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THE ROLE OF LAMININS IN CARTILAGINOUS TISSUES: FROM DEVELOPMENT TO REGENERATION

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Abstract

As a key molecule of extracellular matrix, laminin provides a delicate microenvironment for cell functions. Recent findings suggest that laminins expressed by cartilage-forming cells (chondrocytes, progenitor cells and stem cells) could promote chondrogenesis. However, few papers outline the effect of laminins on providing a favorable matrix microenvironment for cartilage regeneration. In this review, we delineated the expression of laminins in hyaline cartilage, fibrocartilage and cartilage-like tissue (nucleus pulposus) throughout several developmental stages. We also examined the effect of laminins on the biological activities of chondrocytes, including adhesion, migration and survival. Furthermore, we scrutinized the potential influence of various laminin isoforms on cartilage-forming cells' proliferation and chondrogenic differentiation. With this information, we hope to facilitate an understanding of the spatial and temporal interactions between cartilage-forming cells and laminin microenvironment to eventually advance cell-based cartilage engineering and regeneration.

Keywords: Laminin, cartilage, regeneration, stem cell, matrix microenvironment.

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Introduction

Cartilage is a specialized connective tissue with multi-component extracellular matrices (ECMs) that maintain its functionality. The major resident cells, chondrocytes, are responsible for the production of extracellular molecules, such as collagen, laminin and fibronectin (Tavella *et al.*, 1997; Wilusz *et al.*, 2014). Despite the progress in cartilage engineering (Bernhard and Vunjak-Novakovic, 2016), insufficient cartilage regeneration remains a significant clinical challenge, due to an absence of blood supply (Mobasheri *et al.*, 2014; Roelofs *et al.*, 2013).

Chondrocytes in cartilaginous tissues, such as hyaline cartilage, fibrocartilage and cartilage-like tissue (nucleus pulposus), are surrounded by a thin pericellular matrix (PCM), which is different from the territorial matrix and interterritorial matrix in both biochemical and biomechanical properties (Poole *et al.*, 1984; Wilusz *et al.*, 2014). Increasing evidence suggests that PCM contains laminin (LM), collagen type IV, nidogen and perlecan, which form the functional equivalent of a basement membrane (Kvist *et al.*, 2008). Compared to the kidney, an organ highly enriched in glomerular basement membranes, articular chondrocytes exhibited significantly higher expression of LM α4, LM β1 and nidogen-2, despite comparable levels of LM α1, LM α2 and LM α5 (Kvist *et al.*, 2008). The distribution and abundance of basement membrane components in cartilage are age-dependent. A gradual shift is distinct, from a diffuse expression in the territorial and interterritorial

matrices of newborn mice toward a pericellular localization in mature cartilage, which then becomes less distinct once reaching old age (Kvist *et al.*, 2008).

Basement-membrane proteins are present in several tissues and organs including skin and muscle, where they have been reportedly involved as critical components of the stem cell niche, regulating the functions of progenitor cells during healthy and diseased states (Boonen and Post, 2008; Fuchs, 2009). In articular cartilage, the staining of basement membrane proteins in the PCM was most prominent around cells of the cartilage superficial layer (Kvist *et al.*, 2008), which is known as a niche for chondroprogenitors (Candela *et al.*, 2014). Furthermore, a recent study found enhanced LM α1, LM α5 and nidogen-2 in the PCM of osteoarthritic chondrocytes, suggesting that laminin promotes restoration of chondrocyte phenotypes (Schminke *et al.*, 2016). The above evidence indicates that basement membrane components, especially laminin, might play a crucial role in regulating the fate and functions of chondroprogenitors and chondrocytes in cartilage repair and regeneration.

As a critical component in the basement membrane of various tissues, laminins, a family of heterotrimeric glycoproteins, contain one of five α-chains, one of three β-chains and one of three γ-chains (Fig. 1**A**) (Aumailley, 2013; Schéele *et al*, 2007). Laminins were reported to direct various cellular functions, including adhesion, migration, growth, differentiation and apoptosis, through intercommunication with specific cell surface receptors, such as dystroglycan, sulfated glycolipids or particular integrins (Fig. 1**B**) (Aumailley and Rousselle, 1999; Colognato and Yurchenco, 2000; Eble, 2001; Häusler *et al.*, 2002; Hohenester *et al.*, 2013; Vuoristo *et al.*, 2009; Yamada and Sekiguchi, 2015). Recent studies have implicated laminins in various disorders and diseases, such as hepatocellular carcinoma and congenital muscular dystrophy (Hall *et al.*, 2007; Petz *et al.*, 2012).

Increasing evidence indicates that ECMs can influence cartilage regeneration by regulating cell fate and functionality (Connelly *et al.*, 2011; Lynch and Pei, 2014). Currently, the interaction between collagen or fibronectin and cartilage regeneration has drawn much attention (Aigner and Stöve, 2003; Stoffels *et al.*, 2013). However, few review papers about potential roles of laminin and its isoforms on cartilage-forming cells for cartilage regeneration are available. In this review, the expression of laminins was outlined in various developmental stages of cartilage and cartilage-like tissues, including developing, adult and

