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Hyaluronan in Limb Morphogenesis

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Abstract

Hyaluronan (HA) is a large glycosaminoglycan that is not only a structural component of extracellular matrices, but also interacts with cell surface receptors to promote cell proliferation, migration, and intracellular signaling. HA is a major component of the extracellular matrix of the distal subapical mesenchymal cells of the developing limb bud that are undergoing proliferation, directed migration, and patterning in response to the apical ectodermal ridge (AER), and has the functional potential to be involved in these processes. Here we show that the HA synthase *Has2* is abundantly expressed by the distal subridge mesodermal cells of the chick limb bud and also by the AER itself. *Has2* expression and HA production are downregulated in the proximal central core of the limb bud during the formation of the precartilage condensations of the skeletal elements, suggesting downregulation of HA may be necessary for the close juxtaposition of cells and the resulting cell-cell interactions that trigger cartilage differentiation during condensation. Overexpression of *Has2* in the mesoderm of the chick limb bud in vivo results in the formation of shortened and severely malformed limbs that lack one or more skeletal elements. Skeletal elements that do form in limbs overexpressing *Has2* are reduced in length, exhibit abnormal morphology, and are positioned inappropriately. We also demonstrate that sustained HA production in micromass cultures of limb mesenchymal cells inhibits formation of precartilage condensations and subsequent chondrogenesis, indicating that downregulation of HA is indeed necessary for formation of the precartilage condensations that trigger cartilage differentiation. Taken together these results suggest involvement of HA in various aspects of limb morphogenesis.

Keywords

Hyaluronan; Limb development; Cartilage differentiation; Apical ectodermal ridge

Introduction

Hyaluronan (HA) is a linear glycosaminoglycan composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine that is a structural component of the extracellular matrix of a variety of tissues. It is a huge molecule with molecular weight ranging from 10^3 to 10^4 kDa and an extended length of 2–25 μm (see Toole, 1997 see Toole,

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2000a,b for reviews). HA occupies an extensive molecular domain in dilute solution, and has a pronounced hydration capacity (Toole, 1997, 2000a,b). At higher concentrations individual extended HA molecules form an entangled continuous hydrated network. Although several of the functions of HA may be attributable to its physicochemical properties, HA is not simply a passive structural and space-filling component of extracellular matrices. Rather, HA directly interacts with cells and influences their behavior. HA concomitantly promotes the proliferation and migration of cells in a number of model systems (Evanko et al., 1999; Itano et al., 2002; Ward et al., 2003; Rilla et al., 2002) and during embryogenesis (Gakunga et al. 1997; Camenisch et al., 2000; Bakkers et al., 2004; Ori et al., 2005). Moreover, interaction of HA with its cell surface receptors including CD44 directly activates intracellular signaling, and can have instructive effects on cell behavior (Turley et al., 2002). HA is an important modulator of the EGFR/ErbB (Camenisch et al., 2002; Tsatas et al., 2002; Bourguignon et al., 1997; Sherman et al., 2000; Ghatak et al., 2005; Misra et al., 2006), TGF-β (Bourguignon et al., 2002), and BMP (Peterson et al., 2004) signaling networks, and acts in concert with these signaling molecules to regulate a variety of cellular processes.

The outgrowth and patterning of the developing limb is regulated by reciprocal interactions between the apical ectodermal ridge (AER), a thickened cap of ectoderm along the distal periphery of the limb bud, and the underlying distal subridge mesoderm (Saunders, 1948). The distal subridge mesenchymal cells of the developing limb bud, which are undergoing proliferation, directed migration, and patterning in response to the AER and other signaling centers such as the zone of polarizing activity (ZPA) produce high amounts of HA which forms an expansive hydrated extracellular matrix between the cells (Toole, 1972; Kosher et al., 1981; Singley and Solursh, 1981; Knudson and Toole, 1985). HA is also synthesized and secreted by the AER itself (Kosher and Savage, 1981). Thus the cell and tissue interactions controlling the outgrowth and patterning of the limb occur in an environment rich in extracellular and pericellular HA.

One of the functions of the AER is to maintain the subridge mesenchymal cells in a labile undifferentiated condition (Kosher et al., 1979). When the mesenchymal cells in the central core of the limb bud become located outside of the range of AER signaling, they initiate cartilage differentiation, the onset of which is characterized by a transient cellular condensation process in which the cells become closely juxtaposed to one another. During this condensation intimate cell-cell interactions occur which are necessary to trigger chondrogenic differentiation (see Kosher, 1983; Solursh, 1983 for reviews). During the initial formation of precartilage condensations, HA production is strikingly downregulated and the hydrated HA-rich extracellular matrix and pericellular HA surface coats are removed (Kosher et al., 1981; Knudson and Toole, 1985) as a result of the receptor-mediated endocytosis of HA and its intracellular degradation by hyaluronidase, in particular Hyal1 (Kulyk and Kosher, 1987; Nicoll et al., 2002). On the basis of these observations it has been suggested that the HA-rich extracellular matrix elaborated by subridge mesenchymal cells may mediate the antidifferentiative effect of the AER by preventing the intimate cell-cell interactions necessary to trigger chondrogenesis (Kosher et al., 1981). Furthermore, the downregulation of HA production and the removal of extracellular HA that occurs when cells in the central core of the limb bud become located outside of the range of AER signaling may be necessary for the close juxtaposition of cells and the resulting cell-cell interactions that trigger chondrogenesis during the formation of precartilage condensations (Kosher et al., 1981).

