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Hyaluronan and mesenchymal stem cells: from germ layer to cartilage and bone

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1. ABSTRACT

A simple, linear polysaccharide with unique molecular functions, hyaluronan is a glycosaminoglycan whose biomechanical and hydrodynamic properties have been thoroughly characterized. However, the exact role the molecular mechanisms and signaling pathways of hyaluronan play in the regulation of stem cell fate, such as self-renewal and differentiation, remains to be determined. The abundance of hyaluronan in embryonic tissues indicates that it is highly important in developmental processes. Recent studies have focused on understanding the mechanisms of hydrated hyaluronan action and its interaction with neighboring substances. This review is an attempt to elucidate the complex role of hyaluronan signaling in the initialization and regulation of developmental processes, particularly in events dictating the fates of mesenchymal stem cells during the organogenetic phases of chondrogenesis and osteogenesis.

2. INTRODUCTION

Hyaluronic acid (HA), or hyaluronan is a very large, high-molecular-weight, linear glycosaminoglycan composed of between 2,000 to 25,000 disaccharide units of glucuronic acid and N-acetylglucosamine (beta1,4-GlcUA-β1,3-GlcNAc-)$_n$. Unique among glycosaminoglycans, the polysaccharide has an extracellular biosynthesis site (on the plasma-cellular membrane) that lacks a core protein acceptor, a chemical composition devoid of sulphated esters, and unique and diverse biological functions. Particularly, during early development, hyaluronan serves not only as passive space filler, but it also activates and regulates essential biological events by mediating cell behavior via its effects on cellular migration, proliferation, and differentiation. Furthermore, hyaluronan is ubiquitously distributed in the extracellular matrix of invertebrate and vertebrate organisms as a major constituent of tissues, where cells migrate, divide, and

differentiate. In addition, hyaluronan plays a pivotal role in the homeostasis of physiological events such as tissue regeneration, wound healing, and tumorigenesis.

The synthesis of this high-molecular-weight, highly hydrated biopolymer occurs on the inner side of the plasma membrane, and the product is released directly into the extracellular space. Hyaluronan is synthesized by HAS1, HAS2, and HAS3, hyaluronan synthase isoforms that are encoded by three separate, but related, genes (1-4). The HAS genes are located on three different chromosomes, which, in turn, determine different expression patterns, and confer distinct functions on the products obtained (5, 6). HAS2 was suggested to be the predominant enzyme in chondrocyte metabolism (7, 8) and therefore, the major source of hyaluronan production during embryonic development (9, 10). Recently, the regulation of hyaluronan synthesis and degradation was summarized by Bastow *et al.* (11).

At least three fundamentally different functions have been proposed for hyaluronan in the regulation of cell behavior under both normal and pathological conditions:

– With the advent of glycochemistry research, a passive structural role was suggested for hyaluronan (12, 13) based on its unique biomechanical and hydrodynamic properties. Hyaluronan expands the extracellular space by binding salt and water, and it creates a highly hydrated ECM that facilitates cell movement (reviewed in Toole, 2004 (14)).

– Hyaluronan plays the role of supporting element in supermolecular aggregates that are created by specific interactions of between it and other extracellular macromolecules. A good example is cartilage tissue, in which the highest order hyaluronan structures occur in conglomerates of aggrecan and collagen type II, both of which are necessary to maintain the biomechanical properties of the tissue (15-18).
– The most complex but least understood hyaluronan function is its signaling role, i.e., its involvement in intracellular signal transduction and in the activation of biochemical pathways.

The goal of this review, therefore, is to understand how hyaluronan regulates signal transduction during the embryonic development of mesenchymal cells that leads to the formation of bone and cartilage. Beginning with the early morphological events of mesenchymal phenotype development, our analysis also reviews the steps of aggregation and condensation and a series of differentiation steps, all of which contribute to the formation of chondrogenic and osteogenic anlagen. The current knowledge of the mechanisms that dictate the fate of hyaluronan -mediated signaling in mesenchymal cells will be reviewed in human, chicken, rat, mouse, and rabbit embryonic limb model systems.

3. HYALURONAN RECEPTORS

Hyaluronan -mediated signals are transmitted through cell surface receptors, and mesenchymal cells express several of the many possible hyaluronan receptors: CD44 (19, 20), RHAMM (receptor for hyaluronic acid mediated motility) (21) and the toll-like receptor 4 (TLR4) (22, 23). The predominant receptor for hyaluronan, CD44, was suggested to be one of the specific markers for mesenchymal stem cells (24-26). The expression of additional hyaluronan receptors has been identified (27, 28), but their exact signaling roles have not been elucidated yet.

CD44 is a cell surface transmembrane protein that mediates both cell-cell and cell-matrix interactions. Originally identified by Hughes in 1981 (29) in NIH 3T3 fibroblast cells, CD44 was shown to be expressed by a variety of cells and tissues. In mouse embryo, CD44 expression was initially detected in the ectoderm surrounding the limb-bud mesenchyme from the earliest stage of limb-bud outgrowth beginning from day 8 of gestation (30). In rat embryo, CD44 was first detected in the mesenchyme bordering the neural tube on day 10 (31). This receptor regulates a variety of biological functions, such as cell-cell adhesion (20, 32-34), pericellular matrix assembly (35, 36), and cell migration (37-39), and it also participates in hyaluronan endocytosis (40-42), and tumor cell metastasis (43-46).

Although CD44 proteins are all encoded by a single gene, cells that display CD44 express some heterogenic isoforms of this receptor due to posttranslational modifications and to the alternative splicing of ten variant exons in the cell membrane vicinity at the proximal region of the extracellular domain. The cytoplasmic and the amino terminal domains are highly conserved (47). The cytoplasmic tail of CD44 does not contain any actin-binding sites, and its interaction with the cytosketeton is mediated by cytoskeleton-associated proteins, such as merlin, ankyrin, and others (48). In construct with the CD44 receptor that integrates with the cytoskeleton proteins, hyaluronan affects the integrity of the cytoskeletal structures (49). The cytoplasmic domain is required for hyaluronan binding and its internalization (50-52).

It has been suggested that mesenchymal cells mainly express CD44s (CD44N), the standard form of CD44 (53, 54), while tumor cells are associated with the expression of a diversity of CD44 isoforms (55-58).

Research has indicated that low-molecular-weight hyaluronans, practically degraded to oligomers, inhibit the signaling of high-molecular-weight hyaluronan (59, 60). A possible explanation for this phenomenon could be that the activation of a normal hyaluronan signals depends on the simultaneous interaction of many CD44s molecules with the same hyaluronan molecule. Indeed, Hardingham and Muir (61) reported that decasaccharides were the smallest hyaluronan fragments able to bind strongly to CD44, while Lesley *et al.* showed that only oligosaccharides larger than 20 residues could interact with two CD44s simultaneously (62). Actually, the longer the sugar chain, the more linked binding sites for CD44 that are present; alternatively, the higher the overall receptor density, the more binding activity is achieved, and, in turn, the signal strength rises.

The affinity of CD44 to hyaluronan seems to be regulated from inside the cell (63) and can be modulated by cytokines (64) and the hyaluronan-binding protein TSG-6 (the secreted product of tumor necrosis factor-stimulated gene-6) (65). It has been shown that CD44 affinity to hyaluronan requires a very specific glycosylation pattern (62, 66) and helical folding of the hyaluronan-binding domain of CD44 (67).

Another hyaluronan receptor, RHAMM has emerged as a critical regulator of cell motility and focal adhesion turnover (68-70), and it also has multiple isoforms (71, 72). RHAMM is located at the cell membrane and has no transmembrane or cytoplasmic domains. Nonetheless, it was also reported to be present in the cell cytoplasm, where it interacts with microtubules and actin filaments (73). In an *in vitro*, bovine system, embryos produce RHAMM mRNA, as detected, beginning from the 2-cell stage (74). This receptor is generally suggested to be activated during cell migration (70, 75-77). Further experiments revealed that the signaling role of RHAMM is not restricted to this particular function only. As evidenced, inhibition of the *RHAMM* gene during embryogenesis causes loss of pluripotency and cell viability in human embryonic stem cells (78). Furthermore, in CD44 knockout mice, the *RHAMM* gene compensates for the loss of CD44 by supporting up-regulating genes associated with CD44, as assessed by a microarray containing 13,000 cDNA clones (79).

Recently, toll-like receptors (TLRs) for hyaluronan were shown to be involved in the hyaluronan signaling of bone marrow-derived mesenchymal cells. Part of the innate immune system, these transmembrane receptors are used to detect inflammation and initiate host defense response mechanisms (80). Recent studies demonstrated that mesenchymal stem cells of the bone marrow express TLRs (22, 81), and the expression is upregulated in the inflammation zones (82). To date, apart from the study of Chang *et al.* (83), which indicates that hyaluronan inhibits osteoblast differentiation of mouse derived bone marrow cells via TLR4, there is no evidence for the involvement of this receptor in developmental events.

4. ROLE OF HYALURONAN IN DEVELOPMENTAL PROCESSES

4.1. Epithelial-mesenchymal transition (EMT)

Normal developmental processes, organogenesis and growth depend on interactive signaling between cells and tissues. Epithelial-mesenchymal interactions are critical mechanisms employed during embryogenesis that promote cartilage formation from the primary mesenchyme in parallel with the formation of the neural crest from the ectoderm (84). Formation of the primary mesenchyme from the epithelium is known as the process of epithelial-mesenchymal transition (EMT). Primary developmental EMTs are morphogenetic mechanisms that drive germ layer reorganization at the initial primary embryonic epithelium transition during gastrulation and neural crest formation (85-87). The entire series of EMT events are involved in the phenotypic transition of a cell originally destined for the epithelium to a cell with a mesenchymal phenotype, including the corresponding mesenchymal gene expression profile. The most apparent EMT event is the reorganization of the cells, through the formation of new intercellular communications (88-90), from a two-dimensional sheet of epithelial cells to a more complex set of juxtaposed tissues. For the transition to be successful, progenitor cells must be properly positioned to take on the correct temporal and spatial patterning. Indeed, when epithelial cells undergo an EMT, certain phenotypic alterations, such as the loss of cell-cell contact and the acquisition of cell motility, are immediately obvious (91-93). Therefore, some components of the extracellular matrix that contain hyaluronan play a cell-cell adhesion role, and as a result, they are likely to be modified during EMT (94-96).

