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REVIEW

Role of the ECM in notochord formation, function and disease

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ABSTRACT

The notochord is a midline structure common to all chordate animals; it provides mechanical and signaling cues for the developing embryo. In vertebrates, the notochord plays key functions during embryogenesis, being a source of developmental signals that pattern the surrounding tissues. It is composed of a core of vacuolated cells surrounded by an epithelial-like sheath of cells that secrete a thick peri-notochordal basement membrane made of different extracellular matrix (ECM) proteins. The correct deposition and organization of the ECM is essential for proper notochord morphogenesis and function. Work carried out in the past two decades has allowed researchers to dissect the contribution of different ECM components to this embryonic tissue. Here, we will provide an overview of these genetic and mechanistic studies. In particular, we highlight the specific functions of distinct matrix molecules in regulating notochord development and notochord-derived signals. Moreover, we also discuss the involvement of ECM synthesis and its remodeling in the pathogenesis of chordoma, a malignant bone cancer that originates from remnants of notochord remaining after embryogenesis.

KEY WORDS: Basement membrane, Chordoma, Extracellular matrix, Notochord

Introduction

The extracellular matrix (ECM) represents a key component of the microenvironment and is composed of secreted proteins and polysaccharides that are assembled locally into an organized network to which cells adhere (Hynes, 2012). The ECM is well known for its ability to provide structural support for organs and tissues, as well as being a substrate for adhesion and migration for individual cells, and it also forms specialized structures such as basement membranes (BMs). The role of the ECM in cell adhesion and signaling through different receptors, including integrins, has received much attention (Berrier and Yamada, 2007; Legate et al., 2009). The biomechanical properties of the ECM, such as its stiffness and deformability, have also been recognized to provide key inputs for cell behavior (Discher et al., 2009; Gattazzo et al., 2014; Dupont, 2016). Thus, ECM macromolecules and the scaffolds they form have major roles in the proliferation, differentiation, survival, polarity and migration of cells. ECM-derived signals are arguably at least as important as soluble signals in governing different cell processes (Hynes, 2009). As a matter of fact, the ECM is also able to position, concentrate, sequester and store growth factors and other signaling molecules. Possible consequences of these interactions are to restrict or promote access of ligands to related cell surface receptors, as well as to allow for a spatio-temporal modulation of factor release and the organization of morphogen gradients. Indeed, soluble factors can be maintained in the ECM, thereby acting as a reservoir, or be released from the matrix – for example, in the presence of appropriate forces or after proteolytic degradation – for the subsequent binding to their cell surface receptors (Hynes, 2009). In keeping with its various roles and functions, the ECM has a complex and dynamic molecular composition and is mainly composed of fibrous proteins embedded in a gel-like polysaccharide 'ground substance'. In addition to fibrous structural proteins (e.g. collagens and elastin), glycosaminoglycans and proteoglycans, the ECM contains adhesion proteins (e.g. fibronectin, laminins and fibrillins) that link components of the matrix both to one another and to the cell surface (Hynes, 2012). The molecular composition and three-dimensional organization of the ECM vary in specialized organs and tissues, resulting in qualitative and quantitative differences.

The notochord is a transient organ whose major functions include inducing and patterning different tissues during embryogenesis and establishing the antero-posterior body axis of all chordate embryos (for a recent review on notochord structure and function, see Corallo et al., 2015). In vertebrates, the notochord arises from the dorsal organizer, also known as the embryonic shield in zebrafish, and is critical for proper vertebrate development (Shih and Fraser, 1996; Saùde et al., 2000; Stemple, 2005). One of the main regulators of notochord formation is brachyury, a T-box transcription factor involved in mesoderm specification during embryogenesis that has a crucial role in notochord development (Satoh et al., 2012). In teleosts, the notochord is one of the earliest distinguishable features of the embryo. It forms as chordamesoderm cells converge at the midline, thereby creating a rod of stacked cells that then differentiates into two distinct cell populations in a Notch-dependent manner: the outer sheath layer and an inner vacuolated cell layer (Yamamoto et al., 2010). Cells of the outer sheath secrete a thick extracellular peri-notochordal BM, which is composed of several ECM proteins, whereas cells in the inner layer form large fluid-filled intracellular vacuoles (see Box 1). The pressure exerted by vacuolated cells on the peri-notochordal BM provides the proper stiffness and mechanical strength to the notochord (Adams et al., 1990). Moreover, it was recently shown that notochord vacuoles are specialized post-Golgi structures whose biogenesis and maintenance require late endosomal trafficking (Ellis et al., 2013).

Biochemical and immunohistochemistry studies in different chordates have identified several ECM components in the developing notochord (Oettinger et al., 1985; Smith and Watt, 1985; Hayashi et al., 1992; Swiderski and Solursh, 1992; Sandell, 1994; Götz et al., 1995). Besides its major structural role, the notochord is also a source of developmental signals that pattern the surrounding tissues (Stemple, 2005). Being positioned centrally in the embryo with respect to both the dorsal–ventral and left–right axes, the notochord produces secreted factors that instruct the surrounding ectodermal, mesodermal and endodermal tissues to acquire their specific differentiated fates (Corallo et al., 2015; Fig. 1A; for further details see Box 2).