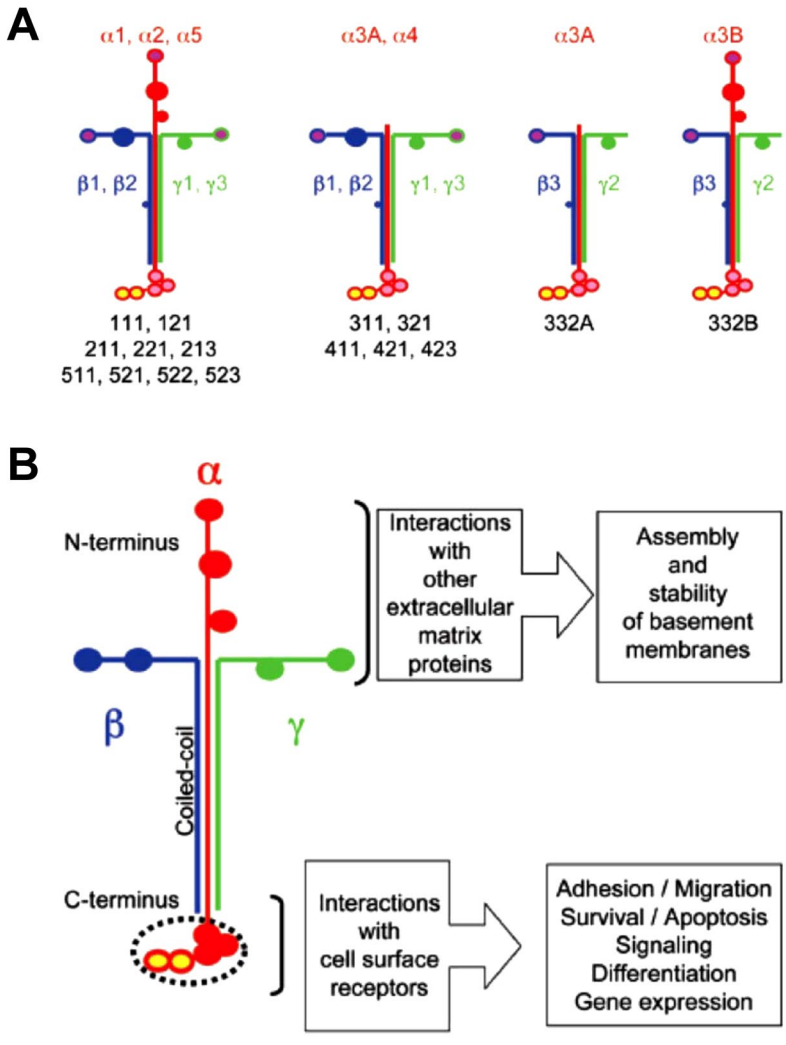

Fig. 1. Laminin family. (**A**) Known and/or predicted laminin heterotrimers. Eleven genes encode five α, three β and three γ chains in the human genome. There are two transcripts for the LM α3 chain, one short α3A and one long α3B transcript. (**B**) Mapping of the major functions of laminins. The laminin short arms (N-terminus) are involved in architectural function within the basement membrane, while the end of the long arm (C-terminus) is typically involved in cellular interactions. Reprinted with permission from Aumailley (2013).

pathological cartilage (Fig. 2). The effect of laminins on the biological activities of chondrocytes was also discussed, as well as stem cell chondrogenesis, in terms of adhesion, migration, survival, proliferation and chondrogenic differentiation (Fig. 3). This review allows an in-depth understanding of the role of laminins in providing a favorable matrix microenvironment for regeneration of cartilaginous tissues.

Stage-dependent expression of laminin in cartilage and cartilage-like tissue

Increasing evidence indicates that chondrocytes are responsible for the production of various laminins (Table 1**a**,**b**) that mainly locate in the PCM of cartilage (Foldager *et al.*, 2014; SundarRaj *et al.*, 1995). The expression of laminins varies during different developmental stages of cartilage and cartilage-like tissues (Fig. 2) (Dürr *et al.*, 1996; Foldager *et al.*, 2016; Lee *et al.*, 1997).

Developing stage

A diverse distribution pattern of laminins was found in different developing stages of cartilage (Dürr *et al.*, 1996; Häusler *et al.*, 2002). Lee *et al.*, (1997) found that laminin chains (α1, α2, β1, β2 and γ1), produced by chicken embryo sternal chondrocytes, exhibited an increased expression in aggregated cells during the maturation stage; LM-111 was detected primarily in the cytoplasm, rather than in the matrix of cartilaginous tissues. Kvist *et al.* (2008) reported that laminin, initially being widespread in newborn cartilage, organized into a pericellular distribution around the chondrocytes in mature cartilage. Similar to the location pattern in the superficial layer of adult articular cartilage, most laminins were detected in the resting zone in epiphyseal cartilage and expression decreased in the proliferating and hypertrophic zones (Dürr *et al.*, 1996; Ustünel *et al.*, 2003a). These findings indicate that laminins are dynamically expressed in a spatiotemporal manner.