Further experiments revealed that hyaluronan plays a crucial role in the transition of epithelia to mesenchyme during embryogenesis. In some cases, hyaluronan plays both an essential and a sufficient role in the induction of EMT (94, 96, 97). HAS2 null mice embryos have been found to totally lack the ability of epithelial-mesenchymal transformation (10, 97).

Studies of the process of EMT have revealed that high levels of hyaluronan appear at the site of neural crest formation (98). Recent findings indicate that hyaluronan is required for two pivotal events in the EMT: the first event is the matrix expansion that occurs during the initiation of the cell migration phase (96, 99), and the second comprises the anchorage-independent cell survival and cell proliferation steps (60, 99, 100). These findings indicate that the EMT program is activated and regulated mainly by hyaluronan.

Both *in vitro* and *in vivo* developmental studies have revealed that the initial stage of EMT, when the epithelial cells begin to lose their cell-cell contact, is mediated by the cadherin-catenin based adherence junctions, as reviewed previously (101-103). After the adherence junctions dissociate, the cells undergo a phenotypic conversion that is expressed in the remodeling of their cytoskeleton and in the formation of alternative focal adhesion sites. This process promotes cellular locomotion and leads to changes in the locations of the cells that cause further cell growth in an anchorage-independent manner.

A number of cytokines such as Wnt, TGF-beta (transforming growth factor beta), SF/HGF (scatter factor/hepatocyte growth factor), EGF, and other ligands all bond to the family of tyrosine kinase receptors known to establish and further induce EMTs (for reviews see (104, 105)). Numerous studies have shown that members of the TGF-beta superfamily are implicated as potent activators of cell motility. They stimulate expression of the hyaluronan receptor RHAMM, located on the cell surface, and elevate hyaluronan synthesis (106-108). Hyaluronan appears to mediate tyrosine phosphorylation of cadherins and catenins which, in turn, leads to a decrease in cell-cell adhesion. Fujita *et al.* (95) demonstrated that hyaluronan mediates

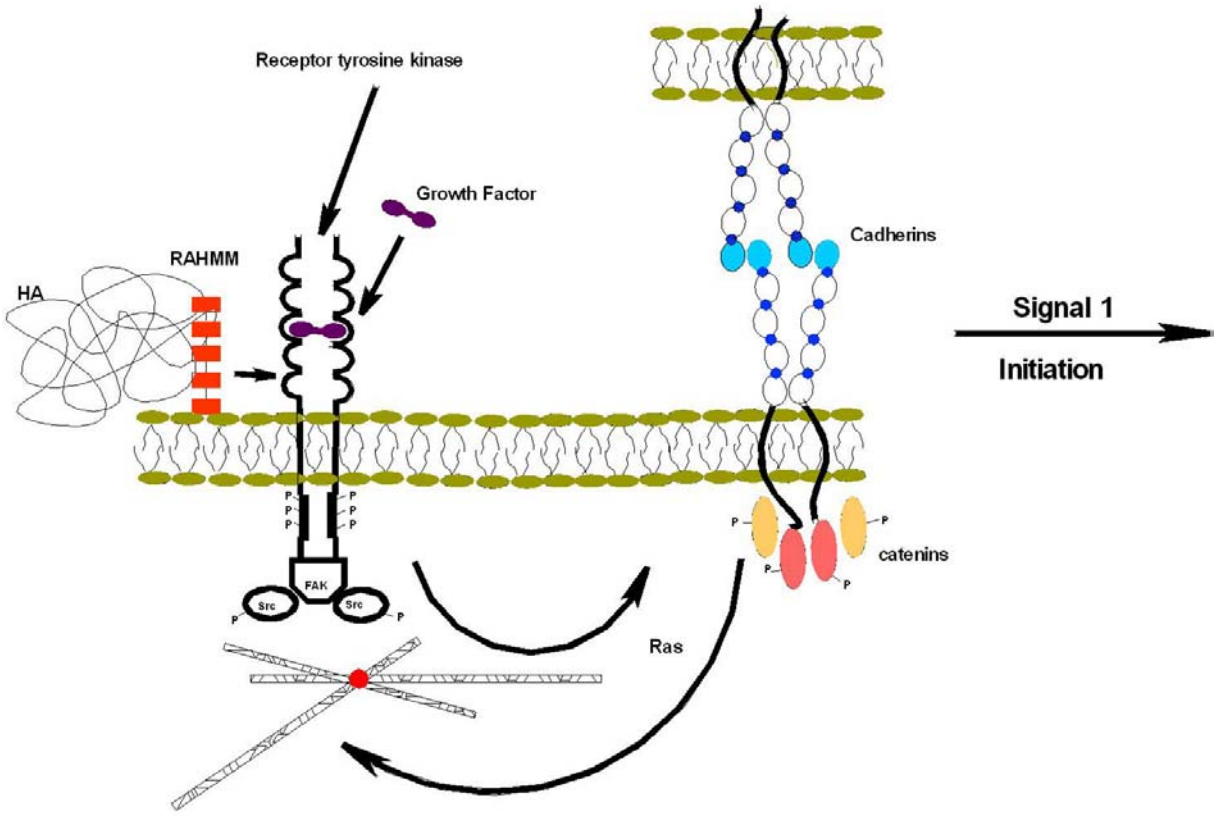

Figure 1. Binding of hyaluronan to cell surface RHAMM activates a signaling cascade that results in dissociation of adherent junctions and cell motility. RHAMM is associated with protein tyrosine kinases and activates Src phoshorylation via focal adhesion kinase and ERK kinases, which leads to the phosphorylation of catenins and a decrease in cell-cell adhesion through the dissociation of adherent junctions. The adherent junctions are composed of clustered cadherins associated with cadherins alpha- and beta-catenins. This results in local actin filament reorganization and the formation of focal adhesion sites, which involves integrins and ECM components, such as fibronectin (see fig. 2) and the activation of Ras signaling.

signaling that induces a rapid, transient protein tyrosine kinase phosphorylation that is quickly followed by a net de-phosphorylation, a pair of events that is confined to focal adhesion of the extended laminae (68). Formation of the focal adhesion sites involves integrins, a large family of transmembrane proteins that associate with extracellular matrix proteins outside the cell and with cytoskeleton proteins in the cytoplasmic domain, which help activate the local organization of actin fibers (109-111). This course of events is supported by Hall *et al.*, who showed that hyaluronan mediates focal adhesion turnover via the RHAMM receptor (69). The focal adhesion site promotes cell survival signals through the dephosphorylation of FAK (focal adhesion kinase), an event that is also RHAMM dependent (77, 112). Hence, RHAMM regulates both the transient phosphorylation and the net dephosphorylation of FAK. The phosphorylation of FAK appears to permit focal adhesion assembly and to eliminate cell motility. Conversely, the disassembly of focal adhesions involves the dephosphorylation of FAK, which enables cell movement across the ECM.

This transient phosphorylation process has also been shown to be CD44-dependent. Indeed, CD44-negative control cells did not respond to hyaluronan stimulation (95), which suggests that there is a structural link between CD44 and FAK and that the formation of the focal adhesion sites stimulates CD44 activation and its association with the tyrosine kinase receptor. Thus, the activity orchestrated by CD44 and additional factors are also associated with the control of FAK stimulation (see Figure 1). This is in accordance with the observations of Hall *et al.* (69) that the focal adhesion kinases $pp125^{FAK}$ and $pp123^{FAK}$ are phosphorylated/dephosphorylated subsequent to hyaluronan stimulation (113). Therefore, the existence was demonstrated of the complex comprising RAHMM and containing Src, the hyaluronan-stimulated protein tyrosine kinase, which colocalize with cytoskeletal elements. The process of cytoskeleton reorganization promotes events that define the mesenchymal phenotype of cells, i.e., the induction of cell motility and anchorage-independent growth and proliferation. Dispersal of the adherent junctions leads to the accumulation of beta-catenin, which, in turn, was reported to stimulate the production of hyaluronan (96). Accompanying the increase of hyaluronan production in epithelial cells is the induction of gelatinase production, especially gelatinase B (MMP-9) (96), which can associate into clusters with CD44 (114).

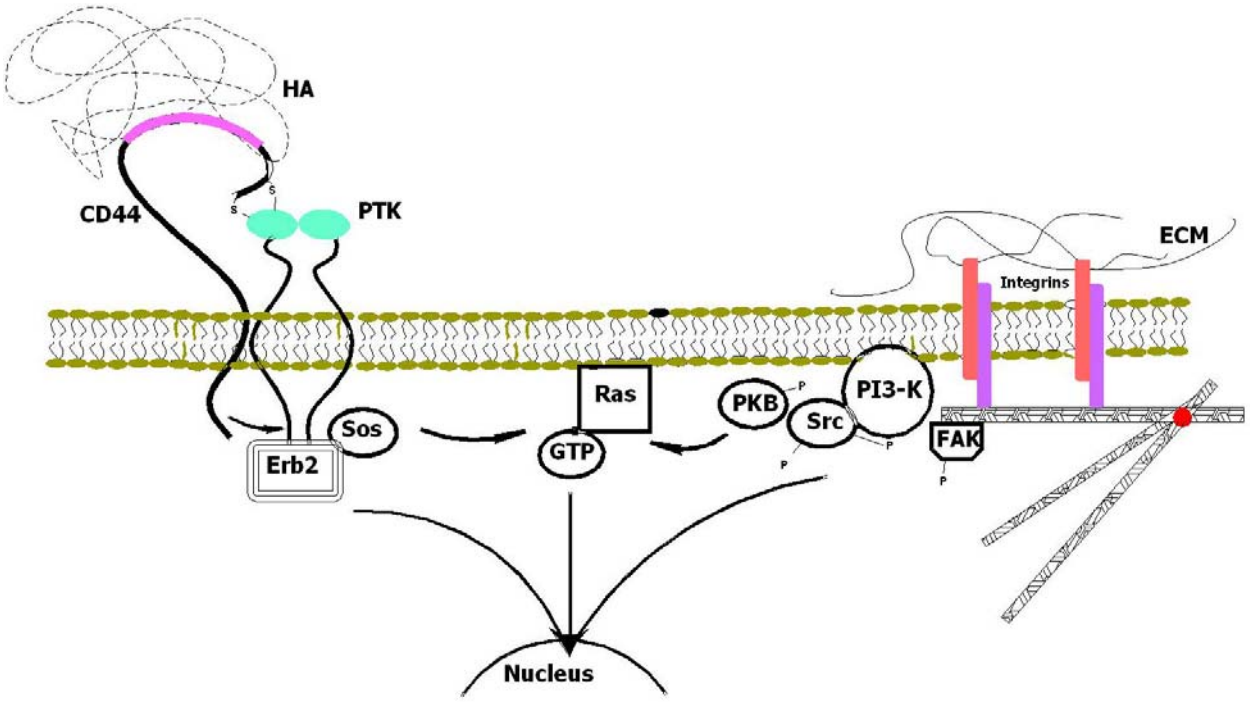

Figure 2. Sequent event following the dispersion of adherent junctions is the formation of focal adhesion sites, which involves integrins and FAK. The integrins interact with fibronectin and other ECM components. Formation of focal adhesion site associates with CD44 and receptors tyrosine kinaze, indicating involvement of cytokines, such as TGF-beta, in the pathway. Activation of CD44 stimulates Ras-ERK signaling via activation of PKB and the receptor tyrosine kinase Erb2, triggering further the survival pathway of the cell. Activation of these signaling pathways in unison leads to survival and anchorage-independent proliferation.