HA is synthesized in a unique fashion. It is made at the inner side of the plasma membrane rather than in the Golgi apparatus and is extruded to the outside of the cell during polymerization (Weigel et al., 1997). HA is synthesized by three HA synthase isoforms called Has1, Has2, and Has3 (Weigel et al., 1997; Spicer and McDonald, 1998). Has2 is the major source of HA during the period of early organogenesis in the mouse embryo when the early

outgrowth and patterning of the limb bud is occurring (Spicer and McDonald, 1998; Camenisch et al., 2000; Tien and Spicer, 2005).

In the present study we demonstrate that *Has2* is indeed abundantly expressed by the distal subridge mesodermal cells of the developing chick limb bud that are undergoing outgrowth and patterning in response to the AER and other signaling centers, and is also expressed by the AER itself. Furthermore, *Has2* expression is downregulated in the proximal central core of the limb bud during the formation of precartilage condensations. Misexpression of *Has2* from the onset of limb development in vivo results in shortened and severely malformed limbs that lack one or more skeletal elements and/or possess skeletal elements that exhibit abnormal morphology or positioning. Finally, we demonstrate that sustained production of HA in micromass cultures of limb mesenchymal cells impairs the formation of precartilage condensations and subsequent chondrogenesis, consistent with the suggestion that downregulation of HA is necessary for the formation of the precartilage condensations that trigger cartilage differentiation.

Materials and methods

Preparation of Has2 adenoviral and retroviral expression vectors and in vivo infection protocol

A recombinant adenoviral expression construct containing a cDNA encompassing the full coding sequence of chicken *Has2* (GenBank accession number **AF106940**) was prepared as previously described (Ward et al., 2003; Zoltan-Jones et al., 2003; Ghatak et al., 2005). Briefly, the cDNA was cloned into the pACCM-V.pLpA shuttle vector, co-transfected into 293 cells with the pJM17 adenovirus, and plaques resulting from successful homologous recombination were chosen, amplified, and purified using cesium chloride gradient centrifugation (Becker et al., 1994; Ward et al., 2003; Zoltan-Jones et al., 2003; Ghatak et al., 2005). The titer of the *Has2* adenovirus was 10^{11} pfu/ml. The *Has2* adenoviral mediated synthesis of HA by cells infected with the *Has2* adenovirus was confirmed by visualization of HA-dependent pericellular coats using a particle dye exclusion assay (Zoltan-Jones et al., 2003) and by a competitive enzyme-linked immunosorbant assay-like method using biotinylated HA-binding protein (HABP) (Seikagaku America, Inc.) as a probe (Gordon et al., 2003; Zoltan-Jones et al., 2003).

The cDNA containing the full coding sequence of chicken *Has2* was also cloned into the avian replication competent retroviral expression vector RCASBP(A), and concentrated *Has2* retrovirus (10^9 cfu/ml) was prepared using standard established protocols as previously described (Ferrari et al., 1998; Ferrari and Kosher, 2002; Fisher et al., 2006). The production of HA by the *Has2* RCAS expression vector was confirmed by biotinylated HABP staining of infected chick limb bud mesenchymal cells as described below.

Concentrated *Has2* adenovirus or *Has2* RCAS retrovirus were microinjected as previously described (Ferrari et al., 1998; Ferrari and Kosher, 2002) into the mesoderm of the right wing buds of stage 18 (Hamburger and Hamilton, 1951) chick embryos or into the prospective limb-forming regions of stage 10 embryos. At various times after microinjection the skeletons of the embryos were stained with Alcian blue and Alizarin Red as previously described (Ferrari and Kosher, 2002) to visualize cartilage and mineralized bone, respectively.

Preparation and infection of micromass cultures

Micromass cultures of mesenchymal cells from stage 22–24 embryonic chick wing buds were established as previously described (Gay and Kosher, 1984) at densities of 2.5, 3.4, and 5×10^4 cells/10 μl of F12 medium containing 10% fetal bovine serum, 1% L-glutamine, and

antibiotics. For retroviral infection of the cultures, 1 μl of concentrated *Has2* RCAS retrovirus or control RCAS retrovirus lacking the *Has2* cDNA insert was added per 10 μl of cell suspensions containing 2.5, 3.4, or 5×10^6 cells/ml, after which 10 μl drops of the suspensions were dispensed onto the surface of 35 mm Nunclon tissues cultures dishes. The retrovirus-containing micromass cultures were incubated for 3 h at 37° C in a humidified CO_2 incubator to allow for cell attachment, after which the cultures were supplied with 2.5 ml of medium. Thus, the micromass cultures were exposed to concentrated *Has2* or control retrovirus for 3 h during the initial plating and attachment of the cells. The morphology of the cultures was monitored daily by phase contrast microscopy.