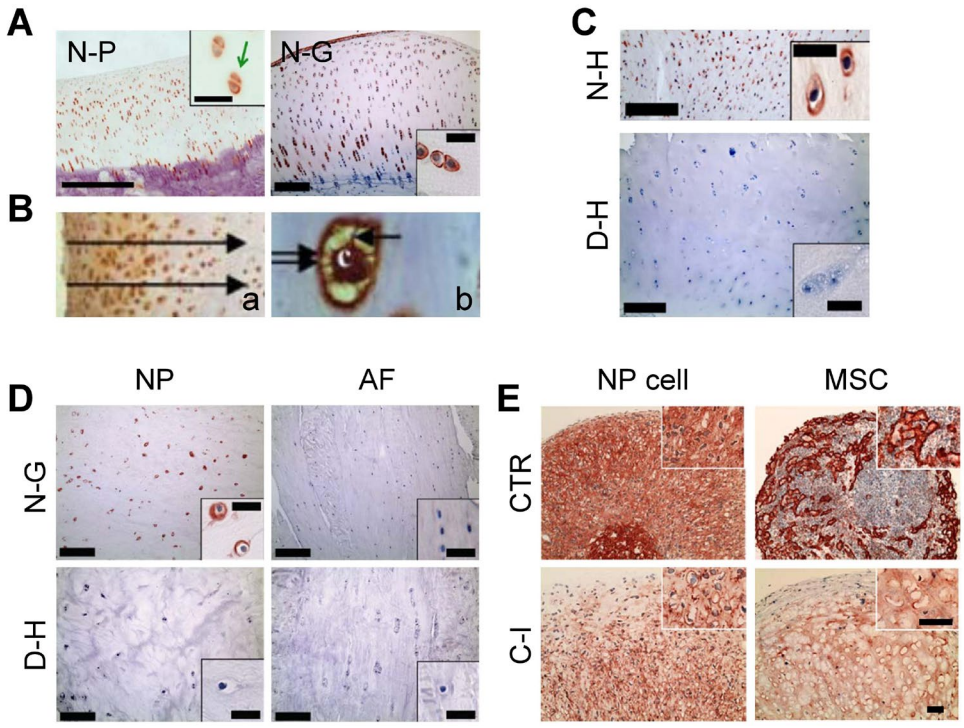

Fig. 2. Laminin expression in hyaline cartilage, fibrocartilage and cartilage-like tissues (NP), as well as cartilage-forming cells, under chondrogenic induction. (**A**) Normal articular cartilage from porcine (N-P) (Foldager *et al.*, 2016) and goat (N-G) (Foldager *et al.*, 2014). Bars: large image = 200 μm, small image = 30 μm (N-P) and 20 μm (N-G). (**B**) A decrease of immunostaining from the periphery to deeper parts of human nasal septal cartilage in the direction of the arrows (a); immunostaining of laminin in chondrocyte cytoplasm (c), projections (arrows) and pericellular rings (double arrows) is strong (b) (Üstünel *et al.*, 2003b). In the original publication, scale bars were not shown but magnification values of (a) ×25 and (b) ×100 were given. (**C**) Laminin-positive pericellular stain was only detectable in normal human (N-H) articular cartilage rather than degenerated human (D-H) articular cartilage (Foldager *et al.*, 2014). Bars: large image = 200 μm, small image = 20 μm. (**D**) Laminin-positive stain was only stained in normal goat (N-G) NP tissue rather than in the degenerated human (D-H) NP tissue and both normal goat and degenerated human AF tissues (Foldager *et al.*, 2014). Bars: large image = 200 μm, small image = 20 μm. (**E**) Immunohistochemistry of laminin in goat NP cell and bone marrow-derived MSC pellets after 14 d of chondrogenic induction (C-I). The group without treatment serves as a control (CTR) (Toh *et al.*, 2013). Scale bar: 50 μm. Reprinted with permission from Foldager *et al.* (2014), Foldager *et al.* (2016), Toh *et al.* (2013) and Üstünel *et al.* (2003b).

During the development of intervertebral disc (IVD), laminins gradually appear but have a shifting pattern in different developmental stages. Using immunofluorescence labelling procedures, Hayes *et al.* (2001) found that laminin was distributed pericellularly in developing nucleus pulposus (NP), annulus fibrosus (AF) and vertebral bodies of rats. Furthermore, Toh *et al.* (2013) cultured goat NP cells in a pellet system to form cartilaginous tissue and found that laminins were expressed with an orderly shift from a diffused distribution to a defined pericellular localization. Interestingly, dramatic differences in laminin expression existed in the cells between the immature NP and AF region. Chen *et al.* (2009) found higher levels of the LM α5 chain and related receptors in immature rat and pig NP regions compared to the AF, but AF regions had more intense expression and, frequently, more LM α1 chains than NP tissue. These studies demonstrate that, similar to the pattern in cartilage, laminins are continuously expressed in all developing stages in cartilage-like tissues and show a region-specific expression pattern.

In addition, laminins were found in tissue engineered cartilaginous constructs. For instance, an extensive expression of laminin was observed *in vitro* following three weeks of chondrogenic culture of bone marrow-derived mesenchymal stem cells (MSCs) in both poly (ethylene glycol) diacrylate (PEGDA) hydrogel (Köllmer *et al.*, 2012) and hyaluronic acid-based hydrogel (Toh *et al.*, 2012). Similarly, Jeng *et al.* (2012, 2013) found widespread expression of laminins throughout the ECM, in both engineered cartilage constructs and reparative tissues, following implantation of chondrocyte-seeded constructs in caprine cartilage defects – although the expression of laminin appeared diffused compared to the pericellular staining pattern observed in normal adult cartilage.

Adult stage

As an important ECM component, in adult cartilage, laminins participate in the organization of the basement membrane-like structure around the chondrocytes. Dürr *et al.* (1996) demonstrated that laminins were mainly located in the PCM of human adult articular cartilage, which was further verified in goat and bovine cartilage (Foldager *et al.*, 2014; Kvist *et al.*, 2008). Ustünel *et al.* (2003b) found a similar pattern of laminin expression in human nasal septal cartilage with higher expression in the periphery of the cartilage, which gradually decreased in deeper zones. Laminins were also found in meniscus and some other fibrocartilage (Chu *et al.*, 2017; Foldager *et al.*, 2014; Salter *et al.*, 1995). However, the expression of laminins displayed a more pericellular diffusion in menisci, which was different from the well-defined pericellular localization of articular cartilage (Foldager *et al.*, 2014). Similarly, in the

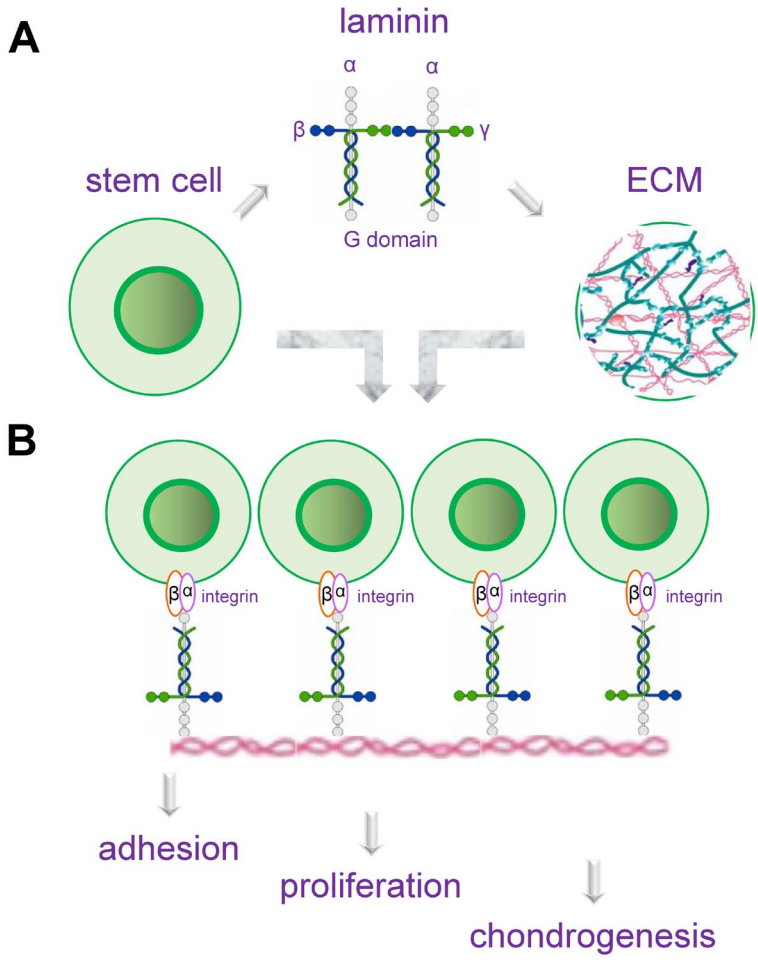

Fig. 3. The expression and function of laminin during cartilaginous tissue regeneration. (**A**) Stem cells produce laminin and form the ECM (Laperle *et al.*, 2015; Rodin *et al.*, 2014). (**B**) After adhesion, laminin promotes stem cell proliferation and regulates chondrogenesis *in vitro* (Hashimoto *et al.*, 2006; Toh *et al.*, 2013).