As the EMT process progresses, the cells succeed in adapting to the emerging conditions, under which they are less dependent on cell-cell interactions, as anchorage-independent growth is among the fundamental characteristics of the cells during EMT. Normal epithelial cells undergo apoptosis if they detach from their underlying substrate, a process termed anoikis (115). Transformed cells, however, evolve specific mechanisms that allow them to survive and grow as anchorage independent cells. Hyaluronan has been shown to play an essential role in the stimulation of such unattached anchorage-independent survival. Upon hyaluronan activation of CD44, the cell forms the focal adhesion site, which involves integrines and focal adhesion kinase (see Fig.2). Although the exact mechanism underlying this effect has not yet been fully elucidated, indications exist that hyaluronan activates Ras-signaling pathways (116) via the stimulation of Akt (protein kinase B), glycogen synthase kinase-3beta (96), and the receptor tyrosine kinase ErbB (97, 117). The activation of Ras can be altered by adding growth factors, which leads to the release of receptors and integrins that trigger the ERK survival pathway. Recently, hyaluronan has also been shown to stimulate a series of events, resulting in activation of the MAPK pathway that involves CD44 (99, 117). Other new findings indicate that some of the CD44 isoforms are associated with the TGF receptor (TGFR) via either its intracellular (118) or extracellular domains (119). Oligomers of hyaluronan reverse the effect of elevated hyaluronan production by inhibiting EMT initiation (120), PI 3-kinase activity (96), and the cell survival pathway (60, 100).

The large number and variety of extracellular stimuli that can initiate EMT events suggest that multiple signaling pathways are involved in this complex process. Although a number of the key signaling pathways have been identified, our understanding of these pathways is far from complete. Tumorogenesis, the process of the initiation of metastasis, in which cells deposit an organized epithelial layer, appears to stimulate the pattern of behavior governed by the above factors and involve the same molecular pathways (104, 111, 121, 122).

4.2. Limb development

The product of mesodermal lateral plate cells that undergo chondrogenesis and osteogenesis, limb organogenesis leads to the development of joints and, through endochondral ossification, of other structures during skeletal development. Joints develop from the mesenchyme via the process of mesenchymal condensation, which is regulated by the interactions of the epithelial-mesenchymal cells (123, 124).

Early mesenchymal formation is regulated by the apical ectodermal ridge (AER), an ectoderm layer found above the mesodermal distal subridge and a zone of

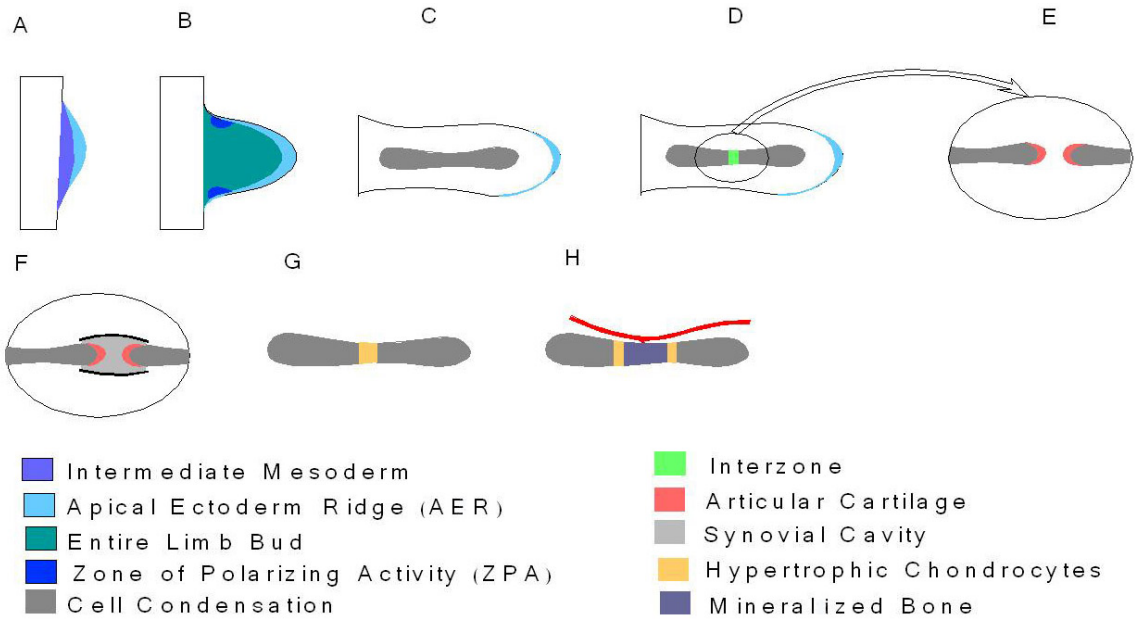

Figure 3. Development of mesenchyme skeletal elements in the embryonic limb. (A) Early limb formation, the intermediate mesenchyme covered by ectoderm. (B) Limb growth regulated by AER and ZPA. (C) Condensation of mesenchymal cells. (D) Interzone formation: mesenchymal cells differentiate into chondrocytes. (E) Separation of the adjacent skeletal anlagen and formation of synovial cavity. (F) Articular cartilage and synovial cavity formed. (G) Chondrocytes that were proliferating at the center of condensation region stop proliferating and become hypertrophic. (H) Hypertrophic chondrocytes direct the formation of mineralized matrix and attract vascular elements. Osteoprogenitor cells arrived with blood vessels form the ossification centers, remove the mineralized cartilage matrix and form bone tissue.

polarizing activity (ZPA) (Figure 3. A, B) that provides the molecular cues required for limb patterning and outgrowth (reviewed in (125)). Studies have shown that both mesenchymal and AER cells secrete large amounts of hyaluronan (126, 127) and hyaluronidase (128), such that the early limb mesenchymal cells are separated by an extensive extracellular matrix, in which hyaluronan is the predominant glycosaminoglycan that undergoes rapid turnover and remodeling. In the early limb bud, the mesodermal zone subjacent to the AER shows patterns of gene activity characteristic of undifferentiated tissues (125). Sherman *et al.* have demonstrated that in AER, the CD44v3 isoform acts as a co-receptor for members of the FGFR family of RTKs (129). Therefore, the current view is that a series of signaling systems are functioning coordinately, and that hyaluronan, together with other factors such as FGF (130, 131), bone morphogenic protein (BMP) (132), and Wnt (133-135), contributes to AER signaling maintenance of the subridge mesenchymal cells in an undifferentiated state and promotes subridge mesenchymal cell migration toward the AER (136). Limb bud growth is accompanied by the gradual distancing of the cells in the apical mesoderm from the AER. Out of range of AER influence, the mesenchymal cells can differentiate independently of the AER.

The first morphological indication that a site is destined to become cartilage is the occurrence of mesenchymal condensation (Figure 3 C), an event that is dependent on cell-cell and cell-matrix interactions. Prior to the condensation process, the early mesenchymal cells are separated by a hyaluronan-rich extracellular matrix. Maleski and Knudson (34) were the first to describe how hyaluronan initiates the formation of cellular aggregates during the precondensation stage by cross-bridging via multivalent interactions with hyaluronan molecules found on adjacent cells. After aggregate formation, hyaluronan is down-regulated and the condensation stage begins. Cells form close contacts with one another, a process that triggers the appearance ofprechondrogenic cells that lack their hyaluronan-rich pericellular coat (137). Mesenchymal cell morphology is round to polygonal, although sometimes the cells have spiky projections, and its nucleo-cytoplasmic ratio is typically high (138). As chondrofication begins, the pericellular matrix appears in the intercellular space, initially as a thin film coating the cells, which then become separated from one another in a manner similar to capsules (lacunae) (139). The pericellular matrix in the vicinity of the cell comprises a thin envelope of proteoglycans with an adjacent layer of collagen fibrils (140). Differentiated (mature) chondrocytes do not express hyaluronan or CD44 (141). However, as cartilage is formed, both hyaluronan and CD44 are detected in the expanded surrounding matrix, and therefore, differentiated cells regain their capacity to form hyaluronan-dependent pericellular matrices (142).

4.3. Joint cavitation

Synovial joint development involves the formation of a cavity, initiated in a presumptive joint area called the interzone, within the condensed mesenchymal cells. An interzone is a three-layered structure formed from a narrow band of densely packed flattened cells from which the joint

cavitates. Although the precise cavitation mechanisms have not been resolved, the leading hypotheses ascribe this process to programmed cell death and mechanical stimulation effected by cell locomotion. Developmentally programmed cavitation involves a reorganization of the intercellular matrix that leads to cellular dissociation, the separation of opposing sides of the joint, and cavity formation. hyaluronan was suggested to play a crucial role in the loss of intercellular integrity necessary for joint formation (143-145).