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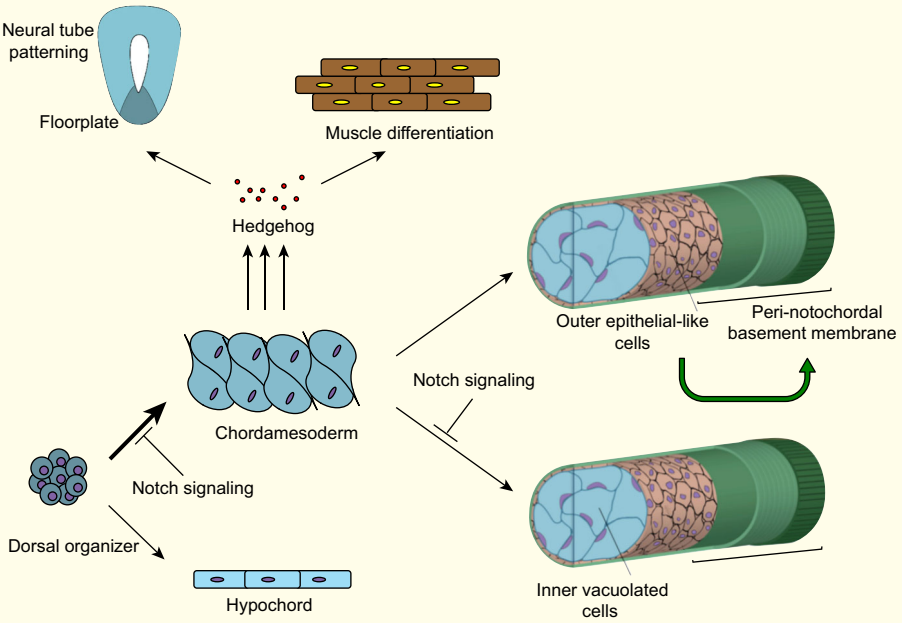
Box 1. Notochord development



In vertebrates, the notochord arises from the dorsal organizer, a transient embryonic structure that appears during gastrulation and has a crucial role in the induction of the embryonic axis. The organizer mediates the dorsal patterning of the mesoderm, regulates the movements of the ectoderm and mesoderm, and provides signals required for the induction of the neuroectoderm along the antero-posterior axis. In teleosts, the dorsal organizer (named embryonic shield) gives rise to three different germ layers: the floor plate, the notochord and the hypochord (Shih and Fraser, 1996; Saùde et al., 2000) (see box figure). In particular, the notochord precursor, called 'chordamesoderm', begins as a rod of stacked cells that converge at the embryonic midline and express specific markers, such as echidna hedgehog (*ehh*), sonic hedgehog (*shh*), collagen II (*col2a1*) and T/Brachyury homolog a (*ta*) (Yan et al., 1995). During the segmentation stage, expression of these early marker genes is turned off and chordamesoderm cells differentiate, through a Notch-dependent mechanism, into two distinct cell subpopulations – an outer 'epithelial-like' cell layer and the inner vacuolated cells (Yamamoto et al., 2010) (see box figure). In this way, the notochord acts as a hydrostatic skeleton during development, driving the elongation of the antero-posterior axis before spine morphogenesis (Adams et al., 1990; Ellis et al., 2013). Interestingly, the notochord persists throughout life in non-vertebrate chordates, whereas in vertebrates, it contributes to the nucleus pulposus of intervertebral discs (Choi et al., 2008).

Several studies in zebrafish have demonstrated that the formation of a notochord sheath is closely linked to the differentiation of notochordal cells, and their reciprocal interactions are fundamental for the proper development and function of the notochord itself (Parsons et al., 2002; Pagnon-Minot et al., 2008; Mangos et al., 2010; Yamamoto et al., 2010). In this review, we will provide an overview of *in vivo* mutation studies, with particular emphasis on those performed in zebrafish, as it is the major animal model used to gain insight into the roles of the different ECM components in regulating notochord development and function, as well as the mechanisms involved in the patterning activity of notochord-derived signals. Furthermore, we will discuss how abnormal ECM deposition contributes to the pathogenesis of human chordoma, a rare but aggressive and life-threatening tumor, which affects the skeleton and arises from notochord remnants.

Role of ECM components in regulating notochord development and the patterning activity of notochord-derived signals

Circumferentially surrounding the notochord is a tri-layered extracellular notochord sheath composed of an inner laminin-rich BM (Parsons et al., 2002), a middle layer of collagen fibers and an outer layer, in which extracellular fibers run perpendicularly to the middle layer (Scott and Stemple, 2004; Grotmol et al., 2006) (Fig. 1). The notochordal sheath and vacuoles have been described in different vertebrate species (Leeson and Leeson, 1958; Bancroft and Bellairs, 1976). However, the detailed molecular composition of the peri-notochordal BM is only partially understood. In zebrafish, several proteins, including laminins, collagens, fibrillins and Emilin3, have been characterized as essential constituents of the notochord sheath. Indeed, ablation of these key ECM components of the notochord sheath results in severe structural and molecular defects of the developing embryo (for references see below and Table 1). Analysis of notochord-related phenotypes in animal models that arise from mutation of genes encoding proteins involved in matrix assembly and structure has provided valuable information on the role of specific ECM components in notochord structure and function. In particular, manipulations in fish models have allowed researchers to bypass some of the difficulties associated with mammals, in which ablation of ECM components often cause embryonic lethality. Several mutants identified by large-scale forward genetic screens in zebrafish (*Danio rerio*) display notochord distortion, and a number of studies in zebrafish have investigated the notochord-related phenotypes that are caused by defects of the components of the extracellular sheath layers, as discussed in the following sections.