Table 1a. Laminin expression pattern in hyaline cartilage and fibrocartilage. Abbreviation: ACI: autologous chondrocyte implantation; AF: annulus fibrosus; DO: day old; MO: month old; NP: nucleus pulposus; OA: osteoarthritis; OARSI: Osteoarthritis Research Society International; WO: week old; YO: year-old.

	Age	Species	LM types	Location pattern	Reference
hyaline cartilage and fibrocartilage	embryo	chicken	LM α1, α2, β1, β2, γ1	sternal cartilage	Lee *et al.*, 1997
	embryo	mouse	LM α1, α2, β1, β2, γ1	14-DO limb bud cartilage	Lee *et al.*, 1997
	embryo/ neonate	rat	LM	AF tissue	Hayes *et al.*, 2001
	fetus	human	LM	16-WO knee joint cartilage (articular, epiphyseal and meniscus)	Salter *et al.*, 1995
	fetus	human	LM α1, β1, γ1	resting-zones of 30-WO tibia epiphyseal cartilage	Dürr *et al.*, 1996
	fetus	human	LM γ2	cartilage at early stages of gestation	Lu *et al.*, 2001
	neonate	mouse	LM	mandibular condyle cartilage	Silbermann *et al.*, 1990
	newborn /adult	mouse	LM α1, α2, α4, α5, β1, γ1	femoral head cartilage	Kvist *et al.*, 2008
	childhood /adolescent	human	LM	resting-zones of growth plate cartilage	Häusler *et al.*, 2002
	immature	rat	LM	60-DO humerus proximal epiphyseal cartilage (articular cartilage and epiphyseal growth plate)	Ustünel *et al.*, 2003a
	immature	rat	LM	temporomandibular joint condylar cartilage and disc tissue	Chu et al., 2017
	immature	porcine	LM α1	3-MO AF tissues	Chen *et al.*, 2009
	immature	rat	LM α1	1-MO AF tissues	Chen *et al.*, 2009
	immature	porcine	LM γ1	AF tissues	Gilchrist *et al.*, 2007
	adult	goat	LM	normal articular cartilage, meniscus and calcified cartilage	Foldager *et al.*, 2014
	adult	bovine	LM	18-MO metacarpophalangeal joint cartilage	Kvist *et al.*, 2008
	adult	human	LM α1, β1, γ1	upper zone of articular cartilage	Dürr *et al.*, 1996
	adult	human	LM	nasal cartilage	SundarRaj *et al.*, 1995
	adult	human	LM	mandibular condyles cartilage degenerative lesion	Ishibashi *et al.*, 1996
	adult	human	LM	nasal septal cartilage	Ustünel *et al.*, 2003b
	adult	human	LM α4	high expression in arthritis cartilage lesions grade IV hypertrophic chondrocyte clusters according to the OARSI criteria for osteoarthritis	Fuerst *et al.*, 2011
	adult	human	LM	normal articular cartilage	Foldager *et al.*, 2014
	adult	human	LM	normal articular cartilage; no expression in traumatically damaged cartilage and clinically failed repair cartilage	Foldager *et al.*, 2016
	adult	human	LM α1, α5	healthy and OA articular cartilage	Schminke *et al.*, 2016
	adult	mouse	LM α1, α2, β1, β2, γ1	3-WO knee joint cartilage	Lee *et al.*, 1997
	adult	porcine	LM	normal articular cartilage/repair tissue after scaffold-seeded ACI	Foldager *et al.*, 2016

Table 1b. Laminin expression pattern in cartilage-like tissues. Abbreviation: ACI: autologous chondrocyte implantation; AF: annulus fibrosus; DO: day old; MO: month old; NP: nucleus pulposus; OA: osteoarthritis; OARSI: Osteoarthritis Research Society International; WO: week old; YO: year-old.

	Age	Species	LM types	Location pattern	Reference
Cartilage-like tissue (NP tissue)	embryo/ neonate	rat	LM	NP and notochordal cell surface	Hayes *et al.*, 2001
	immature	porcine	LM γ1	NP tissue	Gilchrist *et al.*, 2007
	immature	porcine	LM γ2	3-6-MO NP tissue	Gilchrist *et al.*, 2011a
	immature	rat	LM γ2	1-MO NP tissue	Gilchrist *et al.*, 2011a
	2-, 12-, 35-YO	human	LM α5, γ1	age-dependent decrease in NP tissue, particularly LM γ1	Chen *et al.*, 2009
	3-, 24-MO	porcine	LM α1, α5	NP tissues	Chen *et al.*, 2009
	1-, 12-MO	rat	LM α1, α5	NP tissues, particularly LM α5	Chen *et al.*, 2009
	mature	goat	LM	normal NP tissues	Foldager *et al.*, 2014
	mature	goat	LM	NP cells, pellet, and native tissues	Toh *et al.*, 2013

rat temporomandibular joint (TMJ), laminins were found present in the PCM surrounding individual chondrocytes, but were predominately distributed in the proliferative zone of the condylar cartilage (Chu *et al.*, 2017).

In adult cartilage-like tissues, laminins produced by mature goat NP cells in a pellet culture system also formed ECMs with a pericellular distribution (Toh *et al.*, 2013). Moreover, many laminins and some subunits, such as LM α1, α5 and γ1, were located in the PCM of goat, porcine, human and rat NP cells (Chen *et al.*, 2009; Foldager *et al.*, 2014; Toh *et al.*, 2013). Similar to the findings in immature IVD, laminins produced by mature NP cells showed a region-specific expression pattern. Chen *et al.* (2009) found that the LM α5 chain had more significant expression in NP than AF regions, although with lower expression compared to immature NP tissues.