Concomitant with the first signs of cavitation, free hyaluronan molecules become available in the joint interzone (146). Cells within the interzone show high expression levels of hyaluronan (147) and hyaluronan receptors (148) before and during cavitation. Evidence exists that mechanical strains created by movements of the embryo stimulate hyaluronan synthesis (149, 150), which, in turn, initiates joint cavity formation. The application of hyaluronan oligosaccharides close to joint formation disrupts joint formation by blocking cell surface receptors (150, 151). According to a widely accepted theory, joint cavity formation within the interzone is brought about by swelling pressure produced by hyaluronan. However, it is likely that since this mechanism also involves cellular reorganization and cell-cell and cell-matrix interactions, the directional nature of hyaluronan action in cavity formation may be the same as that during the epithelial-mesenchymal transition: the simultaneous disruption of cell-cell interactions and the formation of cell-matrix interactions lead to cellular reorganization and phenotypic transitions.

Three types of tissue develop at the site of joint formation: synovial membranes, joint cavities, and articular cartilage (138). To accomplish this feat, the cells undergo transitional cellular metamorphoses in the interzone (152), a claim that is supported by the finding that ERK/MARK signaling is involved in controlling joint cavity phenotype (150, 153).

4.4. Long bone formation

Cartilage anlagen develop into long bones via a process termed endochondral ossification, in which chondrocytes undergo terminal differentiation via chondrocyte hypertrophy, a process characterized by cartilage matrix calcification, vascular invasion, and ossification (Figure 3, G, H). Hypertropic chondrocytes undergo programmed apoptosis in the zone of erosion, where osteoprogenitor cells migrate into the lacunae, attach to the surface of calcified cartilage, and form woven bone. The calcified cartilage is partially eroded and bone is deposited, replacing the tissue residues penetrated by the vascular mesenchymeal stream. This process of endochondral ossification then spreads from the zone of erosion to both ends of the shaft. The role of hyaluronan in bone development is not yet fully understood, but the accumulated evidence suggests that it plays a critical role in this process.

It has been shown that early in endochondral ossification, when only undifferentiated cells are present, HA expression reaches peak levels (154, 155). Moreover, observations have revealed that hyaluronan is synthesized mainly by hyaluronan synthase 2 (HAS-2), the enzyme capable of synthesizing high molecular weigh hyaluronan (156, 157).

Histochemical analysis has shown that chondrocytes in the hypertrophic zone secrete large amounts of HA (158). Pavasant *et al.* (159) demonstrated that the same factors that stimulate hyaluronan production by chondrocytes during the hyperthrophic phase, such as growth hormone (GH) and insulin-like growth factor-I (IGF-I), also promote their terminal differentiation during the erosion phase.

In comparison to chondroblasts, mature chondrocytes produce larger pericellular coats of hyaluronan. Composed of both hyaluronan and aggrecan, the coats are attached to the surfaces of the chondrocytes via specific hyaluronan receptors and by sustained transmembrane interactions with hyaluronan synthases. The development by Clarris and Fraser (160) of the assay facilitated visualization of the pericellular coat of hyaluronan, showing that the coat extends outward from the plasma membrane of mature chondrocytes by as much as one cell diameter.

Detailed studies comparing hyaluronidase activity during early pericellular matrix accumulation and at the hyperthropic stages have shown that hyaluronidase expression levels were enhanced while the chondrocytes become hypertophic (161, 162). Hyaluronan turnover in the zone of erosion causes an increase in hyaluronan oligosaccharide production, which further stimulates the production of vascular endothelial growth factor (VEGF) (120). Up-regulation of VEGF was shown to stimulate the ingrowth of blood vessels (163, 164) as part of the process of osteogenesis. Recently, Gao *et al.* (165) reported that the initiation of angiogenesis by hyaluronan oligosaccharides was mediated via a RHAMM receptor, but not by CD44. The blood vessels bring chondroclasts, osteoblasts, and osteoclasts into the new ossification centers, which are responsible for the remodeling of the mineralized cartilage matrix and for the formation of bone tissue.

A role of hyaluronan oligosaccharides in the bone formation process was suggested a decade ago by Pilloni and Bernard (166), who reported on the enhancement of mineralization and bone formation by degraded oligosaccharides of hyaluronan (30 – 40D), but not by high-molecular-weight hyaluronan molecules. Recently, Tanne, (167) reported that degraded hyaluronan oligosaccharides have an inhibitory effect on the activity and expression of Runt-related 2 (Runx2) transcription factor, a master factor of chondrocyte differentiation toward hypertrophic chondrocytes (168-170), while high-molecular-weight hyaluronan has no effect on the expression of Runx2. According to the reports of Kim *et al.* (171) and Takeda *et al.* (172), Runx2 expression decreased in late hypertrophic chondrocytes.

Taken together, these findings indicate that the accumulation of low molecular weigh hyaluronan in the

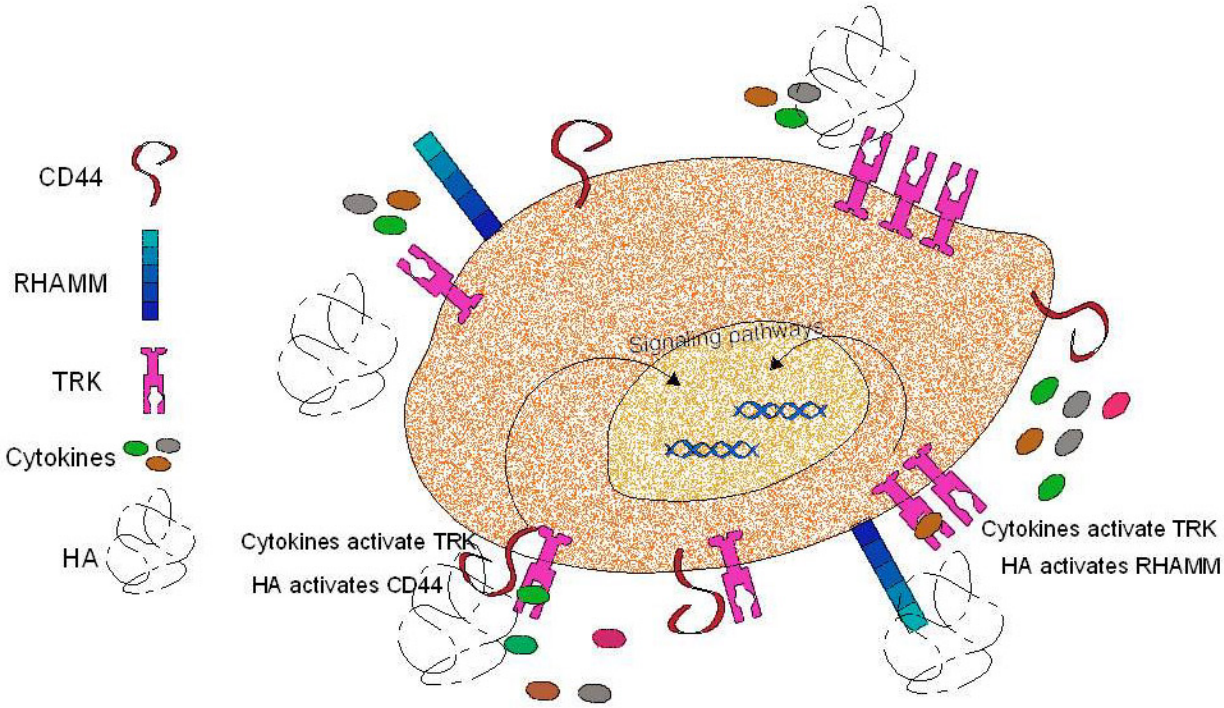

Figure 4. Cell signaling is induced by HA via receptors CD44 and RHAMM and involves TRK activation by cytokines and nonreceptor kinases (Src family). The signaling complexes promote phenotypic transitions, cell survival and cell differentiation pathways. To induce the pathways, a multi-step activation mechanism occurs at the same time as, for example, the activation of TRK by cytokines, the activation of hyaluronan-receptor by hyaluronan, and the formation of complex signaling. Only the intersection of all these activities initiates the complex pathways, which transacted to the cell nucleus where it triggers the corresponding gene to express its profile.

late stages of chondrocyte hypertrophy induces the invasion of blood vessels into the hyperthrophic zone and promotes the terminal differentiation of chondrocytes and their replacement by bone cells. Furthermore, several studies have indicated that hyaluronan also effects the proliferation and differentiation of osteoprogenitor cells to the osteoblastic phenotype (173). Located in the periosteum and bone marrow, osteoprogenitor cells generate the osteoblasts that are responsible for bone formation and functions. Hughes *et al.* (174) observed that both osteoblasts and osteoclasts produce hyaluronan and express the hyaluronan receptor CD44, while Chang *et al.* (83) reported that these cells also express RHAMM and toll-like receptors for hyaluronan.

Cao *et al.* demonstrated that the addition of exogenous hyaluronan increased by 3.6 fold and 3 fold Receptor Activator for Nuclear Factor KB Ligand (RANKL) mRNA and protein levels, respectively, in a dose-dependent manner in murine bone marrow derived stem cells. An osteoclast regulatory factor, RANKL is an important activating cytokine that plays a crucial role in the initiation of the pathway that leads to the differentiation of osteoclast precursor cells to active osteoclasts. On osteoclast precursor cells, RANKL binds to the transmembrane receptor RANK. The stimulation of RANKL by hyaluronan could be blocked with anti-CD44 antibody (175).

5. SUMMARY AND PERSPECTIVE

Increasing in the production and accumulation of hyaluronan prior to and during essential morphological developmental events such as chondrogenesis and osteogenesis suggests that it plays a key role in these events. In recent decades, many research groups attempted to elucidate this role, and their work led to the assumption that the remarkable hydrodynamic properties of hyaluronan influence cell behavior. Indeed, hyaluronan is capable of retaining water, and the pressure exerted by its swelling can cause cells to physically separate structures during such morphological events as EMT and joint cavity formation. In addition, hyaluronan plays a direct signaling role through its interactions with its cognate family of cell surface receptors. In spite of the acceptance that hyaluronan -induced signaling exists, the mechanism of its action has not been fully established.

The initiation of morphological events is preceded by the temporal and spatial up-regulation of hyaluronan, including cellular reorganization and phenotypic transitions during the epithelial-mesenchymal transition, joint cavity formation, and with endochondral ossification. The accumulation of hyaluronan at these sites preserves the cells in an undifferentiated state, while concomitantly stimulating their proliferation. Once cells and matrix reorganization have been accomplished, hyaluronan

undergoes down regulation and degradation via several well-established mechanisms. Hyaluronan behaves similarly at the sites of bone fractions, callus formation, and wound healing, where it assists mesenchymal cell migration to regions in which regeneration is needed (176, 177).