Analytical procedures

The accumulation of cartilage matrix by micromass cultures was monitored histochemically by staining with 1% Alcian blue, pH 1.0 as previously described (Gay and Kosher, 1984). The area of cultures occupied by Alcian blue staining cartilage matrix was quantified as previously described (Fisher et al., 2006) using the automated Threshold and Analyze Particles commands in ImageJ image analysis program (version 1.32j) on images acquired under identical exposure and lighting conditions.

The distribution of HA in RCAS control and *Has2* RCAS infected micromass cultures was determined by histochemical staining with a biotinylated HA binding protein (HABP) from the HA-binding region of aggrecan which binds strongly and specifically to HA in cells and tissue sections (Knudson and Toole, 1985; Asari et al., 1992; Yu et al., 1996; Shibata et al., 2003; Underhill and Zhang, 2000; Toole et al., 2001). Briefly, cultures that had been fixed in 4% paraformaldehyde were blocked by sequential treatment with avidin, biotin, and 0.1% BSA solutions (Avidin/Biotin Blocking Kit, Vector), incubated with 2 μg/ml of biotinylated HABP (Seikagaku America, Inc) overnight at 4° C, washed with PBT, treated with an avidin-biotin HRP complex (Vectastain ABC kit), washed with PBT, and developed with diaminobenzidene/H_2O_2 (Vector). No HABP staining was detectable in cultures pretreated for 2 h with 200 TRU/ml of *Streptomyces hyalurolyticus* hyaluronidase (Seikagaku America, Inc) which specifically degrades HA.

In situ hybridization on serially sectioned limb buds that had been fixed in Bouin's solution was performed as previously described (Coelho et al., 1991) using a ^{33}P-labeled 517 bp chicken *Has2* cDNA probe extending from nucleotides 1490 to 2007 at the 3′ end of the cDNA. The probe was prepared by digesting a pCI-neo vector containing the full coding sequence of chicken *Has2* (GenBank accession number **AF106940**) with XcmI.

Results

Expression of Has2 during early chick limb morphogenesis

Has2 is the major HA synthase that is expressed during the period of early organogenesis in the mouse embryo when the early outgrowth and patterning of the limb bud is occurring (Spicer and McDonald, 1998; Camenisch et al., 2000; Tien and Spicer, 2005). Accordingly, we have examined the temporal and spatial pattern of *Has2* expression by in situ hybridization during the early stages of development of the chick limb bud. *Has2* is expressed in the posterior subridge mesoderm as early as stage 17/18, which is shortly after formation of the limb bud (Fig. 1A). During early limb morphogenesis from stages 20 (Fig. 1B) through stage 25 (Fig. 1C), *Has2* is abundantly expressed by the distal posterior subridge mesenchymal cells of the limb bud that are undergoing outgrowth and patterning in response to the AER and other signaling centers such as the ZPA (Fig. 1C). The distal subridge mesodermal cells that are abundantly expressing *Has2* are characterized by a high rate of HA synthesis and are surrounded by an HA-rich extracellular matrix (Kosher et al., 1981; Singley and Solursh,

1981; Knudson and Toole, 1985). In contrast to the distal subridge mesoderm, little or no *Has2* expression is detectable in the proximal central core of the limb bud where precartilage condensations are forming (Fig. 1C). The downregulation of *Has2* expression in the chondrogenic proximal central core of the limb bud correlates with the downregulation of HA production that occurs during precartilage condensation formation (Kosher et al., 1981; Knudson and Toole, 1985).

At stage 27, *Has2* is expressed at the posterior margin of the autopod and in the distal mesoderm at the tips of the developing digits where outgrowth is occurring in response to the AER (Fig. 1D). In contrast, *Has2* is not expressed in the interdigital mesoderm which underlies the portions of the distal ectoderm in which AER activity has ceased (Fig. 1D). *Has2* is also not expressed in the precartilage condensations of the developing digits (Fig. 1D).

As shown in Fig. 1E and F, during early limb development, in addition to being expressed in the distal subridge mesoderm, *Has2* is also expressed by the AER itself, which is producing relatively high amounts of HA as it is directing the outgrowth, directed migration, and patterning of the underlying mesoderm (Kosher and Savage, 1981). However, little *Has2* expression is detectable in nonridge dorsal/ventral limb ectoderm (Fig. 1E,F), which exhibits relatively little HA synthesis (Kosher and Savage, 1981).