Pathological stage

Disorders of ECM formation are the significant characteristics of cartilage degradation, which can influence subsequent reactive processes for degeneration (Lu *et al.*, 2011; Moazedi-Fuerst *et al.*, 2016). In most studies, the expression of laminins significantly decreased or disappeared in degenerative articular cartilage and menisci (Foldager *et al.*, 2014; Foldager *et al.*, 2016; Ishibashi *et al.*, 1996). Also, the expression of laminins showed an age-related change in degenerative cartilage (Ishibashi *et al.*, 1996). Considering the prominent expression in developing and normal cartilage, as well as the negative expression in traumatically-damaged cartilage and cartilage that failed clinical repair (Foldager *et al.*, 2016), laminin could serve as an early marker for cartilage degeneration by observing its dynamic expression. Interestingly, the diverse expression of laminins in degenerative cartilage also indicate their role in cartilage degeneration (Moazedi-Fuerst *et al.*, 2016; Schminke *et al.*, 2016). For example, LM α4, with significantly higher expression in severely degenerated sites, compared with mild areas, in human osteoarthritic cartilage, co-localized with syndecan-4 around hypertrophic chondrocytes and perpetuated cartilage damage in osteoarthritic cartilage, which suggested that LM α4 played a deleterious role in cartilage degeneration (Fuerst *et al.*, 2011). Therefore, laminins may be a possible regulator in degenerative cartilage.

Similar findings have been uncovered in degenerative cartilage-like tissues. Chen *et al.* (2009) reported that laminin chains decreased in older specimens compared with immature NP, which was inferred as a cause of decreased cellularity in older tissues. Foldager *et al.* (2014) found significant expression of laminin in healthy goat NP, but failed to find laminin expression in human degenerative NP tissue using immunohistochemical analysis. Collectively, these findings suggest that laminins are altered in expression pattern and/or decreased in quantity in conjunction with the degeneration of the cartilage-like tissues, implicating the role of laminin in cartilage degeneration.

The effect of laminin on chondrocyte function

Laminins are important cell-adhesive ligands and are mainly present around chondrocytes. The interactions between laminins and chondrocytes can regulate many cell-biological functions, such as adhesion, migration and survival (Bulić, 1996; Francisco *et al.*, 2014; SundarRaj *et al.*, 1995).

Cell adhesion

As major adhesive molecules of ECM in cartilaginous tissues, laminins exert a prominent role in regulating cell-cell or cell-matrix interactions. Many studies demonstrated that LM-511, LM-332 and LM-111 have strong cell attachment capabilities in

chondrocytes and NP cells (Dürr *et al*., 1996; Gilchrist *et al*., 2011a; Gilchrist *et al*., 2011b). For example, Dürr *et al*. (1996) demonstrated that human fetal chondrocytes could attach to the E8 fragment of LM-111, mainly depending on the interaction with integrin $\alpha 6\beta 1$. Moreover, Francisco *et al*. (2013) found that supplementation with LM-111 in an injectable functionalized hydrogel promoted adhesion of porcine NP cells. Multiple cell surface receptors mediated the adhesion of laminins with chondrocytes and NP cells (Dürr *et al*., 1996; Gilchrist *et al*., 2007; Nettles *et al*., 2004), especially through integrins (Loeser, 2014). Blocking studies indicated that integrin β1 or $\alpha 6\beta 1$ were primary receptors for strong cell attachment in human chondrocytes and NP cells (Bridgen *et al*., 2013; Dürr *et al*., 1996). These results demonstrate that laminins can potentiate the strength of cell adhesion by binding to integrins. Furthermore, adhesion capacity is significantly different among various types of laminin. For instance, Gilchrist *et al*. (2011a) demonstrated by analysis of adherent numbers and detachment strength in immature porcine NP cells, that LM-511 and LM-332 displayed stronger effects than other matrices, such as LM-111, type II collagen and fibronectin. Diminished NP cell adhesion on LM-111 is consistent with its relatively low expression in porcine, rat or human immature NP tissues (Chen *et al*., 2009; Gilchrist *et al*. 2011a). The discrepancy in adhesion capacities of laminins may be explained by receptor binding differences; in other words, a prominent integrin subunit may exist in special cell types and regions.

Cell migration

Under stimuli of various matrices, chondrocytes are able to migrate to regulate biological activities. Bulić (1996) demonstrated that laminin and laminin-derived peptides promoted maximal bovine articular chondrocyte migration, but migration subsequently decreased when subjected to a higher compound concentration. Moreover, Moazedi-Fuerst *et al*. (2016) found that *in vitro* blocking of LM α4 significantly decreased cluster formation of human osteoarthritic chondrocytes. Interestingly, they found that LM $\alpha 4$ was important for targeted migration but did not inhibit movement. Furthermore, immature porcine NP cells could attach rapidly to LM-511 and LM-332 substrates, suggesting the positive role of laminin in regulating migration of NP cells (Gilchrist *et al*., 2011a). These studies suggest that laminins have a regulatory effect on migration of cartilage-forming cells.

Even though laminins are actively involved in cell migration (Gorfu *et al*., 2008; Nguyen-Ngov *et al*., 2012), the detailed mechanisms underlying the effects of laminins on chondrocyte migration are still unclear. Matrix metalloproteinases (MMPs), which are responsible for the degradation of collagen type II and digestion of proteoglycan and other non-collagen proteins in osteoarthritis, exerted regulative roles in cell migration (Laurent *et al*., 2003; Li *et al*., 2013; Moazedi-Fuerst *et al*., 2016). Laminins might control chondrocyte migration by regulating MMPs; for example, LM $\alpha 4$ blockade could downregulate MMP3 and upregulate MMP16 (Fuerst *et al*., 2011; Moazedi-Fuerst *et al*., 2016).