The functional link between the family of transmembrane proteins (such as TRK ERBB) and hyaluronan receptors (activated by cytokines) leads to the activation of a variety of downstream signaling cascades, involving Akt, Rac-1, ERK, and FAK, all of which are critical for cell motility. A variety of co-receptors for CD44 have been described, including among others, EGFR (178), c-Met, VEGFR (179), and FGFR (118). One possible, multi-step activation mechanism occurs at the same time as, for example, the activation of TRK by cytokines, the activation of hyaluronan-receptor by hyaluronan, and the formation of signaling complexes (see Figure 4). In this manner, hyaluronan activates the PI3K-AKT signaling pathway, which promotes cell survival, the ERBB/Ras-MARK signaling pathway, which promotes selective cell differentiation, and the Wnt-beta-catenin signaling pathway that is critical for cell differentiation.

The complex task of establishing the regulatory role of hyaluronan still needs to be clarified. The similarities that exist between hyaluronan-activated and mediated mechanisms in developmental events and cancer metastasis, in the latter of which hyaluronan plays a crucial role in cell survival, invasiveness, and EMT, dictate that hyaluronan and its mechanisms of action continue to be studied.

6. ACKNOWLEDGEMENTS

All authors equally contributed to this article.

7. REFERENCES

1. N. Itano and K. Kimata: Hyaluronan synthase: a scenario for remodeling the extracellular matrix. *Seikagaku* 70, 1171-1175 (1998)

2. A. Shyjan, P. Heldin, E. Butcher, T. Yoshino and M. Briskin: Functional cloning of the cDNA for a human hyaluronan synthase. *J.Biol.Chem.* 271, 23395-23399 (1996)

3. A. P. Spicer and J. A. McDonald: Characterization and molecular evolution of a vertebrate hyaluronan synthase gene family. *J.Biol.Chem.* 273,1923-1932 (1998)

4. A. Spicer, J. Olson and J. A. McDonald: Molecular cloning and characterization of a cDNA encoding the third putative mammalian hyaluronan synthase. *J.Biol.Chem.* 272:8957-8961 (1997)

5. N. Itano, T. Sawai, M. Yoshida, P. Lenas, Y. Yamada, M. Imagawa, T. Shinomura, M. Hamaguchi, Y. Yoshida, Y. Ohnuki, S. Miyauchi, A. P. Spicer, J. A. McDonald and K. Kimata: Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J.Biol.Chem.* 274, 25085-25092 (1999)

6. A. Recklies, C. White, L. Melching and P. Roughley: Differential regulation and expression of hyaluronan synthases in human articular chondrocytes, synovial cells and osteosarcoma cells. *Biochem.J.* 354, 17-24 (2001)

7. D. Hiscock, B. Caterson and C. Flannery: Expression of hyaluronan synthases in articular cartilage. *Osteoarthritis Cartilage* 8, 120-126 (2000)

8. S. Ohno, K. Tanimoto, K. Fujimoto, C. Ijuin, K. Honda, N. Tanaka, T. Doi, M. Nakahara and K. Tanne: Molecular cloning of rabbit hyaluronic acid synthases and their expression patterns in synovial membrane and articular cartilage. *Biochim.Biophys.Acta* 1520, 71-78 (2001)

9. J. Tien and A. Spicer: Three vertebrate hyaluronan synthases are expressed during mouse development in distinct spatial and temporal patterns. *Dev.Dyn.* 233, 130-141 (2005)

10. T. Camenisch, A. Spicer, T. Brehm-Gibson, J. Biesterfeldt, M. Augustine, A. Calabro Jr, S. Kubalak, S. Klewer and J. McDonald: Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J.Clin.Invest.* 106, 349-360 (2000)

11. E. Bastow, S. Byers, S. Golub, C. Clarkin, A. Pitsillides and A. Fosang: Hyaluronan synthesis and degradation in cartilage and bone. *Cell Mol.Life Sci.* 65, 395-413 (2008)

12. B. Toole, M. Okayama, R. Orkin, M. Yoshimura, M. Muto and A. Kaji: Developmental roles of hyaluronate and chondroitin sulfate proteoglycans. *Soc.Gen.Physiol.Ser.* 32, 139-154 (1977)

13. B. Toole: Extracellular events in limb development. *Birth Defects Orig.Artic.Ser.* 10, 187-191 (1974)

14. B. Toole: Hyaluronan: from extracellular glue to pericellular cue. *Nat.Rev.Cancer.* 4, 528-539 (2004)

15. H. Watanabe, H. Watanabe and K. Kimata: The roles of proteoglycans for cartilage. *Clin.Calcium* 16, 1029-1033 (2006)

16. C. Knudson and W. Knudson: Cartilage proteoglycans. *Semin.Cell Dev.Biol.* 12, 69-78 (2001)

17. C. Knudson: Hyaluronan and CD44: strategic players for cell-matrix interactions during chondrogenesis and matrix assembly. *Birth Defects Res.C.Embryo.Today* 69, 174-196 (2003)

18. A. Day: The structure and regulation of hyaluronan-binding proteins. *Biochem.Soc.Trans.* 27, 115-121 (1999)

19. R. Ghilzon, C. McCulloch and R. Zohar: Stromal mesenchymal progenitor cells. *Leuk.Lymphoma* 32, 211-221 (1999)

20. L. Bendall and D. Gottlieb: CD44 and adhesion of normal and leukemic CD34+ cells to bone marrow stroma. *Leuk.Lymphoma* 32, 427-439 (1999)

21. J. Ponting and S. Kumar: Localisation and cellular origin of hyaluronectin. *J.Anat.* 187 (Pt 2), 331-346 (1995)

22. F. Liotta, R. Angeli, L. Cosmi, L. Fili, C. Manuelli, F. Frosali, B. Mazzinghi, L. Maggi, A. Pasini, V. Lisi, V. Santarlasci, L. Consoloni, M. L. Angelotti, P. Romagnani, P. Parronchi, M. Krampera, E. Maggi, S. Romagnani and F. Annunziato: Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells* 26, 279-289 (2008)

23. L. Shi, J. Wang, X. Liu, X. Hu and Q. Fang: Upregulated functional expression of Toll like receptor 4 in mesenchymal stem cells induced by lipopolysaccharide. *Chin.Med.J.(Engl)* 120, 1685-1688 (2007)

24. B. Zhu, J. Ren, X. Wang, X. Li and J. Nie: Biological properties of mesenchymal stem cells derived from bone marrow of patients with multiple myeloma. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 14, 1138-1142 (2006)

25. Z. Guo, J. Yang, X. Liu, X. Li, C. Hou, P. Tang and N. Mao: Biological features of mesenchymal stem cells from human bone marrow. *Chin.Med.J.(Engl)* 114, 950-953 (2001)

26. Y. Hu, L. Ma, G. Ma, X. Jiang and C. Zhao: Comparative study of human fetal and adult bone marrow derived mesenchymal stem cells. *Zhonghua Xue Ye Xue Za Zhi* 23, 645-648 (2002)

27. N. Grammatikakis, A. Grammatikakis, M. Yoneda, Q. Yu, S. Banerjee and B. Toole: A novel glycosaminoglycan-binding protein is the vertebrate homologue of the cell cycle control protein, Cdc37. *J.Biol.Chem.* 270, 16198-16205 (1995)

28. C. Rao, T. Deb, S. Gupta and K. Datta: Regulation of cellular phosphorylation of hyaluronan binding protein and its role in the formation of second messenger. *Biochim.Biophys.Acta* 1336, 387-393 (1997)

29. E. Hughes and J. August: Characterization of plasma membrane proteins identified by monoclonal antibodies. *J.Biol.Chem.* 256, 664-671 (1981)

30. S. Wheatley, C. Isacke and P. Crossley: Restricted expression of the hyaluronan receptor, CD44, during postimplantation mouse embryogenesis suggests key roles in tissue formation and patterning. *Development* 119, 295-306 (1993)

31. A. Delpech and B. Delpech: Expression of hyaluronic acid-binding glycoprotein, hyaluronectin, in the developing rat embryo. *Dev.Biol.* 101, 391-400 (1984)

32. S. Green, G. Tarone and C. Underhill: Aggregation of macrophages and fibroblasts is inhibited by a monoclonal antibody to the hyaluronate receptor. *Exp.Cell Res.* 178, 224-232 (1988)

33. E. Turley: Proteoglycans and cell adhesion. Their putative role during tumorigenesis. *Cancer Metastasis Rev.* 3, 325-339 (1984)

34. M. Maleski and C. Knudson: Hyaluronan-mediated aggregation of limb bud mesenchyme and mesenchymal condensation during chondrogenesis. *Exp.Cell Res.* 225, 55-66 (1996)

35. C. Knudson: Hyaluronan receptor-directed assembly of chondrocyte pericellular matrix. *J.Cell Biol.* 120, 825-834 (1993)

36. M. Maleski and C. Knudson: Matrix accumulation and retention in embryonic cartilage and *in vitro* chondrogenesis. *Connect.Tissue Res.* 34, 75-86 (1996)

37. N. Boudreau, E. Turley and M. Rabinovitch: Fibronectin, hyaluronan, and a hyaluronan binding protein contribute to increased ductus arteriosus smooth muscle cell migration. *Dev.Biol.* 143, 235-247 (1991)

38. L. Thomas, H. R. Byers, J. Vink and I. Stamenkovic: CD44H regulates tumor cell migration on hyaluronate-coated substrate. *J.Cell Biol.* 118, 971-977 (1992)

39. K. Svee, J. White, P. Vaillant, J. Jessurun, U. Roongta, M. Krumwiede, D. Johnson and C. Henke: Acute lung injury fibroblast migration and invasion of a fibrin matrix is mediated by CD44. *J.Clin.Invest.* 98, 1713-1727 (1996)

40. Q. Hua, C. Knudson and W. Knudson: Internalization of hyaluronan by chondrocytes occurs via receptor-mediated endocytosis. *J.Cell.Sci.* 106 (Pt 1), 365-375 (1993)

41. D. Aguiar, W. Knudson and C. Knudson: Internalization of the hyaluronan receptor CD44 by chondrocytes. *Exp.Cell Res.* 252, 292-302 (1999)