Glycoproteins

The major components of basal lamina in different tissues are the laminins (Miner and Yurchenco, 2004). The structure of these

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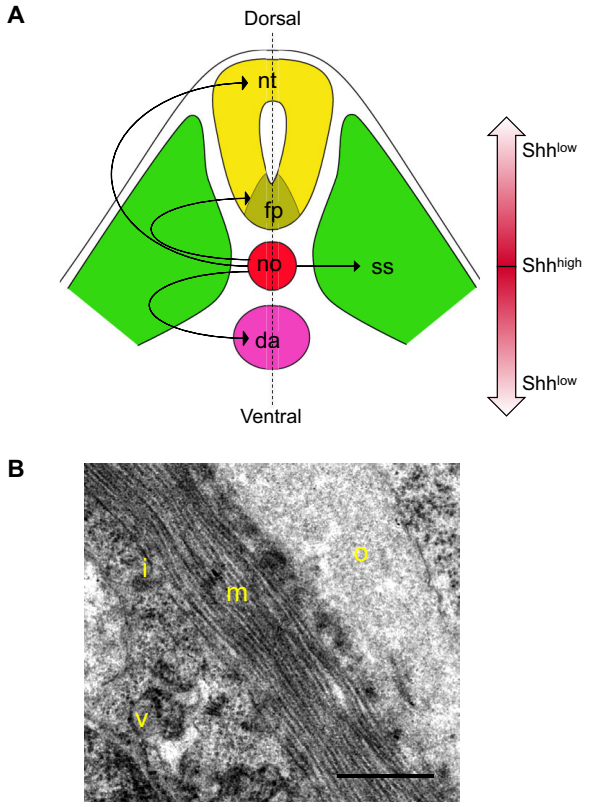

Fig. 1. The notochord and the peri-notochordal basement membrane. (A) Schematic diagram of a transverse section through the trunk of a vertebrate embryo, depicting the tissue organization and signaling during embryogenesis. The notochord cells (no) secrete Shh (arrows) and a gradient of Shh signal is established in the dorso-ventral axis. This signal is critical for the proper patterning of the surrounding tissues, including the neural tube (nt), the floor plate (fp), the somites (ss) and the dorsal aorta (da). The dashed line marks the midline of the dorso-ventral axis. (B) Transmission electron micrograph of the peri-notochordal basement membrane of a zebrafish embryo at 30 h post-fertilization. The peri-notochordal basement membrane is composed of three parts: the inner (i) basal lamina, surrounded by a medial (m) layer of collagen fibrils that run parallel to the notochord, and an outer (o) granular layer of loosely organized ECM that is mostly oriented in a perpendicular manner to the notochord. The peri-notochordal sheath counteracts the pressure generated by intracellular vacuoles of the notochordal cells (v), providing rigidity and correct stiffness to this structure. Scale bar: 10 µm. The image in B has been adapted with permission from Corallo et al., 2013.

glycoproteins is well known and exemplifies the typical multidomain structure and the multimeric complexes that are common in many ECM proteins. Laminins comprise a family of cross-shaped heterotrimeric glycoproteins generated by three different α, β and γ chains. There are multiple isoforms for each of the laminin chains encoded by distinct genes, leading to more than ten different trimer combinations, and laminin molecules are named according to their chain composition. The trimeric laminins connect cell membranes and ECM molecules, and also bind to each other to form sheets in the basal lamina (Colognato and Yurchenco, 2000). Since a mouse null for laminin γ1 (*Lamc1*) is embryonic lethal owing to failure of endoderm differentiation (Smyth et al., 1999), the zebrafish became the first model for investigating laminin function during notochord development (Parsons et al., 2002). Characterization of laminin deposition during zebrafish gastrulation demonstrated a circumferential pattern around a portion of the notochord that overlapped with fibronectin (Latimer and Jessen, 2010). In the grumpy (*gup*) and sleepy (*sly*) zebrafish mutants, which have mutations in either the laminin β1 or laminin γ1 chains, the proper organization of all three layers of the peri-notochordal BM is prevented, further indicating that laminins might serve as a scaffold for the assembly of other BM proteins. Moreover, these notochord cells fail to properly fill vacuoles, resulting in a significant shortening of the main embryonic axis in the mutants, in agreement with a mechanical role for the BM in sustaining the hydrostatic pressure of vacuolated cells (Parsons et al., 2002). In addition, the persistent expression of genes that are usually only expressed early in notochord development in these mutants, e.g. collagen II (*col2a1*), sonic hedgehog (*shh*) and echidna hedgehog (*ehh*; also known as *ihhb*), suggests that signals originating from extracellular sheath proteins are required for notochord differentiation. Indeed, notochord differentiation could be rescued by exogenous sources of the missing laminin chain, as demonstrated by the ability of wild-type shield cells transplanted into mutant hosts to form normal notochords (Parsons et al., 2002). Moreover, notochordal sources of laminin are also sufficient for rescuing the mutant phenotype, because shields from *gup* or *sly* mutants that have been transplanted into wild-type hosts resulted in secondary axis formation and a phenotypically normal notochord. Therefore, laminin β1 and laminin γ1 can non-autonomously rescue notochord differentiation. Hence, functional laminin chains can be supplied to the notochord from either autonomous or non-autonomous cell sources (Parsons et al., 2002). Interestingly, in *sly* mutants, hedgehog (Hh) signaling is not affected, whereas ectopic bone morphogenic protein (BMP) activity was observed in the adjacent myotome (Dolez et al., 2011). These findings suggest that there is a complex feedback mechanism between Hh, BMP and laminin-111 (containing α1, β1 and γ1 chains, also called laminin 1), which is involved in the correct deposition of the peri-notochordal sheath and myogenesis.