Cell survival

Laminins are known to promote cell survival in several cell types by mediating cell-laminin interactions in response to various environmental conditions *in vitro* (Ekblom *et al*., 2003; Gu *et al*., 2002). Bulić (1996) demonstrated that IKVAV sequence-containing peptide derived from the laminin could promote proliferation of bovine articular chondrocytes, suggesting a positive role of laminin in promoting chondrocyte survival. Furthermore, an increasing number of studies have demonstrated that laminin had the same effect in promoting NP cell survival (Francisco *et al*., 2014; Gilchrist *et al*., 2011a). Francisco *et al*. (2014) found that LM-111 could significantly increase viability of NP cells in a three-dimensional (3D) poly (ethylene glycol) (PEG)-laminin hydrogel compared to blank gels – despite the inhibition of cell viability by PEG hydrogel alone – suggesting that LM-111 retained the bioactivity of the native protein in 3D PEG hydrogels and cell survival was mainly mediated by cell-LM-111 interactions. These results also suggest that laminin may be a survival ligand for NP cells. However, contrary reports also exist showing that laminin may have different effects on chondrocyte survival. Chu *et al*. (2017) demonstrated that, as opposed to collagen types IV and VI, laminin had no effect on cell viability and proliferation of rat TMJ condylar and disc chondrocytes. In another example, Fuerst *et al*. (2011) found that MMP3 expression was significantly downregulated in human chondrocytes from mild osteoarthritis after neutralizing LM $\alpha 4$. Considering the damaging effect of MMP3 on cartilage, the results demonstrate that LM $\alpha 4$ has the opposite effect on chondrocyte survival and may aggravate cartilage damage in osteoarthritis. This disparity in chondrocyte survival response to laminins may be explained by different responses of chondrocytes to various laminin isoforms.

The effect of laminin on stem cell proliferation

Cartilage-forming cells, such as stem cells and progenitor cells, are able to differentiate into a chondrogenic lineage under specific stimulation and they play important roles in cartilage regeneration (Li *et al*., 2014; Pizzute *et al*., 2016; Toh *et al*., 2014; Zhang *et al*., 2015). Due to the increasing loss of proliferation capacity and risk of spontaneous differentiation resulting from replicative senescence, it is difficult to obtain a sufficient number of stem cells to differentiate into chondrocytes for autologous transplantation (Li and Pei, 2012; Toh *et al*., 2016b). Therefore, acquisition of a sufficient number of stem cells by increasing proliferation before inducing differentiation is a critical step for cartilage regeneration (Pei, 2017).

Positive effects

Although there are few reports on the effect of laminins on chondrocyte survival, recent studies have shown the roles of laminins in regulating proliferation and apoptosis of chondroprogenitor cells, such as mouse teratocarcinoma-derived chondrogenic cell line (ATDC5). Choi *et al.* (2010) demonstrated that laminin could significantly increase proliferation and decrease apoptosis of ATDC5 cells, when cultured on laminin-derived peptide-coated surfaces of hybrid mussel adhesive proteins (fp-151) compared with those on non- and bare fp-151-coated surfaces. These results indicate that activation of integrin signaling by laminin might be responsible for enhanced cell survival in ATDC5 cells. In addition, LM-111, LM-332 and Matrigel (the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, BD Biosciences, San Jose, CA, USA) could efficiently promote proliferation of mouse neural progenitor cells derived from induced pluripotent stem cells (iPSCs) (Komura *et al.*, 2015). Considering that hyaline cartilage, such as nasal septal cartilage, emerges from neural crest progenitor/stem cells (Crane and Trainor, 2012; Somoza *et al.*, 2014), regulation of progenitor cells' proliferation by laminins may be a promising approach to ensure substantial cell quantity for applications in regenerative medicine (Leiton *et al.*, 2015; Ortinau *et al.*, 2010).

As an important molecule of ECM, many studies have demonstrated the positive effect of laminins on promoting proliferation in various stem cells (Table 2). Increasing evidence suggests that specific laminin isoforms could enhance the proliferation capacity of adult stem cells when cultured on a coated or soluble laminin environment (He *et al.*, 2013; Lam *et al.*, 2012; Lindner *et al.*, 2010; Mathews *et al.*, 2012). Indeed, previous studies have shown that laminins are secreted by various stem cell types, implying the role of laminins in regulating stem cell renewal and differentiation (Toh *et al.*, 2013; Toh *et al.*, 2016a). Hashimoto *et al.* (2006) found that LM-332 and LM-511/521, but not LM-111 and LM-211/221, could promote the adhesion of human MSCs with LM-332 having the largest number of cells attached and spread well within 10 min. Also, they found that LM-332 promoted proliferation of human MSCs through interactions with integrins $\alpha 3\beta 1$ and $\alpha 6\beta 1$.

Pluripotent stem cells (PSCs), consisting of embryonic stem cells (ESCs) and iPSCs, have significant abilities to differentiate into chondrocytes (Ko *et al.*, 2014; Toh *et al.*, 2010). Multiple studies have shown that laminins, normally expressed by these PSCs, were indicative of the cells' functions (Laperle *et al.*, 2015; Vuoristo *et al.*, 2009). For example, laminins promoted strong renewal and stimulated efficient proliferation of PSCs as a robust substratum (Lam *et al.*, 2012; Rodin *et al.*, 2010; Rodin *et al.*, 2014; Vuoristo *et al.*, 2009). By comparing the effects on mouse ESC proliferation of different laminin isoforms, Domogatskaya *et al.* (2008) found that LM-511 and LM-332 could promote ESC proliferation, while LM-111, Matrigel and gelatin caused rapid differentiation. The finding was later confirmed in a study by Miyazaki *et al.* (2012), who reported that the E8 fragments of LM-511 and LM-332 interacted with integrin $\alpha 6\beta 1$ to promote robust proliferation for up to ten passages of human ESCs and iPSCs in their undifferentiated state.

Although the above studies verify that laminins are effective in promoting stem cell proliferation, the results from the two-dimensional (2D) environment *in vitro* could be different from those of the 3D ECM microenvironment *in vivo*, in which stem cells mainly reside (Pei *et al.*, 2011). Thus, scaffold modification to incorporate laminins could be applied to promote bioactivity of scaffolds in the 3D culture system (Brynda *et al.*, 2009; Heydarkhan-Hagvall *et al.*, 2012; Kang *et al.*, 2012). He *et al.* (2013) utilized laminin to modify poly(l-lactide) scaffolds and found that larger amounts of laminins could promote proliferation of mouse neonatal stem cells. The study also suggested that laminin modification was essential for stem cells to build up a 3D growth microenvironment and that laminin concentration could affect cell proliferation. Taken together, laminins exert significant effects on promoting stem cell proliferation in both the 2D and 3D microenvironment.