Overexpression of Has2 in the mesoderm of the developing chick limb bud in vivo perturbs limb morphogenesis

In a functional study to explore the possible role of HA in early limb development, we overexpressed *Has2* via adenoviral and RCAS replication competent retroviral vectors in the mesoderm of the developing chick limb bud in vivo to determine if upregulated and sustained HA production perturbs limb morphogenesis.

As shown in Fig. 2, microinjection of *Has2* adenovirus into the mesoderm of the developing limb bud at stage 18, which is shortly after the initial formation of the limb bud, results in the formation of shortened and severely malformed limbs. The phenotype of the *Has2* infected limbs is complex and variable, very likely reflecting differences in the extent of infection of different samples as is typical of in vivo misexpression studies (Ferrari et a., 1998;Omi et al., 2005). There are, however, several consistent defects in the *Has2* infected limbs. In 42% (11 of 26) of the *Has2* infected limbs one or more cartilaginous skeletal elements are completely or partially absent (Fig. 2). Furthermore, 92% (24 of 26) of the *Has2* infected limbs are shorter than those of contralateral control limbs (Fig. 2).

The skeletal elements of the limbs infected with *Has2* adenovirus are not only reduced in length, but also 65% (17 of 26) of the *Has2* infected limbs possess skeletal elements that are misshapen and exhibit abnormal morphology (Fig. 2). Indeed, 35% (9 of 26) of the *Has2* infected limbs contain skeletal elements that exhibit little resemblance to normal skeletal elements, or are inappropriately arranged or positioned (15%, 4 of 26). In some cases, a rudimentary radius, ulna, or an unidentifiable skeletal element is fused to and growing out of the shaft of the humerus (Fig. 2C, E, F). In 27% (7 of 26) of the *Has2* infected limbs the autopod contains only a digit 3, and digits 4 and 2 are absent.

Microinjection of a *Has2* RCAS retroviral expression vector into the prospective limb forming region at stage 10 also results in severely shortened and abnormal limbs. In the *Has2*-RCAS infected limb shown in Fig. 3, all of the segments of the limb are malformed. The stylopod (femur) is severely shortened and misshapen, and the zeugopod consists of a single abnormal skeletal element that articulates with two extremely small digits that bear no resemblance to normal digits (Fig. 3).

Studies on the role of HA in regulation of limb chondrogenesis in vitro

During the initial formation of precartilage condensations in the proximal central core of the developing limb, HA production and *Has2* expression are strikingly downregulated and extracellular HA is removed (Kosher et al., 1981; Knudson and Toole, 1985). It has been suggested that this downregulation of HA may be necessary for the close juxtaposition of cells and the critical cell-cell interactions that trigger chondrogenic differentiation during the formation of the precartilage condensations of the skeletal elements (Kosher et al., 1981; Knudson and Toole, 1985). Consistent with this possibility, as described above, we have found that the differentiation of cartilaginous limb skeletal elements is impaired in response to overexpression of *Has2* in the mesoderm of the developing chick limb in vivo. In the present study we have investigated this hypothesis further and more directly by determining if sustained HA production in response to retroviral misexpression of *Has2* impairs the ability of limb mesenchymal cells to undergo condensation and chondrogenesis in vitro.

In these studies micromass cultures of stage 22–24 limb bud mesenchymal cells were infected for 3 hours at the onset of culture with our *Has2* RCAS retroviral expression vector or with a control RCAS retroviral vector lacking the *Has2* cDNA inset. Since replication competent RCAS retrovirus needs to be internalized and integrated into the genome of the cells, it should take about 15–24 hours after infection for the *Has2* transgene to be expressed. For this reason, in these studies we used micromass cultures established at various relatively low cell densities ($2.5–5 \times 10^4$ cells/10 μl) at which cartilage differentiation progresses more slowly than in standard high density micromass cultures (2×10^5 cells/10 μl). This was done to ensure that the *Has2* transgene would be expressed before or during the formation of precartilage condensations which at these lower densities do not form until day 2 or later after the onset of culture (Rodgers et al., 1989; Seghatoleslami et al., 1996).

As shown in Fig. 4, low density micromass cultures infected with the control RCAS vector form numerous precartilage condensations of closely packed rounded cells detectable by phase contrast microscopy on day 2–3 of culture (Fig. 4A and E). These precartilage aggregates subsequently enlarge and differentiate into cartilage nodules characterized by the deposition of a refractile matrix detectable by phase contrast microscopy (Fig. 4C and G). In contrast, only a few very small condensations form in cultures infected with the *Has2* RCAS vector (Fig. 4B and F), and this is subsequently reflected in a striking decease in the number of refractile cartilage nodules that differentiate in the *Has2* infected cultures compared to controls (Fig. 4D and H). As shown at higher magnification in Fig. 5, whereas control cultures form numerous precartilage condensations or nodules of closely juxtaposed rounded cells (Fig. 5A), most of the cells of the *Has2* infected cultures are large, flattened and spatially separated (Fig. 5B). These results indicate that sustained expression of *Has2* inhibits the formation of precartilage condensations and subsequent chondrogenesis.