In contrast to the laminin zebrafish mutants described above, ablation of the laminin α1 chain in the *bashful* (*bal*) mutant causes a milder notochord phenotype as here only the anterior region of the notochord fails to form (Pollard et al., 2006). Noteworthy, *bal* mutants display residual laminin-111 deposition in the posterior notochord, suggesting that other laminin isoforms might fulfill a compensatory role in posterior notochord development. Indeed,

Box 2. Notochord signaling

One of the main signals secreted by notochordal cells are members of the Hh family (Fig. 1A; for a recent review on Hh signaling, see Lee et al., 2016). The Hh pathway is a key regulator of embryonic development that controls the patterning of adjacent tissues, including neural tube (Yamada et al., 1991), somites (Pourquié et al., 1993) and pancreas (Roy et al., 2001). Shh, in particular, induces a range of ventral spinal cord fates in a graded fashion, simultaneously suppressing the expression of characteristically dorsal genes (Yamada et al., 1991; Placzek et al., 1991). Notochord signals are also involved in establishing left-right asymmetry, and notochord ablation in *Xenopus* gastrulae results in randomization of asymmetry (Danos and Yost, 1995; Lohr et al., 1997). In teleosts, notochord-derived Hh signals control the formation of the horizontal myoseptum, as well as specify slow-twitch muscle fates (Devoto et al., 1996; Barresi et al., 2000). Moreover, notochord-derived signals are critical for the formation of the dorsal aorta (Fekany et al., 1999; Cleaver et al., 2000; Lawson et al., 2002), as well as for the specification of the cardiac field (Goldstein and Fishman, 1998). Finally, the notochord is also important for the proper development of early endoderm and pancreas (Cleaver and Krieg, 2001).

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Table 1. Summary of currently available morphant, mutant and knockout *in vivo* models for ECM proteins or genes involved in notochord formation and function

Protein	Zebrafish models (ZFIN database)	Mouse models (MGI database)	Other models
Laminin α1	*bashful* (*bal*) (Pollard et al., 2006)	*Lama1* null mice (embryonic lethal)	
Laminin α4 and α5	*lama4* and *lama5* morphants (Pollard et al., 2006)	*Lama4* null (impaired motor control, prenatal hemorrhages) *Lama5* null (lethality during late gestation)	Ascidian (*C. intestinalis*) *chongmague* (*cmg*) mutant (Veeman et al., 2008)
Laminin β1	*grumpy* (*gup*) (Parsons at al., 2002)	*Lamb1* null (fail to survive past E5.5)	
Laminin γ1	*sleepy* (*sly*) (Parsons at al., 2002)	*Lamc1* null (embryonic lethal)	
Emilin3	*emilin3a* and *emilin3b* morphants (Corallo et al., 2013)	*Emilin3* null (no defects)	
Fibronectin	*fn1a* and *fn1b* mutants/morphants (no notochord phenotype)	*Fn1* mutant (Georges-Labouesse et al., 1996)	Ascidian (*C. intestinalis*) *Cs-Fn* knockdown (Segade et al., 2016)
Fibrillin-2	*puff daddy* (*pfd*) mutants *fbn2* morphants (Gansner et al., 2008)	*Fbn2* null (bilateral syndactyly with fusion of both soft and hard tissues)	Frog (*Xenopus*) XF morphants (Skoglund et al., 2006)
Chondroitin 6-sulfotransferases		*Chst3* null (behavioral and hematopoietic defects)	Ascidian *(C. intestinalis*) *Ci-C6ST-like-1* and *Ci-C6ST-like-6* (Nakamura et al., 2014)
Collagen II	*Col2a1* morphants (Gansner et al., 2007)	*Col2a1* null (Aszódi et al., 1998)	Human *COL2A1* mutation (Codsi et al., 2015)
Collagen VIII	*gulliver* (*gul*) and *leviathan* (*lev*) (Gansner and Gitlin, 2008; Gray et al., 2014)	*Col8a1* null, *Col8a2* null (eye abnormalities)	
Collagen XI	*Col11a1* morphants (Baas et al., 2009)	*Col11a1* null (perinatal lethality, weak cartilages)	
Collagen XV	*Col15a1* morphants (Pagnon-Minot et al., 2008)	*Col15a1* null (heart and skeletal muscle abnormalities)	
Collagen XXVII	*col27a1a* and *col27a1b* morphants (Christiansen et al., 2009)	*Col27a1* null (neonatal lethality, severe chondrodysplasia)	
Lysyl oxidases	*loxl1* and *loxl5b* morphants (Gansner et al., 2007)	*Lox*, *Loxl1*, *Loxl2* and *Loxl3* null (perinatal lethality, cardiovascular defects)	

References in brackets refer to animal models whose notochord-related phenotype is discussed in the text. For mouse-null models displaying early lethality or unrelated defects, the main phenotypic features are provided in brackets.

concurrent knockdown of the laminin α4 and α5 chains by injection of antisense morpholino oligonucleotides (MOs) in *bal* mutants results in a more-severe notochord phenotype that is comparable to that observed in *gup* and *sly* mutants (Pollard et al., 2006). In particular, when *bal* embryos were injected with laminin α4 MO, vacuoles failed to inflate properly; *ehh* expression, characteristic of the early stages of notochord development, was also abnormally persistent and laminin-111 immunoreactivity was severely reduced. Injection of laminin α5 MO in *bal* embryos led to the same abnormal and persistent expression of *ehh* along the notochord concurrently with a failure of cellular inflation and loss of laminin immunoreactivity (Pollard et al., 2006). Taken together, these results point to a redundant role for laminin-411 (containing α4, β1 and γ1 chains, also called laminin 8) and laminin-511 (containing α5, β1 and γ1 chains, also called laminin 10) in notochord development.

Further studies in other chordate organisms confirmed that laminins play key roles in notochord morphogenesis. Characterization of the *chongmague* (*cmg*) mutant in the ascidian *Ciona savignyi* identified a mutation in the ortholog gene of vertebrate laminin α chains, and revealed an essential role for laminin in boundary formation and in convergence and extension movements during notochord development (Veeman et al., 2008). Work in *Xenopus laevis* showed that dystroglycan, a cell surface laminin receptor, is required for proper laminin assembly, cell polarization and vacuole differentiation during notochord development, and identified an adhesome consisting of laminin, dystroglycan and myosin IIA as being crucial for maintaining the shape of notochordal cells (Buisson et al., 2014).

