after an initial developmental delay, synovial joints of normal appearance do form in the digits of these mice. Together, data presented by both groups argue that the Hif1α-null digit phenotype reflects delayed joint specification caused by aberrant differentiation of hypoxic chondrocytes.

By demonstrating the persistence of ectopic joints in Jaws−/− digits, our studies identify JAWS as a novel regulator in the establishment of the interphalangeal joint axes. Because cells in the ectopic Jaws−/− joint express appropriate early interzone markers (Fig. 3B,C), we conclude that the molecular specification and the spatial positioning of the synovial joint interzone are genetically separable processes. Similarly, the restriction of ectopic joints to digits and the lack of knee joints in Jaws−/− embryos support the notion that distinct JAWS-dependent mechanisms govern synovial joint development at different sites in the embryonic skeleton. Overall, Jaws−/− mice provide a unique animal model for exploring the tightly coordinated processes of chondrogenesis and joint morphogenesis, and for better understanding the etiology of joint degeneration leading to debilitating diseases such as osteoarthritis.

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