To confirm and quantify the apparent inhibition of chondrogenesis detectable by phase contrast microscopy in the *Has2* infected cultures, we examined the accumulation of cartilage matrix in the RCAS control and *Has2* RCAS infected cultures by staining with Alcian blue, pH 1.0. As shown in Fig. 6, there is a striking decrease in the extent of Alcian blue staining cartilage matrix in the *Has2* infected cultures compared to controls. The area occupied by Alcian blue cartilage matrix as determined by image analysis is reduced by 61–71% in the *Has2* infected cultures established at various low cell densities compared to cultures infected with the control RCAS vector (Table 1).

To confirm and examine the extent of ectopic HA production in the *Has2* infected cultures, we examined the distribution of HA by histochemical staining with a biotinylated HA binding protein (HABP) which binds strongly and specifically to HA in cells and tissue sections (Knudson and Toole, 1985; Asari et al., 1992; Yu et al., 1996; Shibata et al., 2003; Underhill

and Zhang, 2000; Toole et al., 2001). As shown in Fig. 7, relatively little HABP staining is detectable in RCAS control cultures (Fig. 7A), whereas widespread HABP staining is present throughout the *Has2* infected cultures (Fig. 7B). No HABP staining is detectable in cultures pretreated with *Streptomyces* hyaluronidase which specifically degrades HA. These results confirm that there is widespread ectopic HA production in the *Has2* RCAS infected cultures in which precartilage condensation and cartilage differentiation is inhibited.

Discussion

HA in Limb Morphogenesis

Consistent with important roles for HA in limb morphogenesis, we have found that overexpression of *Has2* in the mesoderm of the chick limb bud in vivo results in the formation of severely malformed limbs. Limbs overexpressing *Has2* exhibit complete or partial absence of one or more cartilaginous skeletal elements. This suggests that sustained localized production of HA impairs chondrogenic differentiation, and is consistent with a role for HA in the regulation of the onset of chondrogenesis (see below for further discussion).

Furthermore, overexpression of *Has2* results in limbs and skeletal elements that are considerably shorter than normal (92%). This suggests the possibility that HA may be involved in growth of the limb. HA promotes cell proliferation in several model systems (Evanko et al., 1999; Itano et al., 2002; Ward et al., 2003; Rilla et al., 2002), and is present in high amounts in the distal subridge mesoderm of the limb bud which is undergoing directed outgrowth and proliferation in response to the AER. The possible role of HA in limb growth will require further study.

It is also noteworthy that directed proximodistal outgrowth of the limb not only depends on cell proliferation in response to AER signals, but also is facilitated by the directed distal migration of subridge mesenchymal cells towards the AER in response to chemotactic influences (Li et al., 1996; Li and Muneoka, 1999). HA is an important regulator of cell migration in several developing systems (Camenisch et al., 2000; Bakkers et al., 2004; Ori et al., 2005), and can act as a chemotactic signal to promote directional cell migration (Tzircotis et al., 2005).

A relatively high percentage of limbs overexpressing *Has2* exhibit patterning defects. In particular, 65% of the *Has2* infected limbs have skeletal elements that are misshapen and exhibit abnormal morphology, and 35% have skeletal elements that bear little or no resemblance to normal skeletal elements. Inappropriate positioning of skeletal elements also occasionally occurs. These defects suggest the possibility that HA may be involved in the patterning of the developing limb. Although this possibility will require further study, it is noteworthy that HA is an important co-stimulator of the EGFR/ErbB signaling network, and acts in concert with the ErbB family of tyrosine kinase receptors and ligands to regulate a variety of cellular processes (Camenisch et al., 2002; Tsatas et al., 2002; Bourguigon et al., 1997; Sherman et al., 2000; Ghatak et al., 2005; Misra et al., 2006). This is of particular interest since the EGFR/ErbB signaling network has been implicated in the outgrowth and patterning of the developing limb bud (Omi et al., 2005).

Possible role of HA in AER activity

In the present study we have found that, in addition to being expressed by the distal subridge mesoderm of the developing limb bud, *Has2* is also expressed by the AER itself, as the AER is promoting the proliferation and directed migration of the underlying subridge mesenchymal cells. However, little *Has2* expression is detectable in nonridge dorsal/ventral limb ectoderm. This observation is consistent with our previous biochemical studies showing that the rate of

HA production by the AER is 3–4 fold greater than that of nonridge limb ectoderm (Kosher and Savage, 1981). We suggest that the relatively large amount of HA that is synthesized and secreted by the AER may play an important role in facilitating the interactions between the AER and underlying mesenchymal cells that result in the directed PD elongation of the limb.