42. M. Culty, H. Nguyen and C. Underhill: The hyaluronan receptor (CD44) participates in the uptake and degradation of hyaluronan. *J.Cell Biol.* 116, 1055-1062 (1992)

43. Y. Matsumura and D. Tarin: Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet* 340, 1053-1058 (1992)

44. C. Underhill: CD44: the hyaluronan receptor. *J.Cell.Sci.* 103 (Pt 2), 293-298 (1992)

45. B. Haynes, H. Liao and K. Patton: The transmembrane hyaluronate receptor (CD44): multiple functions, multiple forms. *Cancer Cells* 3, 347-350 (1991)

46. B. Zetter: Adhesion molecules in tumor metastasis. *Semin.Cancer Biol.* 4, 219-229 (1993)

47. L. Goldstein and E. Butcher: Identification of mRNA that encodes an alternative form of H-CAM(CD44) in

lymphoid and nonlymphoid tissues. *Immunogenetics* 32, 389-397 (1990)

48. R. Thorne, J. Legg and C. Isacke: The role of the CD44 transmembrane and cytoplasmic domains in co-ordinating adhesive and signalling events. *J.Cell.Sci.* 117, 373-380 (2004)

49. B. Lacy and C. Underhill: The hyaluronate receptor is associated with actin filaments. *J.Cell Biol.* 105, 1395-1404 (1987)

50. H. Jiang, C. Knudson and W. Knudson: Antisense inhibition of CD44 tailless splice variant in human articular chondrocytes promotes hyaluronan internalization. *Arthritis Rheum.* 44, 2599-2610 (2001)

51. H. Jiang, R. Peterson, W. Wang, E. Bartnik, C. Knudson and W. Knudson: A requirement for the CD44 cytoplasmic domain for hyaluronan binding, pericellular matrix assembly, and receptor-mediated endocytosis in COS-7 cells. *J.Biol.Chem.* 277, 10531-10538 (2002)

52. V. Lokeshwar, N. Fregien and L. Bourguignon: Ankyrin-binding domain of CD44(GP85) is required for the expression of hyaluronic acid-mediated adhesion function. *J.Cell Biol.* 126, 1099-1109 (1994)

53. D. Salter, J. Godolphin, M. Gourlay, M. Lawson, D. Hughes and E. Dunne: Analysis of human articular chondrocyte CD44 isoform expression and function in health and disease. *J.Pathol.* 179, 396-402 (1996)

54. H. Terpe, H. Stark, P. Prehm and U. Gunthert: CD44 variant isoforms are preferentially expressed in basal epithelial of non-malignant human fetal and adult tissues. *Histochemistry* 101, 79-89 (1994)

55. A. Bartolazzi, D. Jackson, K. Bennett, A. Aruffo, R. Dickinson, J. Shields, N. Whittle and I. Stamenkovic: Regulation of growth and dissemination of a human lymphoma by CD44 splice variants. *J.Cell.Sci.* 108 (Pt 4), 1723-1733 (1995)

56. D. Jackson, T. Schenker, R. Waibel, J. Bell and R. Stahel: Expression of alternatively spliced forms of the CD44 extracellular-matrix receptor on human lung carcinomas. *Int.J.Cancer Suppl.* 8, 110-115 (1994)

57. K. Wirth, R. Arch, C. Somasundaram, M. Hofmann, B. Weber, P. Herrlich, S. Matzku and M. Zoller: Expression of CD44 isoforms carrying metastasis-associated sequences in newborn and adult rats. *Eur.J.Cancer* 29A, 1172-1177 (1993)

58. U. Gunthert: CD44: a multitude of isoforms with diverse functions. *Curr.Top.Microbiol.Immunol.* 184, 47-63 (1993)

59. V. Hascall and D. Heinegard: Aggregation of cartilage proteoglycans. II. Oligosaccharide competitors of the proteoglycan-hyaluronic acid interaction. *J.Biol.Chem.* 249, 4242-4249 (1974)

60. S. Ghatak, S. Misra and B. Toole: Hyaluronan oligosaccharides inhibit anchorage-independent growth of tumor cells by suppressing the phosphoinositide 3-kinase/Akt cell survival pathway. *J.Biol.Chem.* 277, 38013-38020 (2002)

61. T. Hardingham and H. Muir: Binding of oligosaccharides of hyaluronic acid to proteoglycans. *Biochem.J.* 135, 905-908 (1973)

62. J. Lesley, V. Hascall, M. Tammi and R. Hyman: Hyaluronan binding by cell surface CD44. *J.Biol.Chem.* 275, 26967-26975 (2000)

63. G. Tzircotis, R. Thorne and C. Isacke: Chemotaxis towards hyaluronan is dependent on CD44 expression and modulated by cell type variation in CD44-hyaluronan binding. *J.Cell.Sci.* 118, 5119-5128 (2005)

64. J. Cichy and E. Pure: Cytokines regulate the affinity of soluble CD44 for hyaluronan. *FEBS Lett.* 556, 69-74 (2004)

65. J. Lesley, I. Gal, D. Mahoney, M. Cordell, M. Rugg, R. Hyman, A. Day and K. Mikecz: TSG-6 modulates the interaction between hyaluronan and cell surface CD44. *J.Biol.Chem.* 279, 25745-25754 (2004)

66. D. Naor, R. Sionov and D. Ish-Shalom: CD44: structure, function, and association with the malignant process. *Adv.Cancer Res.* 71, 241-319 (1997)

67. S. Banerji, A. Wright, M. Noble, D. Mahoney, I. Campbell, A. Day and D. Jackson: Structures of the Cd44-hyaluronan complex provide insight into a fundamental carbohydrate-protein interaction. *Nat.Struct.Mol.Biol.* 14, 234-239 (2007)

68. J. Entwistle, C. Hall and E. Turley: HA receptors: regulators of signalling to the cytoskeleton. *J.Cell.Biochem.* 61, 569-577 (1996)

69. C. Hall, C. Wang, L. Lange and E. Turley: Hyaluronan and the hyaluronan receptor RHAMM promote focal adhesion turnover and transient tyrosine kinase activity. *J.Cell Biol.* 126, 575-588 (1994)

70. C. Hall and E. Turley: Hyaluronan: RHAMM mediated cell locomotion and signaling in tumorigenesis. *J.Neurooncol.* 26, 221-229 (1995)

71. J. Entwistle, S. Zhang, B. Yang, C. Wong, Q. Li, C. Hall, M. Mowat, A. Greenberg and E. Turley:Characterization of the murine gene encoding the hyaluronan receptor RHAMM. *Gene* 163, 233-238 (1995)

72. C. Wang, J. Entwistle, G. Hou, Q. Li and E. Turley: The characterization of a human RHAMM cDNA:

conservation of the hyaluronan-binding domains. *Gene* 174, 299-306 (1996)

73. V. Assmann, D. Jenkinson, J. Marshall and I. Hart: The intracellular hyaluronan receptor RHAMM/IHABP interacts with microtubules and actin filaments. *J.Cell.Sci.* 112 (Pt 22), 3943-3954 (1999)

74. M. Stojkovic, O. Krebs, S. Kölle, K. Prelle, V. Assmann, V. Zakhartchenko, F. Sinowatz and E. Wolf: Developmental Regulation of Hyaluronan-Binding Protein (RHAMM/IHABP) Expression in Early Bovine Embryos. *Biol.Repr.* 68, 60-66 (2003)

75. L. Collis, C. Hall, L. Lange, M. Ziebell, R. Prestwich and E. Turley: Rapid hyaluronan uptake is associated with enhanced motility: implications for an intracellular mode of action. *FEBS Lett.* 440, 444-449 (1998)

76. E. A. Turley, L. Austen, D. Moore and K. Hoare: Ras-transformed cells express both CD44 and RHAMM hyaluronan receptors: only RHAMM is essential for hyaluronan-promoted locomotion. *Exp.Cell Res.* 207, 277-282 (1993)

77. S. Samuel, R. Hurta, M. Spearman, J. Wright, E. Turley and A. Greenberg: TGF-beta 1 stimulation of cell locomotion utilizes the hyaluronan receptor RHAMM and hyaluronan. *J.Cell Biol.* 123, 749-758 (1993)

78. M. Choudhary, X. Zhang, P. Stojkovic, L. Hyslop, G. Anyfantis, M. Herbert, A. Murdoch, M. Stojkovic and M. Lako: Putative role of hyaluronan and its related genes, HAS2 and RHAMM, in human early preimplantation embryogenesis and embryonic stem cell characterization. *Stem Cells* 25, 3045-3057 (2007)

79. S. Nedvetzki, E. Gonen, N. Assayag, R. Reich, R. Williams, R. Thurmond, J. Huang, B. Neudecker, F. Wang, E. Turley and D. Naor: RHAMM, a receptor for hyaluronan-mediated motility, compensates for CD44 in inflamed CD44-knockout mice: a different interpretation of redundancy. *Proc.Natl.Acad.Sci.U.S.A.* 101, 18081-18086 (2004)

80. E. Kopp and R. Medzhitov: The Toll-receptor family and control of innate immunity. *Curr.Opin.Immunol.* 11, 13-18 (1999)

81. S. Tomchuck, K. Zwezdaryk, S. Coffelt, R. Waterman, E. Danka and A. Scandurro: Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. *Stem Cells* 26, 99-107 (2008)

82. K. Scheibner, M. Lutz, S. Boodoo, M. Fenton, J. Powell and M. Horton: Hyaluronan fragments act as an endogenous danger signal by engaging TLR2. *J.Immunol.* 177, 1272-1281 (2006)

83. E. Chang, H. Kim, J. Ha, H. Kim, J. Ryu, K. Park, U. Kim, Z. Lee, H. Kim, D. Fisher and H. Kim: Hyaluronan inhibits osteoclast differentiation via Toll-like receptor 4. *J.Cell.Sci.* 120, 166-176 (2007)

84. J. Duband, F. Monier, M. Delannet and D. Newgreen: Epithelium-mesenchyme transition during neural crest development. *Acta Anat.(Basel)* 154, 63-78 (1995)

85. E. Hay: An overview of epithelio-mesenchymal transformation. *Acta Anat.(Basel)* 154, 8-20 (1995)

86. C. Viebahn: Epithelio-mesenchymal transformation during formation of the mesoderm in the mammalian embryo. *Acta Anat.(Basel)* 154, 79-97 (1995)