Another ECM glycoprotein that is localized in the peri-notochordal sheath during early stages of zebrafish development is Emilin3 (Corallo et al., 2013). Although its mutant phenotype is not as severe as in laminin mutants, ablation of both Emilin3 paralogs (*emilin3a* and *emilin3b*) leads to a marked distortion of the notochord in zebrafish embryos, a phenotype associated with significant shortening of the main embryonic axis, disorganization of the medial layer of the peri-notochordal sheath and persistent expression of early chordamesoderm markers. This further supports the concept

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that structural defects in the peri-notochordal sheath also influence the gene expression profile of the developing notochord cells. However, and different from other experimental models of notochord disruption, *shh* and *ehh* are enriched only in the expression field of *emilin3a* and *emilin3b* paralogs, at the level of the chordoneural hinge (Corallo et al., 2013), suggesting the intriguing hypothesis that notochord cells can respond to extracellular signals in a region-specific manner. *In vitro* and *in vivo* mechanistic studies have revealed that Emilin3 modulates the availability of Hh ligands by interacting with Scube2, a secreted permissive factor for Hh signaling (Creanga et al., 2012; Tukachinsky et al., 2012), in the notochord sheath (Corallo et al., 2013). This supports the novel notion that specific ECM proteins are involved in the fine-tuning of the Hh activity that arises from the notochord.

Fibronectin is another ECM protein with essential roles during embryogenesis, and mice lacking fibronectin die between embryonic day (E)9.5 and E10.5 with a range of defects. Fibronectin contributes to gastrulation, axis elongation, germ layer specification, axial patterning and morphogenesis of mesodermal tissues, including the notochord and somites (George et al., 1993; Pulina et al., 2011; Cheng et al., 2013). Studies in fibronectin-null ($Fn1^{-/-}$) mice showed that functional inactivation of fibronectin leads to mesodermal defects, and embryos fail to develop notochord and somites (Georges-Labouesse et al., 1996). Analysis with lineage markers demonstrated that both the notochord and the somite lineages were induced at the correct times and places. Moreover, notochord precursor cells showed extensive cell migration; however, neither notochord nor somites condensed properly in the absence of fibronectin, indicating that the specification of notochordal and somitic lineages are independent of fibronectin, whereas the correct morphogenesis of these structures requires fibronectin (Georges-Labouesse et al., 1996). Further studies in *Fn1*-null mice confirmed that fibronectin is not required for cell fate specification of the notochordal plate, and indicated that the node- and notochord-derived canonical Wnt and Shh signaling are not perturbed by the absence of fibronectin (Pulina et al., 2011). However, work carried out in the ascidian *Ciona intestinalis* in targeted knockdown experiments in the notochord lineage suggests that fibronectin is required for the proper convergent extension and intercalation of notochord precursor cells (Segade et al., 2016).

Fibrillins are a type of ECM protein whose expression during embryogenesis is involved in notochord patterning. The first identified *Xenopus* fibrillin homolog (XF), which is most closely related to fibrillin-2 (*Fbn2*), is expressed in the embryonic organizer and is the earliest ECM component that is deposited at the developing notochord–somite boundary (Skoglund et al., 2006). Functional studies in *Xenopus* showed that inhibition of fibrillin during embryogenesis perturbs normal gastrulation and directed extension, pointing to a role for fibrillin in regulating directed convergence and extension in the developing notochord (Skoglund and Keller, 2007). A forward genetic screen in zebrafish unveiled a key role for fibrillin-2 in notochord morphogenesis (Gansner et al., 2008). In agreement with the spatio-temporal expression of fibrillin-2 in zebrafish embryos, fibrillin-2 mutants (*puff daddy*) and morphant embryos display notochord distortion. This phenotype is accompanied by a marked reduction of the outer layer of the peri-notochordal sheath, while the other two layers appear to be organized normally (Gansner et al., 2008).

Collagens

Collagens are the most abundant components of the ECM and are fibrous structural proteins made of three subunits (or α chains) that contain a triple-helical region. There are seven classes of collagens, which are encoded by more than 42 genes that generate the subunits of at least 28 different types of collagens (for a recent review, see Gordon and Hahn, 2010). The collagen network is thought to form the scaffold that integrates other BM and ECM components, such as laminins, perlecan and fibronectin, into highly organized supramolecular architectures (Myllyharju and Kivirikko, 2004; Mienaltowski and Birk, 2014). Collagen II is a fibril-forming collagen that is mainly expressed in cartilage, and it is one of the major ECM proteins of the notochord of all chordates. A homolog protein of vertebrate collagen II is the main component of the peri-notochordal sheath of hagfish and lancelet cephalochordates (Yong and Yu, 2016). Initial studies in zebrafish showed that the *col2a1* gene is dynamically expressed along the embryonic axis in three rows of cells, namely the notochord, the floor plate of the central nervous system and the hypochord (Yan et al., 1995). *Col2a1*-null mice produce structurally abnormal cartilage and develop a skeleton without endochondral bone formation, but, interestingly, they are unable to dismantle the notochord, a defect that is associated with the inability to develop intervertebral discs (Aszódi et al., 1998). In contrast to what is seen in wild-type mice, where the nucleus pulposus of intervertebral discs forms from a regional expansion of the notochord that is removed in the developing vertebral bodies, in *Col2a1*-null mice the notochord is not removed and persists as a rod-like structure until birth. Notably, experimental data obtained in wild-type mice has revealed that differential proliferation and apoptosis play no role in notochord degeneration during normal embryogenesis, suggesting that instead the cartilage ECM exerts mechanical forces, which induce the removal of the notochord. In agreement with this, collagens I and III are ectopically expressed in *Col2a1*-null mice, and collagen fibrils appear irregular and disorganized, pointing to a structurally weakened ECM that is unable to constrain osmotic swelling pressure (Aszódi et al., 1998). Interestingly, a recent clinical study reported notochord persistence in a human fetus with a *COL2A1* mutation; this not only confirms the findings obtained with *Col2a1*-null mice, but also points to human developmental disorders being associated with perturbations of collagen II function in the notochord (Codsi et al., 2015).