The basal lamina subjacent to the AER is diffuse and considerably less well organized than elsewhere in the limb (Tomasek et al., 1980; Newman et al., 1981), and it is likely that the HA produced by the AER contributes to the unique disorganization of the basal lamina, which in turn may be important for facilitating interactions between the AER and underlying mesenchymal cells. The unique disorganization and composition of the AER basal lamina may result in the diminished adherence of the basal surface of AER cells to the diffuse underlying substratum, thus promoting the unique wedge-shaped configuration of the AER which, in turn, creates a sublaminar space in which HA produced by the AER accumulates (Newman et al., 1981). Such a localized HA rich sublaminar space may facilitate directional outgrowth of the mesenchyme by providing a region of low mechanical resistance (Newman et al., 1981). In particular, a localized HA rich sublaminar space may facilitate the directed migration of the mesenchymal cells towards the AER thus encouraging the PD elongation of the limb, and may also facilitate direct contact between the migrating cells and the AER. As mentioned above, HA can act as a chemoattractant, and a chemotactic HA gradient can promote directional cell migration (Tzircotis et al., 2005). Thus, HA synthesized and secreted by the AER may contribute to an HA gradient that promotes the directional migration of the subridge mesenchymal cells towards the AER.

Role of HA in regulation of the onset of limb chondrogenesis

One of the functions of the AER is to maintain the mesenchymal cells directly subjacent to it in a labile undifferentiated condition (Kosher et al., 1979). It has been suggested that the HA rich extracellular matrix produced by the subridge mesenchymal cells may mediate this antidifferentiative effect by keeping the cells largely separated from on another, thus preventing the intimate cell-cell interactions that are necessary to trigger chondrogenic differentiation (Kosher et al., 1981). During the initial formation of precartilage condensations that occurs when the cells in the central core of the limb bud become located outside of the range of AER signaling, *Has2* expression and HA production are strikingly downregulated (Kosher et al., 1981; the present study) and concomitantly the HA rich extracellular matrix and pericellular HA rich surface coats are removed (Knudson and Toole, 1985; Kulyk and Kosher, 1987; Nicoll et al., 2002). Thus, it has been suggested that the downregulation of HA production and the removal of extracellular HA may be necessary for the close juxtaposition of cells and the critical cell-cell interactions that trigger chondrogenic differentiation during the formation of precartilage condensations (Kosher et al., 1981). Consistent with this possibility, in the present study we have found that the differentiation of cartilaginous limb skeletal elements is impaired in response to misexpression of *Has2* in the mesoderm of the developing chick limb in vivo. Moreover, we demonstrate that sustained production of HA in response to misexpression of *Has2* in micromass cultures of limb mesenchymal cells inhibits the formation of precartilage condensations and subsequent chondrogenesis. These results indicate that downregulation of HA is indeed necessary for the formation of the precartilage condensations that trigger cartilage differentiation.

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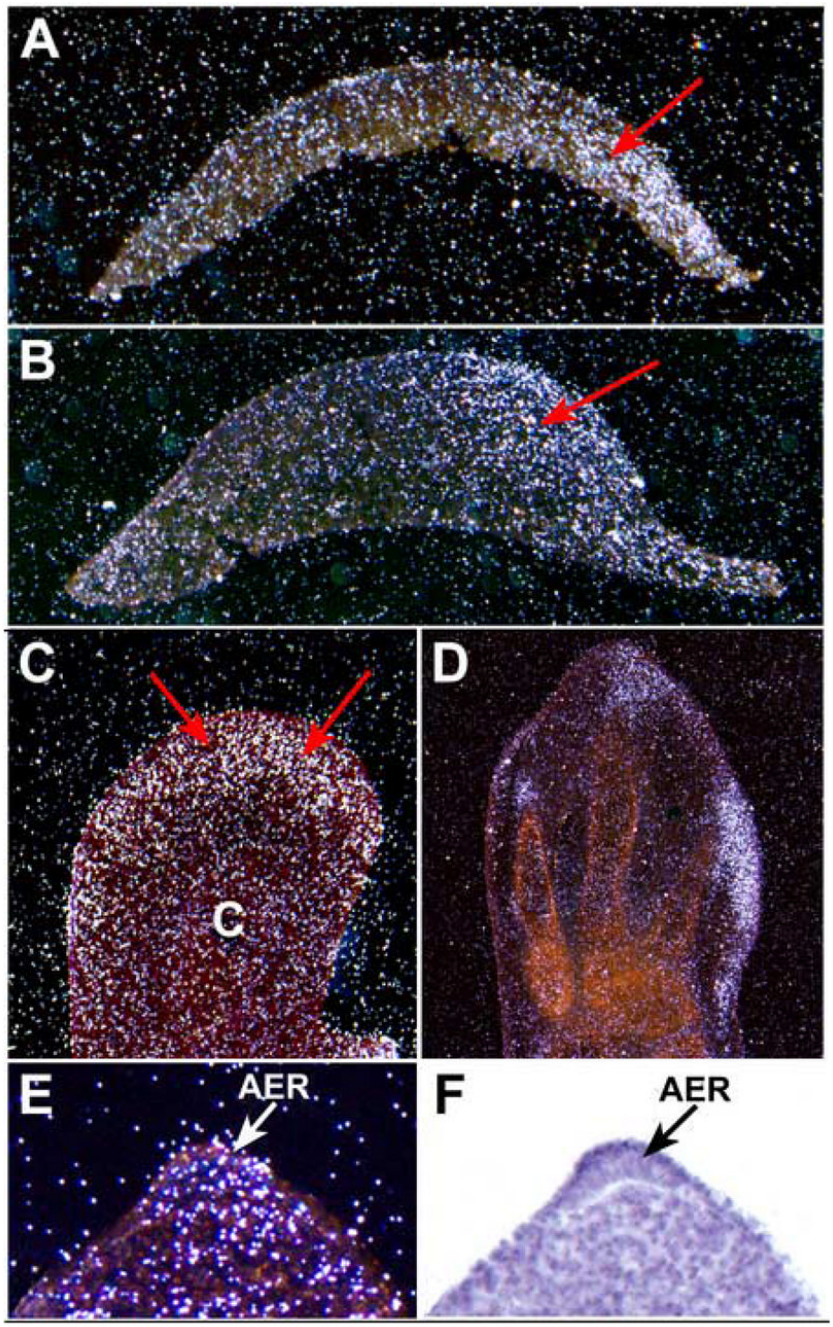