87. M. Veini and R. Bellairs: Early mesoderm differentiation in the chick embryo. *Anat.Embryol.(Berl)* 183, 143-149 (1991)

88. W. Franke, C. Grund, B. Jackson and K. Illmensee: Formation of cytoskeletal elements during mouse embryogenesis. IV. Ultrastructure of primary mesenchymal cells and their cell-cell interactions. *Differentiation* 25, 121-141 (1983)

89. E. Hay: Interaction of embryonic surface and cytoskeleton with extracellular matrix. *Am.J.Anat.* 165, 1-12 (1982)

90. K. Crossin and G. Edelman: Mechanisms of cell adhesion in epithelial-mesenchymal transformations. *Prog.Clin.Biol.Res.* 226, 81-92 (1986)

91. J. Duband, F. Monier, M. Delannet and D. Newgreen: Epithelium-mesenchyme transition during neural crest development. *Acta Anat.(Basel)* 154, 63-78 (1995)

92. B. Boyer and J. Thiery: Epithelium-mesenchyme interconversion as example of epithelial plasticity. *APMIS* 101, 257-268 (1993)

93. C. Bonneton, J. Sibarita and J. Thiery: Relationship between cell migration and cell cycle during the initiation of epithelial to fibroblastoid transition. *Cell Motil.Cytoskeleton* 43, 288-295 (1999)

94. B. Toole, A. Zoltan-Jones, S. Misra and S. Ghatak: Hyaluronan: a critical component of epithelial-mesenchymal and epithelial-carcinoma transitions. *Cells Tissues Organs* 179, 66-72 (2005)

95. Y. Fujita, M. Kitagawa, S. Nakamura, K. Azuma, G. Ishii, M. Higashi, H. Kishi, T. Hiwasa, K. Koda, N. Nakajima and K. Harigaya: CD44 signaling through focal adhesion kinase and its anti-apoptotic effect. *FEBS Lett.* 528, 101-108 (2002)

96. A. Zoltan-Jones, L. Huang, S. Ghatak and B. Toole: Elevated hyaluronan production induces mesenchymal and transformed properties in epithelial cells. *J.Biol.Chem.* 278, 45801-45810 (2003)

97. J. McDonald and T. Camenisch: Hyaluronan: genetic insights into the complex biology of a simple polysaccharide. *Glycoconj.J.* 19, 331-339 (2002)

98. R. Poelmann, A. Gittenberger-de Groot, M. Mentink, B. Delpech, N. Girard and B. Christ: The extracellular matrix during neural crest formation and migration in rat embryos. *Anat.Embryol.(Berl)* 182, 29-39 (1990)

99. U. Rauch, S. Hirakawa, T. Oohashi, J. Kappler and G. Roos: Cartilage link protein interacts with neurocan, which shows hyaluronan binding characteristics different from CD44 and TSG-6. *Matrix Biol.* 22, 629-639 (2004)

100. L. Alaniz, M. Garcia, C. Gallo-Rodriguez, R. Agusti, N. Sterin-Speziale, S. Hajos and E. Alvarez: Hyaluronan oligosaccharides induce cell death through PI3-K/Akt pathway independently of NF-kappaB transcription factor. *Glycobiology* 16, 359-367 (2006)

101. Y. Khew-Goodall and C. Wadham: A perspective on regulation of cell-cell adhesion and epithelial-mesenchymal transition: known and novel. *Cells Tissues Organs* 179, 81-86 (2005)

102. P. Savagner: Leaving the neighborhood: molecular mechanisms involved during epithelial-mesenchymal transition. *Bioessays* 23, 912-923 (2001)

103. D. Shook and R. Keller: Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. *Mech.Dev.* 120, 1351-1383 (2003)

104. J. Yang and R. Weinberg: Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev.Cell.* 14, 818-829 (2008)

105. B. Boyer, A. Valles and N. Edme: Induction and regulation of epithelial-mesenchymal transitions. *Biochem.Pharmacol.* 60, 1091-1099 (2000)

106. C. Knudson, S. Munaim and B. Toole: Ectodermal stimulation of the production of hyaluronan-dependent pericellular matrix by embryonic limb mesodermal cells. *Dev.Dyn.* 204, 186-191 (1995)

107. S. Munaim, M. Klagsbrun and B. Toole: Hyaluronan-dependent pericellular coats of chick embryo limb mesodermal cells: induction by basic fibroblast growth factor. *Dev.Biol.* 143, 297-302 (1991)

108. D. Kimelman: Mesoderm induction: from caps to chips. *Nat.Rev.Genet.* 7, 360-372 (2006)

109. J. Thiery and J. Sleeman: Complex networks orchestrate epithelial-mesenchymal transitions. *Nat.Rev.Mol.Cell Biol.* 7, 131-142 (2006)

110. A. Moustakas and C. Heldin: Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. *Cancer.Sci.* 98, 1512-1520 (2007)

111. C. Hall, B. Yang, X. Yang, S. Zhang, M. Turley, S. Samuel, L. Lange, C. Wang, G. Curpen, R. Savani, A. Greenberg and E. Turley: Overexpression of the hyaluronan receptor RHAMM is transforming and is also required for H-ras transformation. *Cell*. 82, 19-26 (1995)

112. V. Lokeshwar and M. Selzer: Differences in hyaluronic acid-mediated functions and signaling in arterial, microvessel, and vein-derived human endothelial cells. *J.Biol.Chem.* 275, 27641-27649 (2000)

113. C. Isacke and H. Yarwood: The hyaluronan receptor, CD44. *Int.J.Biochem.Cell Biol.* 34, 718-721 (2002)

114. S. Frisch and R. Screaton: Anoikis mechanisms. *Curr.Opin.Cell Biol.* 13, 555-562 (2001)

115. T. Camenisch and J. McDonald: Hyaluronan: is bigger better? *Am.J.Respir.Cell Mol.Biol.* 23, 431-433 (2000)

116. S. Misra, B. Toole and S. Ghatak: Hyaluronan constitutively regulates activation of multiple receptor tyrosine kinases in epithelial and carcinoma cells. *J.Biol.Chem.* 281, 34936-34941 (2006)

117. L. Bourguignon, P. Singleton, H. Zhu and B. Zhou: Hyaluronan promotes signaling interaction between CD44 and the transforming growth factor beta receptor I in metastatic breast tumor cells. *J.Biol.Chem.* 277, 39703-39712 (2002)

118. L. Bourguignon, H. Zhu, B. Zhou, F. Diedrich, P. Singleton and M. Hung: Hyaluronan promotes CD44v3-Vav2 interaction with Grb2-p185(HER2) and induces Rac1 and Ras signaling during ovarian tumor cell migration and growth. *J.Biol.Chem.* 276, 48679-48692 (2001)

119. L. Rodgers, S. Lalani, K. Hardy, X. Xiang, D. Broka, P. Antin and T. Camenisch: Depolymerized hyaluronan induces vascular endothelial growth factor, a negative regulator of developmental epithelial-to-mesenchymal transformation. *Circ.Res* .99, 583-589 (2006)

120. W. Birchmeier and C. Birchmeier: Epithelial-mesenchymal transitions in development and tumor progression. *EXS* 74, 1-15 (1995)

121. J. Thiery: Epithelial-mesenchymal transitions in development and pathologies. *Curr.Opin.Cell Biol.* 15, 740-746 (2003)

122. L. Dunlop and B. Hall: Relationships between cellular condensation, preosteoblast formation and epithelial-mesenchymal interactions in initiation of osteogenesis. *Int.J.Dev.Biol.* 39, 357-371 (1995)

123. B. Hall and T. Miyake: The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. *Anat.Embryol.(Berl)* 186, 107-124 (1992)

124. J. Saunders: Apical ectodermal ridge in retrospect. *J.Exp.Zool.* 282, 669-676 (1998)

125. C. Knudson and B. Toole: Epithelial-mesenchymal interaction in the regulation of hyaluronate production during limb development. *Biochem.Int.* 17, 735-745 (1988)

126. C. Singley and M. Solursh: The spatial distribution of hyaluronic acid and mesenchymal condensation in the embryonic chick wing. *Dev.Biol.* 84, 102-120 (1981)

127. W. Kulyk and R. Kosher: Temporal and spatial analysis of hyaluronidase activity during development of the embryonic chick limb bud. *Dev.Biol.* 120, 535-541 (1987)

128. L. Sherman, D. Wainwright, H. Ponta and P. Herrlich: A splice variant of CD44 expressed in the apical ectodermal ridge presents fibroblast growth factors to limb mesenchyme and is required for limb outgrowth. *Genes Dev.* 12, 1058-1071 (1998)

129. S. Li, R. Anderson, A. Reginelli and K. Muneoka: FGF-2 influences cell movements and gene expression during limb development. *J.Exp.Zool.* 274, 234-247 (1996)

130. S. Li and K. Muneoka: Cell migration and chick limb development: chemotactic action of FGF-4 and the AER. *Dev.Biol.* 211, 335-347 (1999)

131. R. Tuan: Biology of developmental and regenerative skeletogenesis. *Clin.Orthop.Relat.Res.* (427 Suppl), S105-17 (2004)

132. N. Wada, Y. Kawakami, R. Ladher, P. Francis-West and T. Nohno: Involvement of Frzb-1 in mesenchymal condensation and cartilage differentiation in the chick limb bud. *Int.J.Dev.Biol.* 43, 495-500 (1999)

133. L. Fischer, G. Boland and R. Tuan: Wnt signaling during BMP-2 stimulation of mesenchymal chondrogenesis. *J.Cell.Biochem.* 84, 816-831 (2002)

134. R. Tuli, S. Tuli, S. Nandi, X. Huang, P. Manner, W. Hozack, K. Danielson, D. Hall and R. Tuan: Transforming growth factor-beta-mediated chondrogenesis of human mesenchymal progenitor cells involves N-cadherin and mitogen-activated protein kinase and Wnt signaling cross-talk. *J.Biol.Chem.* 278, 41227-41236 (2003)

135. Y. Li, B. Toole, C. Dealy and R. Kosher: Hyaluronan in limb morphogenesis. *Dev.Biol.* 305, 411-420 (2007)

136. C. Knudson and B. Toole: Changes in the pericellular matrix during differentiation of limb bud mesoderm. *Dev.Biol.* 112, 308-318 (1985)