Other collagen proteins have also been reported to play roles in notochord morphogenesis. Loss of the zebrafish α1 chain of collagen VIII (*col8a1*) generates early and late notochord-related phenotypes. A study aimed at characterizing one zebrafish mutant with notochord distortion, called *gulliver* (*gul*), demonstrated that the phenotype arises from mutation of the *col8a1* gene, which leads to defective collagen VIII deposition and disorganization of collagen fibers in the medial layer of the notochord sheath (Gansner and Gitlin, 2008). Interestingly, in the *gul* mutant, notochordal cells form inflated vacuoles and survive despite the accumulation of protein aggregates in the rough endoplasmic reticulum. It is reasonable to speculate that the undulated shape of the notochord in *gul* mutants may be due to a defective peri-notochordal BM architecture that is not able to sustain the hydrostatic pressure generated from vacuolated cells (Gansner and Gitlin, 2008). More recently, a study demonstrated that the disruption of the *col8a1* gene in zebrafish *leviathan* (*lev*) mutants is sufficient to generate vertebral malformation and scoliosis in the adult fish, which resulted from aberrant bone deposition at regions of misshapen notochord tissue (Gray et al., 2014).

Collagen XV is abundantly deposited in the peri-notochordal BM, and ablation of collagen XV by morpholino (MO)-mediated knockdown of the *col15a1* gene in zebrafish embryos leads to disorganization of the peri-notochodal BM, with lower amounts of less-dense collagen fibers in the medial and outer layers, and

disruption of the inner layer (Pagnon-Minot et al., 2008). This phenotype is also associated with defects in notochord differentiation, as indicated by the reduction of the mean size of vacuoles and the persistent expression of early chordamesodermal markers (Pagnon-Minot et al., 2008). Based on the substantial increase in the number of medial fast-twitch muscle fibers, the authors suggested that the loss of BM integrity due to ablation of collagen XV may lead to an increased diffusion of Hh ligands originating from the notochord, thereby causing an altered differentiation program of muscle fibers. Moreover, the distinctive expansion of medial fast fibers in *col15a1* morphant embryos correlates with the onset of collagen XV expression after Shh-mediated induction of adaxial and muscle pioneer cells (Pagnon-Minot et al., 2008).

Studies in zebrafish have shown that expression of the *col11a1* gene, which encodes the α1 chain of collagen XI, is transiently restricted to notochord during development (Baas et al., 2009). MO-mediated *col11a1* knockdown in zebrafish embryos leads to defects in craniofacial cartilage and notochord morphology (Baas et al., 2009). The ECM organization of the peri-notochordal sheath is also altered and causes notochord distortion in collagen XI-deficient embryos (Baas et al., 2009). Collagen XXVII also has a role in notochord morphogenesis, and the two zebrafish paralog genes (*col27a1a* and *col27a1b*) are dynamically expressed in the notochord with a pattern similar to that of *col2a1* (Christiansen et al., 2009). MO-mediated knockdown of *col27a1a* and *col27a1b* causes notochord curvature at early developmental stages. Indeed, ablation of collagen XXVII leads to a weak peri-notochordal sheath, which is unable to withstand the pressure generated by vacuolated cells within the notochord. Notably, this early embryonic phenotype is also accompanied by subsequent phenotypes, including dysmorphic vertebrae, delayed vertebral mineralization and scoliosis (Christiansen et al., 2009). This suggests that collagen XXVII is not only required during early notochord development, but is also critical for the formation of subsequent skeletal structures.

Lysyl oxidases and copper metabolism

A chemical genetic screen in zebrafish embryos pointed to a role for lysyl oxidases in the final stages of notochord development (Anderson et al., 2007). Lysyl oxidases are Cu^{2+}-dependent enzymes that catalyze the cross-linking of elastin and collagens, thus stabilizing the ECM and maintaining notochord sheath integrity (Geach and Dale, 2005; Gansner et al., 2007). Studies in *Xenopus* and zebrafish embryos have revealed that there is a varied timing of expression and localization for different lysyl oxidase transcripts, but all of them are present in the notochord (Geach and Dale, 2005; Gansner et al., 2007). Notably, pharmacological inhibition of lysyl oxidases in zebrafish potently induces an undulating notochord phenotype, which is reminiscent of the mutants identified in large-scale mutagenesis screens (Anderson et al., 2007). Notochord undulations appear during late somitogenesis, the phase of cell vacuolation and notochord elongation, and this phenotype is accompanied by disrupted organization of collagen fibrils in the peri-notochordal sheath. A similar phenotype was observed after concurrent knockdown of *loxl1* and *loxl5b* in zebrafish (Gansner et al., 2007). These findings led to the conclusion that an impaired lysyl oxidase activity prevents the differentiation of vacuolated cells, as suggested by the persistent expression of chordamesoderm markers (Gansner et al., 2007), which is highly reminiscent of the sustained expression observed in the genetic models of peri-notochordal sheath disorganization described above. It is reasonable to speculate that this altered gene expression pattern stems from the loss of notochord strength and stiffness. Hence, it is likely that the ECM sheath is required to sense or transduce notochord differentiation signals, although a detailed mechanistic explanation is still missing.