Fig. 1.

Expression of *Has2* during early chick limb bud development analyzed by in situ hybridization. (A–D) Frontal sections through stage 17/18 (A), stage 20 (B), stage 25 (C), and stage 27 (D) limb buds. In each panel the posterior border of the limb bud is on the right and the anterior border on the left. (E, F) Dark field (E) and bright field (F) images of a sagittal section through the AER of a stage 20 limb bud. At stages 17/18 (A), 20 (B), and 25 (C) *Has2* is expressed in the distal posterior subridge mesoderm (red arrows). Little or no expression is detectable in the proximal central core (c in panel C) where precartilage condensations are forming. At stage 27 (D) *Has2* is expressed in the distal mesoderm at the tips of the digits which is undergoing outgrowth, but is not expressed in the interdigital mesoderm or in the precartilage

condensations of the digits. During early limb morphogenesis *Has2* is also expressed in the AER (arrows in E, F).

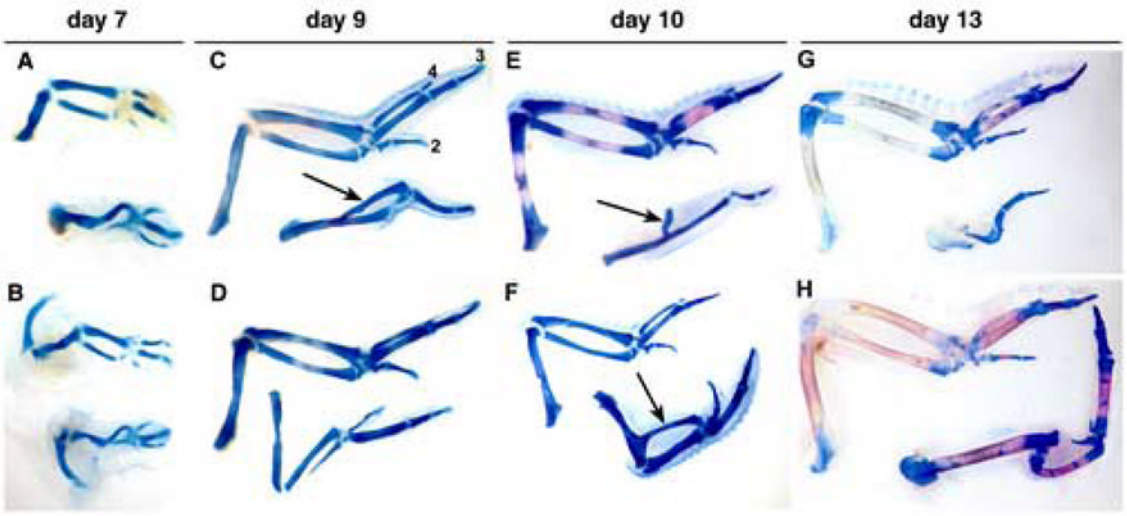

Fig. 2.