Negative effects

Although most reports demonstrate that laminins can promote stem cell proliferation, some studies show that laminins have, to a certain degree, inhibitory effects on stem cell and progenitor cell proliferation, by reducing cell number and viability during expansion (Abay *et al.*, 2016; Celebi *et al.*, 2011; Heydarkhan-Hagvall *et al.*, 2012; Ode *et al.*, 2010; Qian and Saltzman, 2004). Seeger *et al.* (2015) found that LM-211, LM-411, LM-511 and LM-521 inhibited proliferation of undifferentiated MSCs, but upon myogenic differentiation, only LM-521 significantly enhanced proliferation of myogenically differentiating cells. In another study, LM-111 was found to inhibit proliferation by triggering ESC differentiation within two weeks, while LM-411 failed to support survival of ESCs (Domogatskaya *et al.*, 2008). These findings suggest that laminins may have negative effects on stem cell proliferation by activating differentiation and/or reducing the adhesion of stem cells. Furthermore, Arulmoli *et al.* (2016) found that human neural stem/progenitor cells grew well in fibrin and combination scaffolds with hyaluronic acid, but the addition of laminin could not significantly increase cell proliferation by quantitation of the Ki-67 immunostaining-positive cells. Interestingly, Matrigel, which contains laminins, collagens, heparin sulfate proteoglycans and growth factors, induced significant improvement in cell proliferation (Addington *et al.*, 2014; Vuoristo *et al.*, 2009), suggesting that a combination of ECM components and inherent growth factors affects cell proliferation.

Table 2a. Positive effect of laminins on stem cells' proliferation. Abbreviation: ADSCs: adipose derived MSCs; BMMNCs: bone marrow mononuclear cells; BMSCs: bone marrow derived MSCs; C17.2 cell: neonatal mouse cerebellum stem cell; CFU-F: colony-forming unit fibroblast; ECM gel: basement membrane extracellular matrix protein gel, from Sigma-Aldrich; ESCs: embryonic stem cells; HUCB: human umbilical cord blood; iPSCs: induced pluripotent stem cells; LDH: lactate dehydrogenase; LM: laminin; NSCs: neural stem cells; NSPCs: neural stem/precursor cells; PLLA: poly-L-lactide; Matrigel: the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells.

Age	Species	Stem cell type	Assay	Results	Reference
adult	human	ADSCs	cell counting	LM coating promoted cell proliferation	Lam *et al.*, 2012
adult	human	ADSCs	LDH assay	LM coating increased cell proliferation	Keller *et al.*, 2016
adult	human	BMSCs	cell counting	LM-111, LM-332 or ECM gel promoted cell proliferation	Lindner *et al.*, 2010
adult	human	BMSCs	cell counting	LM coating promoted cell proliferation	Mathews *et al.*, 2012
adult	human	BMSCs	cell counting	LM-332 coating promoted cell growth; LM-511/521 or LM-111 slightly promoted growth	Hashimoto *et al.*, 2006
adult	human	STRO-1(+) BMMNCs	CFU-F efficiency	LM coating increased the amount and size of colonies	Gronthos *et al.*, 2001
neonate	mouse	C17.2 cell	MTS cell proliferation assay	LM modified PLLA nanofibrous scaffolds enhanced cell proliferation	He *et al.*, 2013
postnatal	human	NSPCs	neurosphere and cell counting	LM coating increased cell number and neurosphere size	Flanagan *et al.*, 2006
fetus	human	NSCs	neurosphere counting	LM coating increased primary neurosphere formation	Hall *et al.*, 2008
fetus	human	HUCB-derived NSCs	Ki67+ cells counting	LM coating promoted cell proliferation rate	Szymczak *et al.*, 2010
embryo	human	ESCs	colony size	LM-511 or Matrigel rather than LM-111 promoted cell proliferation	Vuoristo *et al.*, 2009
embryo	human	ESCs	cell counting and average contact area	LM-511 coating promoted cell proliferation for at least 28 passages	Rodin *et al.*, 2010
embryo	human	ESCs	growth curve and colony counting	LM-521 coating promoted robust renewal and the addition with E-cadherin permitted efficient clonal expansion	Rodin *et al.*, 2014
embryo	human	ESCs/iPSCs	cell counting	LM coating promoted cell proliferation	Lam *et al.*, 2012
embryo	human	ESCs/iPSCs	cell counting	LM-E8 fragments from LM isoforms supported cell proliferation	Miyazaki *et al.*, 2012
embryo	human	ESCs/iPSCs	cell counting and Ki67+ cells	Knockdown of LM-α5 gene diminished cell proliferation	Laperle *et al.*, 2015
embryo	mouse	NSPCs	neurosphere and cell counting	LM coating increased cell number and neurosphere size	Flanagan *et al.*, 2006
embryo	mouse	ESCs	cell counting	LM-511 or LM-332 promoted cell proliferation	Domogatskaya *et al.*, 2008
embryo	mouse	ESCs	proliferation index by flow cytometry	LM-111 coating promoted cell proliferation	Suh *et al.*, 2012