137. R. Stockwell: Biology of Cartilage Cells. Eds: R. Harrison and R. McMinn. *Cambridge University Press*, GB, (1979)

138. C. Howlett: The fine structure of the proximal growth plate and metaphysis of the avian tibia: endochondral osteogenesis. *J.Anat.* 130, 745-768 (1980)

139. P. Eggli, W. Herrmann, E. Hunziker and R. Schenk: Matrix compartments in the growth plate of the proximal tibia of rats. *Anat.Rec.* 211, 246-257 (1985)

140. K. Noonan, J. Stevens, R. Tammi, M. Tammi, J. Hernandez and R. Midura: Spatial distribution of CD44 and hyaluronan in the proximal tibia of the growing rat. *J.Orthop.Res.* 14, 573-581 (1996)

141. S. Shibata, K. Fukada, H. Imai, T. Abe and Y. Yamashita: *In situ* hybridization and immunohistochemistry of versican, aggrecan and link protein, and histochemistry of hyaluronan in the developing mouse limb bud cartilage. *J.Anat.* 203, 425-432 (2003)

142. C. Archer and P. Francis-West: The chondrocyte. *Int.J.Biochem.Cell Biol.* 35, 401-404 (2003)

143. M. Pacifici, E. Koyama and M. Iwamoto: Mechanisms of synovial joint and articular cartilage formation: recent advances, but many lingering mysteries. *Birth Defects Res.C.Embryo.Today* 75, 237-248 (2005)

144. A. Pitsillides, C. Archer, P. Prehm, M. Bayliss and J. Edwards: Alterations in hyaluronan synthesis during developing joint cavitation. *J.Histochem.Cytochem.* 43, 263-273 (1995)

145. F. Craig, M. Bayliss, G. Bentley and C. Archer: A role for hyaluronan in joint development. *J.Anat.* 171, 17-23 (1990)

146. J. Shepard, H. Krug, B. LaFoon, S. Hoffman and A. Capehart: Versican expression during synovial joint morphogenesis. *Int.J.Biol.Sci.* 3, 380-384 (2007)

147. J. Edwards, L. Wilkinson, H. Jones, P. Soothill, K. Henderson, J. Worrall and A. Pitsillides: The formation of human synovial joint cavities: a possible role for hyaluronan and CD44 in altered interzone cohesion. *J.Anat.* 185 (Pt 2), 355-367 (1994)

148. G. Dowthwaite, A. Ward, J. Flannely, R. Suswillo, C. Flannery, C. Archer and A. Pitsillides: The effect of mechanical strain on hyaluronan metabolism in embryonic fibrocartilage cells. *Matrix Biol.* 18, 523-532 (1999)

149. A. Pitsillides: Identifying and characterizing the joint cavity-forming cell. *Cell Biochem.Funct.* 21, 235-240 (2003)

150. G. Dowthwaite, J. Edwards and A. Pitsillides: An essential role for the interaction between hyaluronan and hyaluronan binding proteins during joint development. *J.Histochem.Cytochem.* 46, 641-651 (1998)

151. M. Ito and M. Kida: Morphological and biochemical re-evaluation of the process of cavitation in the rat knee joint: cellular and cell strata alterations in the interzone. *J.Anat.* 197 Pt 4, 659-679 (2000)

152. J. Lewthwaite, E. Bastow, K. Lamb, J. Blenis, C. Wheeler-Jones and A. Pitsillides: A specific mechanomodulatory role for p38 MAPK in embryonic joint

articular surface cell MEK-ERK pathway regulation. *J.Biol.Chem.* 281, 11011-11018 (2006)

153. P. Gakunga, Y. Kuboki and L. Opperman: Hyaluronan is essential for the expansion of the cranial base growth plates. *J.Craniofac.Genet.Dev.Biol.* 20, 53-63 (2000)

154. A. Suzuki, K. Tanimoto, S. Ohno, Y. Nakatani, K. Honda, N. Tanaka, T. Doi, M. Ohno-Nakahara, K. Yoneno, M. Ueki and K. Tanne: The metabolism of hyaluronan in cultured rabbit growth plate chondrocytes during differentiation. *Biochim.Biophys.Acta* 1743, 57-63 (2005)

155. C. Magee, M. Nurminskaya and T. Linsenmayer: UDP-glucose pyrophosphorylase: up-regulation in hypertrophic cartilage and role in hyaluronan synthesis. *Biochem.J.* 360, 667-674 (2001)

156. Y. Nishida, C. Knudson, J. Nietfeld, A. Margulis and W. Knudson: Antisense inhibition of hyaluronan synthase-2 in human articular chondrocytes inhibits proteoglycan retention and matrix assembly. *J.Biol.Chem.* 274, 21893-21899 (1999)

157. P. Pavasant, T. Shizari and C. Underhill: Distribution of hyaluronan in the epiphysial growth plate: turnover by CD44-expressing osteoprogenitor cells .*J.Cell.Sci.* 107 (Pt 10), 2669-2677 (1994)

158. P. Pavasant, T. Shizari and C. Underhill: Hyaluronan contributes to the enlargement of hypertrophic lacunae in the growth plate. *J.Cell.Sci.* 109 (Pt 2), 327-334 (1996)

159. B. Clarris and J. Fraser: On the pericellular zone of some mammalian cells *in vitro*. *Exp.Cell Res.* 49, 181-193 (1968)

160. S. Nicoll, O. Barak, A. Csoka, R. Bhatnagar and R. Stern: Hyaluronidases and CD44 undergo differential modulation during chondrogenesis. *Biochem.Biophys.Res.Commun.* 292, 819-825 (2002)

161. K. Tanimoto, A. Suzuki, S. Ohno, K. Honda, N. Tanaka, T. Doi, M. Nakahara-Ohno, K. Yoneno, Y. Nakatani, M. Ueki, T. Yanagida, R. Kitamura and K. Tanne: Hyaluronidase expression in cultured growth plate chondrocytes during differentiation. *Cell Tissue Res.* 318, 335-342 (2004)

162. R. Montesano, S. Kumar, L. Orci and M. Pepper: Synergistic effect of hyaluronan oligosaccharides and vascular endothelial growth factor on angiogenesis *in vitro*. *Lab.Invest.* 75, 249-262 (1996)

163. D. West, I. Hampson, F. Arnold and S. Kumar: Angiogenesis induced by degradation products of hyaluronic acid. *Science* 228, 1324-1326 (1985)

164. F. Gao, C. Yang, W. Mo, Y. Liu and Y. He: Hyaluronan oligosaccharides are potential stimulators to angiogenesis via RHAMM mediated signal pathway in wound healing. *Clin.Invest.Med.* 31, E106-16 (2008)

165. A. Pilloni and G. Bernard: The effect of hyaluronan on mouse intramembranous osteogenesis *in vitro*. *Cell Tissue Res.* 294, 323-333 (1998)

166. Y. Tanne, K. Tanimoto, N. Tanaka, M. Ueki, Y. Lin, S. Ohkuma, T. Kamiya, E. Tanaka and K. Tanne: Expression and activity of Runx2 mediated by hyaluronan during chondrocyte differentiation. *Arch.Oral Biol.* 53, 478-487 (2008)

167. E. Mackie, Y. Ahmed, L. Tatarczuch, K. Chen and M. Mirams: Endochondral ossification: how cartilage is converted into bone in the developing skeleton. *Int.J.Biochem.Cell Biol.* 40, 46-62 (2008)

168. M. Goldring, K. Tsuchimochi and K. Ijiri: The control of chondrogenesis. *J.Cell.Biochem.* 97, 33-44 (2006)

169. S. Adams, A. Cohen and L. Lassova: Integration of signaling pathways regulating chondrocyte differentiation during endochondral bone formation. *J.Cell.Physiol.* 213, 635-641 (2007)

170. I. Kim, F. Otto, B. Zabel and S. Mundlos: Regulation of chondrocyte differentiation by Cbfa1. *Mech.Dev.* 80, 159-170 (1999)

171. S. Takeda, J. Bonnamy, M. Owen, P. Ducy and G. Karsenty: Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev.* 15, 467-481 (2001)

172. L. Huang, Y. Cheng, P. Koo, K. Lee, L. Qin, J. Cheng and S. Kumta: The effect of hyaluronan on osteoblast proliferation and differentiation in rat calvarial-derived cell cultures. *J.Biomed.Mater.Res.A.* 66, 880-884 (2003)

173. D. Hughes, D. Salter and R. Simpson: CD44 expression in human bone: a novel marker of osteocytic differentiation. *J.Bone Miner.Res.* 9, 39-44 (1994)

174. J. Cao, P. Singleton, S. Majumdar, B. Boudignon, A. Burghardt, P. Kurimoto, T. Wronski, L. Bourguignon and B. Halloran: Hyaluronan increases RANKL expression in bone marrow stromal cells through CD44. *J.Bone Miner.Res.* 20, 30-40 (2005)

175. R. Poulsom: CD44 and hyaluronan help mesenchymal stem cells move to a neighborhood in need of regeneration. *Kidney Int.* 72, 389-390 (2007)

176. H. Zhu, N. Mitsuhashi, A. Klein, L. Barsky, K. Weinberg, M. Barr, A. Demetriou and G. Wu: The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. *Stem Cells* 24, 928-935 (2006)

177. Y. Kim, Y. Lee, J. Choe, H. Lee, Y. Kim and D. Jeoung: CD44-epidermal growth factor receptor interaction mediates hyaluronic acid-promoted cell motility by

activating protein kinase C signaling involving Akt, Rac1, Phox, reactive oxygen species, focal adhesion kinase, and MMP-2. *J.Biol.Chem.* 283, 22513-22528 (2008)

178. V. Orian-Rousseau, L. Chen, J. Sleeman, P. Herrlich and H. Ponta: CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev.* 16, 3074-3086 (2002)

Abbreviations: HA, hyaluronan; Has1, HAS2, HAS3, hyaluronan synthases; RHAMM, receptor for hyaluronic acid mediated motility; TLR, toll-like receptor; EMT, epithelial-mesenchymal transition; TGF-β, transforming growth factor β; SF/HGF, scatter factor/hepatocyte growth factor; EGF, epithelial grown factor; FGF, fibroblast growth factor; FAK, focal adhesion kinase; RTK, receptor tyrosine kinase; BMP, bone morphogenic protein; Runx2, Runt-related 2; RANKL, receptor activator of nuclear factor

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