The above data also provide an interesting basis for investigating the relationships between ECM sheath organization and copper metabolism disorders, in agreement with the essential role of copper for notochord development (Mendelsohn et al., 2006). In fact, zebrafish models for Menkes disease, a rare X-linked disorder of copper metabolism caused by mutations in the gene coding for the ATP7A copper transporter (Menkes, 1988), display an undulating notochord phenotype. Indeed, the zebrafish *calamity* (*cal*, which is associated with a loss-of-function mutation in the zebrafish ATP7A ortholog) and *catastrophe* (*cto*, which carries an inactivating mutation in the vacuolar ATPase) mutants represent a valuable tool to study the effects of developmental copper deprivation (Madsen and Gitlin, 2008). In support of this notion, downregulation or inactivation of collagen II, collagen VIII or fibrillin-2, which are three substrates for ECM lysyl oxidases and notochord sheath proteins, sensitizes embryos to notochord distortion after suboptimal copper nutrition or partial lysyl oxidase inhibition, indicating that there is a complex balance between ECM gene expression and nutrient availability that is critical for notochord formation (Gansner et al., 2007, 2008; Gansner and Gitlin, 2008).

Proteoglycans

The peri-notochordal sheath of different chordates also contains various types of sulfated proteoglycans, in particular chondroitin sulfate proteoglycans (Oettinger et al., 1985; Smith and Watt, 1985; Sandell, 1994). A study in the ascidian *Ciona intestinalis* showed a high expression of genes coding for chondroitin 6-sulfotransferases in the developing notochord, and disruption of these genes affects the convergent extension movement of notochordal cells, indicating that proper sulfation of proteoglycans is required for notochord morphogenesis (Nakamura et al., 2014).

Notochordal ECM alterations in human diseases and in chordoma

Notochord and notochord-derived cells play a key role in the formation of intervertebral discs and in disc homeostasis. Disc degeneration is the most common cause of back pain and is thought to initiate with changes in the cellular microenvironment, a process shown to be associated with increased ECM breakdown and abnormal ECM synthesis (McCann and Séguin, 2016). During the late stages of vertebrate development, the notochord degenerates in the region of the vertebral bodies or centra, while small areas of notochord tissue persist in the center of the intervertebral discs where notochord cells condense to form nuclei pulposi (Fig. 2A) (Choi et al., 2008). Additionally, a few isolated embryonic notochord cells were also found to reside in the vertebrae between the intervertebral discs. The location of these cells is characteristic of 'notochordal remnants', which in humans have been proposed to give rise to chordoma, a class of rare and malignant bone tumors (Fig. 2B) (Choi et al., 2008; Yakkioui et al., 2014). Thus, the thorough dissection of the molecular mechanisms underlying notochord development could provide valuable information not only for increasing our understanding on vertebrate biology and development, but also for obtaining further insight into the origin and pathogenesis of chordomas. Typically, these neoplasms grow slowly and display a low tendency to metastasize (Heery, 2016). Although surgical excision and radiotherapy are currently the primary treatments for chordoma, there is a need for novel therapies

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Journal of Cell Science (2017) 130, 3203-3211 doi:10.1242/jcs.175950

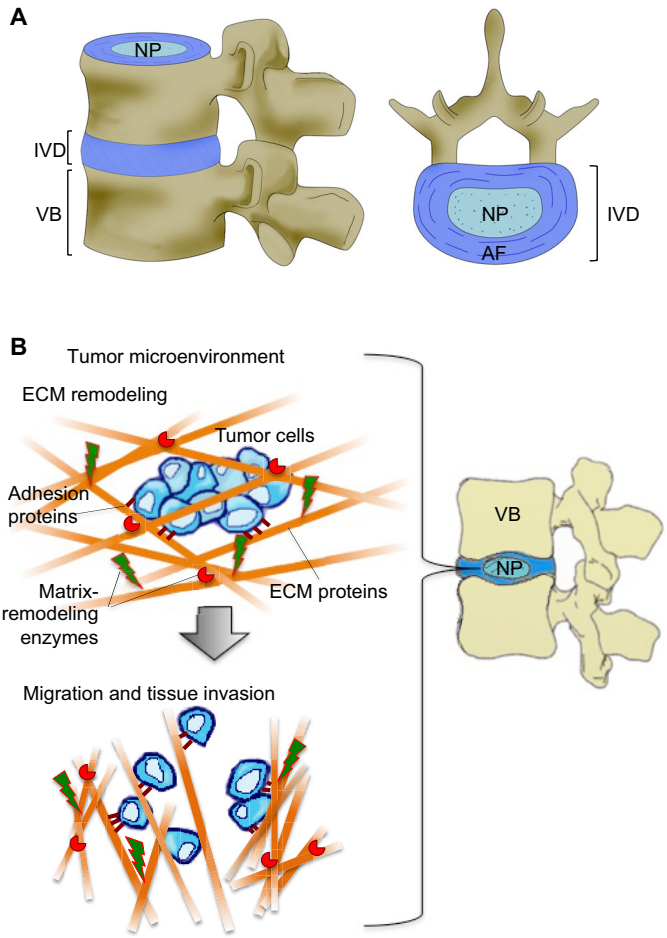

Fig. 2. Intervertebral disks and chordoma development. (A) Schematic drawing of lateral (left panel) and top (right panel) views of a human vertebra, showing its main features. Although the notochord completely regresses in the vertebral bodies (VB), small areas of notochord tissue persist in the center of the intervertebral disks (IVD), where notochord cells condense to form the nucleus pulposus (NP), which is surrounded by smaller fibroblast-like cells of the annulus fibrosus (AF). (B) Potential effects of abnormal ECM secretion and remodeling in the progression of human chordomas. Several ECM components, ECM-associated adhesion proteins and matrix remodeling enzymes are overexpressed in chordoma samples, resulting in abnormal ECM organization. Abnormal ECM dynamics is associated with tumor recurrence and poor prognosis, suggesting that matrix components and ECM architecture affects the metastatic capability of chordoma cancer cells.

owing to the fact that they have a high tendency to recur after treatment, their critical location in the body and their high resistance to chemotherapy (Walcott et al., 2012; Heery, 2016). A better mechanistic knowledge regarding the onset and progression of chordomas might allow the development of novel immunotherapy-based strategies for the targeted treatment of chordoma (Patel and Schwab, 2016). Indeed, proteins that are important for cell mobilization and cell–ECM interactions could constitute alternative therapeutic targets (Schwab et al., 2009).