The effect of laminin on chondrogenesis

It is well known that chondrocytes maintain phenotype and function by regulating specific markers, such as collagen type II and sulfated glycosaminoglycans (GAGs) (von der Mark and Conrad, 1979). Schminke *et al*. (2016) found that laminin could significantly upregulate the level of *COL2A1* (collagen type II gene) and reduce the level of *COL1A1* (collagen type I gene) in healthy and osteoarthritic chondrocytes. Laminin-presenting hydrogels can markedly promote the production of sulfated GAGs in NP cells (Francisco *et al*., 2014; Gilchrist *et al*., 2011b). In a pellet culture system, Toh *et al*. (2013) reported an orderly spatiotemporal shift in expression of laminin from a diffuse territorial and interterritorial distribution to a defined pericellular localization, following chondrogenic induction of bone marrow-derived MSCs. Further studies also showed that laminins directly upregulated *COL2A1* expression in human chondrogenic progenitor cells and GAG content in human MSCs (Lindner *et al*., 2010; Schminke *et al*., 2016), indicating that laminins have essential roles in promoting chondrogenesis of cartilage-forming cells. However, some studies found that laminins were differentially expressed with an obvious trend: an increase in cell aggregation during development followed by a decrease during chondrogenesis (Tavella *et al*., 1997; Toh *et al*., 2013). Moreover, the expression of laminins exists in developing and normal cartilage but disappears in degenerative, traumatically-damaged cartilage and in cartilage that fails clinical repair, suggesting a spatiotemporal distribution and function of laminins in chondrogenesis (Foldager *et al*., 2004; Foldager *et al*., 2016). Growing evidence shows that ECM components can induce chondrogenic differentiation in chick embryo limb-bud mesenchymal cells and human MSCs, but laminin alone fails to drive chondrogenic activity (Bradham *et al*., 1995; Matsubara *et al*., 2004), suggesting that laminins might participate in the process of chondrogenesis with other regulatory factors. For instance, LM-332 promotes proliferation but suppresses chondrogenic differentiation (Lindner *et al*., 2010) by regulating integrin $\alpha 3\beta 1$ activities in human MSCs and mouse ATDC5 cells (Hashimoto *et al*., 2005; Hashimoto *et al*., 2006), while favorably enhancing osteogenesis *via* an integrin/FAK/ERK1/2 signaling pathway (Salasznyk *et al*., 2007). Despite these studies that suggest the roles of laminins in chondrogenesis, the dynamic expression of various laminin isoforms and their functions during chondrogenesis has not been fully delineated. It is likely that the expression of laminins is highly regulated during proliferation and differentiation and specific laminin isoforms could be involved in lineage-specific differentiation. Looking ahead, a better understanding of laminin expression and its functions would likely enable better control of chondrogenesis.

Conclusions and perspectives

As critical components of ECM, laminins play important roles in providing a favorable microenvironment for cartilage regeneration. In this review, there is increasing evidence showing that laminins, secreted by chondrocytes and primarily located in the PCM in cartilage and cartilage-like tissues, are involved in the regulation of chondrocyte activities, such as adhesion, migration and survival. Furthermore, the role of laminins in stem cell proliferation and chondrogenic differentiation was fully discussed. Also, recent studies have shown that modification of scaffolds with laminins can improve the biological activity of cartilage-forming cells for tissue engineering and applications (Francisco *et al*., 2014; Gilchrist *et al*., 2011b). Despite efforts to delineate the expression of laminins during chondrogenesis, our understanding of laminins in terms of their regulation, expression and function during chondrogenesis is still limited. Looking ahead, elucidating the spatiotemporal expression and function of specific laminin isoforms and their receptors in stem cell proliferation and lineage-specific differentiation would enable better control of chondrogenesis and greatly benefit the future clinical exploration of laminins in cell therapy for cartilage injuries and osteoarthritis (Toh *et al*., 2016a).

Some limitations exist to prevent further investigations in cell-laminin interaction and potential clinical application. For example, laminins in various isoforms are present in low concentrations and are highly cross-linked within the ECM, making it difficult to extract them from tissues or purify them from cell supernatant. Due to their large size and higher-order structure, recombinantly expressed laminins, different from their native form, are not easily obtained. Thus, the exploration of native laminin to uncover the "real" roles of laminins in cartilage regeneration is necessary. Fortunately, a recent report shows that recombinant E8 fragments of laminin isoforms (LM-E8s), which are the minimum fragments conferring integrin-binding activity, promote more robust adhesion of human ESCs and iPSCs than Matrigel and intact laminin isoforms (Miyazaki *et al*., 2012). LM-E8s maintain long-term self-renewal, high-level expression of pluripotency markers and differentiation capacity into all three germ layers (Miyazaki *et al*., 2012). Since LM-E8s are much smaller and easier to produce recombinantly and purify than intact laminins, this finding indicates that LM-E8s, the minimum structure harboring the full integrin-binding activity of laminins, are remarkable substrates for the long-term culture of human ESCs, with a significant advantage over intact laminin isoforms, such as LM-511 and LM-332 (Miyazaki *et al*., 2012).

In addition, the focus of current efforts is mainly on the use of a laminin-coated 2D culture environment,

which is different from *in vivo* 3D chondrogenesis. Increasing evidence indicates that decellularized extracellular matrix (dECM), deposited by stem cells and primary cells, provides an excellent *in vitro* 3D model, mimicking the organization of native ECM *in vivo* and can rejuvenate stem cell proliferation and chondrogenic differentiation (Pei *et al*., 2011). The *in vitro* genetic modification model, which uses overexpression and knockout of targeted genes, can facilitate investigation of the functionality of specific laminin isoforms in a 3D environment on stem cell biological activity, such as proliferation and chondrogenic differentiation.

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Discussion with Reviewer

Roberto Narcisi: Are there any reports indicating the role of mechanical stimulation on laminin expression?
Authors: Few reports are available to indicate the role of mechanical stimulation in laminin expression. One is by Ulbrich *et al.* (2010), who found that microgravity did not influence laminin and collagen type IV expression in human chondrocytes.

Roberto Narcisi: Is there any evidence that TGFβ signaling can influence laminin expression in stem cells or chondrocytes?
Authors: Yes. Some reports indicate that TGFβ signaling can influence laminin expression in stem cells or chondrocytes. For example, Toh *et al.* (2013) found that, under chondrogenic induction with TGFβ1, the percentages of NP cells and bone marrow stromal cells (BMSCs) in pellets with pericellular staining of laminin increased compared with no TGFβ1 treatment. Korecki *et al.* (2010) found that the expression level of laminin β1 increased when human BMSCs were exposed to chondrogenic medium with TGFβ3 or notochordal cell-conditioned medium.

Roberto Narcisi: Are there any data available on the effect of laminin silencing or overexpression on stem cell differentiation and proliferation?
Authors: Yes, but only a few publications. Laperle *et al.* (2015) indicated that inducible shRNA knockdown and Cas9-mediated disruption of *LAMA5* dramatically reduced hPSC self-renewal and increased apoptosis without affecting the expression of pluripotency markers. Increased self-renewal and survival was restored to wild-type levels by culturing LAMA5-deficient cells on exogenous laminin-521.

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Editor's note: The Scientific Editor responsible for this paper was Martin Stoddart.