Some studies have carried out a molecular characterization of chordomas using gene expression profile analysis, with the aim to determine their molecular signatures and successfully translate them into more efficient therapies. Actually, chordomas do not only have microscopic features resembling the embryonic notochord, but also express brachyury, the founding member of the T-box family of transcription factors, which is necessary for the specification of mesodermal identity and regulates notochord formation (Showell et al., 2004). Although the pathogenesis of chordoma has not been fully elucidated yet, recent advances in the understanding of the pathophysiology and molecular mechanisms of this tumor have implicated brachyury in chordoma cell initiation and progression (Vujovic et al., 2006; Sun et al., 2015). Interestingly, chordomas are characterized by the abnormal production of ECM components, which also contribute to their histological identification (Gottschalk et al., 2001; Schwab et al., 2009). In particular, a gene expression profile of human chordoma samples revealed that most of the overexpressed genes were involved in either the synthesis of ECM components, the remodeling of the ECM or in forming cell–ECM interactions (Schwab et al., 2009). This raised the hypothesis that the aberrantly high ECM synthesis of chordomas might protect tumor cells from the systemic delivery of chemotherapy agents. Furthermore, chordomas are characterized by the overexpression of several metalloproteinases and enzymes involved in ECM remodeling (Fig. 2B). For example, the matrix metalloproteinase 2 (MMP-2), which degrades denatured collagen and BM proteins, is strongly expressed in primary lesions at sites of tumor infiltration in the bone. Moreover, high expression levels of MMP-2 have been associated with a lower survival of patients affected by chordoma (Naka et al., 2004, 2008). Expression of MMP-9, which was shown to be limited to a few cells near the bone invasion front (Naka et al., 2008; Yakkioui et al., 2014), could also be correlated with tumor recurrence (Rahmah et al., 2010) and poor prognosis (Chen et al., 2011; Froehlich et al., 2012; Yakkioui et al., 2014). Similarly, other matrix metalloproteinases such as MMP-1, cysteine proteinases, such as cathepsin B and K, and serine proteinases including urokinase plasminogen activator are overexpressed in chordoma samples (Haeckel et al., 2000; Naka et al., 2004, 2008), suggesting the involvement of multiple matrix-degrading enzymes in the tumorigenesis and invasive potential of chordomas (Fig. 2B). Thus, the ECM might also function as a scaffold through which malignant cells can metastasize (Yakkioui et al., 2014). For this reason, inhibition of cell migration through the ECM by blocking proteases or proteins involved in cell mobilization may be a feasible alternative approach to conventional systemic therapies (Schwab et al., 2009).

Moreover, it is also worth considering that the proteolytic ability in chordomas might be increased in those tumor cells that need to degrade bone tissue to allow the invasion of the surrounding sites, whereas it could be reduced in those regions where the bone tissue has been already replaced with malignant tissue (Haeckel et al., 2000; Naka et al., 2008). For these reasons, targeted molecular therapies against MMPs represent a field of intense research, although turnover of collagen and other ECM components also occurs through intracellular non-MMP pathways (Overall and Kleifeld, 2006). Interestingly, a zebrafish genetic model for chordoma has been reported that is based on stable notochord-specific expression of H-Ras^{Val12} (Burger et al., 2014). This chordoma model could help us to dissect in detail the pathophysiology and molecular mechanisms of this tumor, and, moreover, might allow the high-throughput screening of potential therapeutic compounds (Burger et al., 2014).

Conclusions and perspectives

The correct deposition of an ECM network around notochordal cells is a crucial developmental process for at least three reasons. First, it confers an appropriate rigidity to the developing embryo, thus providing the mechanical support needed to withstand the hydrostatic pressure that originates from vacuoles. Secondly, the ECM–cell-dependent adhesion between the paraxial mesoderm and the notochord is a main trigger for trunk elongation during tissue morphogenesis, thereby influencing the behavior of notochord cells

by regulating different molecular programs, such as their proliferation and differentiation. Finally, the correct organization of a peri-notochordal ECM sheath is critical for the proper release of signals that are secreted by the notochord and the patterning of surrounding tissues. The availability of mutants generated by means of *in vivo* mutagenesis screens and the development of several tools that are able to interfere in a specific manner with *in vivo* gene function have allowed us to begin to dissect the roles of specific ECM components in notochord structure and function. Genetic and molecular analysis of these and other notochord mutants will be useful to gain additional insights into the mechanism of signaling related to the extracellular sheath and notochord morphogenesis. Furthermore, the rapid development of new approaches for genome editing in different vertebrate organisms, such as those based on the CRISPR/Cas9 technology, will allow to generate novel models for dissecting the mechanisms underlying notochord development and function as well as related diseases, such as chordomas.

Acknowledgements
We apologize to colleagues whose studies were not cited owing to space restrictions.

Competing interests
The authors declare no competing or financial interests.

Funding
Our work was supported by grants from the Università degli Studi di Padova and the Ministero dell'Istruzione, dell'Università e della Ricerca (FIRB Strategic Project RBAP11Z3YA_003 and PRIN grant 2015FBNB5Y to P.B.). D.C. was supported by a fellowship granted by Associazione Italiana per la Lotta al Neuroblastoma and by a post-doctoral fellowship granted by the Fondazione Umberto Veronesi.

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