Adenoviral overexpression of *Has2* in the mesoderm of the developing chick limb bud in vivo perturbs limb morphogenesis. (A–H) Alcian blue/Alizarin Red-stained skeletal elements of day 7 (A, B), day 9 (C, D), day 10 (E, F), and day 13 (G, H) contralateral control forelimbs (top of each panel) and *Has2* infected forelimbs (bottom of each panel) resulting from microinjection of *Has2* adenovirus at the onset of limb formation (stage 18). Digits 2, 3, and 4 in the day 9 control limb in panel C are indicated. *Has2* infected limbs are shortened (92%) and several (42%) exhibit partial or complete absence of one or more skeletal elements. Skeletal elements that do form in the *Has2* infected limbs are often misshapen and exhibit abnormal morphology (65%), and/or are positioned inappropriately (15%). In some cases, a rudimentary skeletal element is fused to and growing out of the shaft of the humerus (arrows in C, E, F), and in some cases (27%) the autopod contains only a digit 3, and digits 4 and 2 are absent.

Fig. 3.

Alcian blue/Alizarin Red-stained skeletal elements of a day 13 contralateral control hindlimb (top) and *Has2* RCAS retrovirus infected hindlimb (bottom) resulting from microinjection of the *Has2* retroviral vector into the prospective leg forming region at stage 10. The femur in the *Has2* infected limb is shortened and misshapen, and the zeugopod consists of a single abnormal skeletal element that articulates with two extremely small digits that bear no resemblance to normal digits.

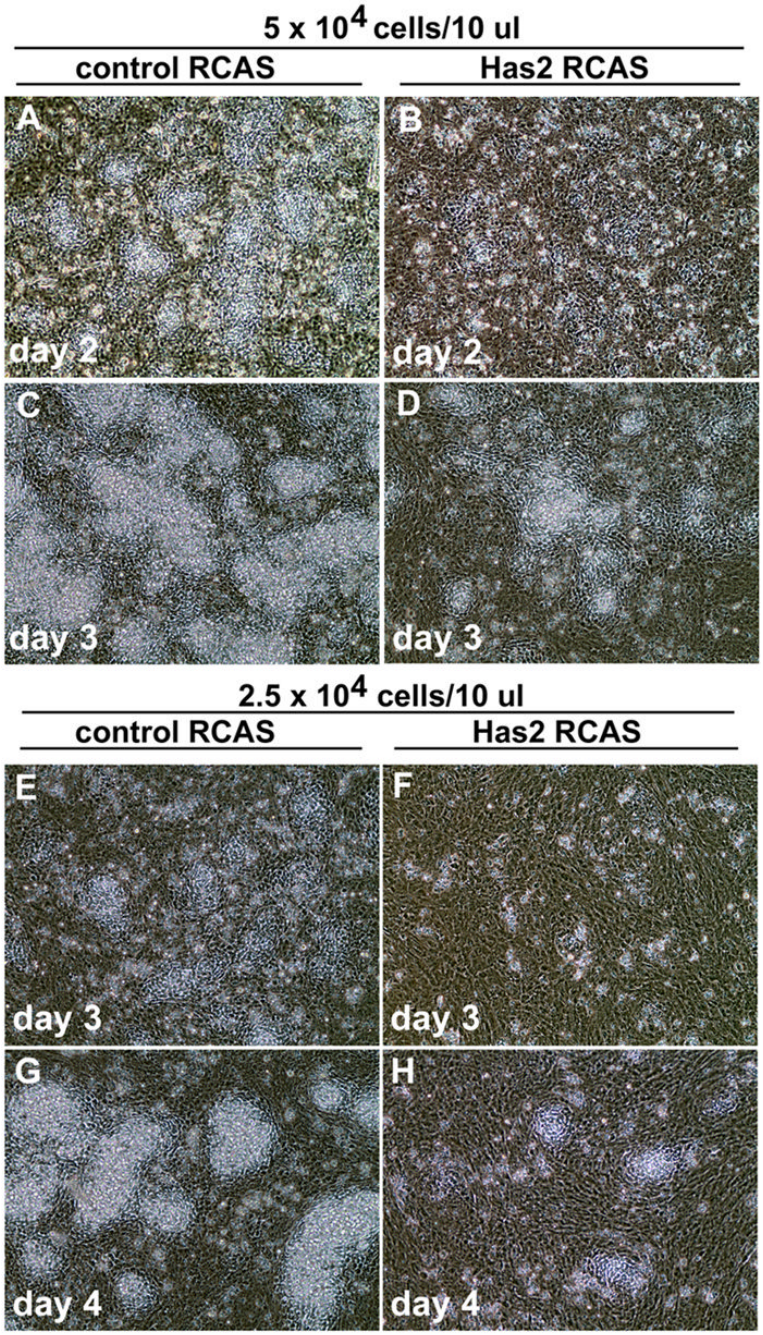

Fig. 4.

Overexpression of *Has2* impairs formation of precartilage condensations and chondrogenesis by limb mesenchymal cells in vitro. (A–H) Phase contrast microscopy images of micromass cultures of stage 22–24 limb bud mesenchymal cells established at 5×10^4 cells/10 μl (A–D) or 2.5×10^4 cells/10 μl (E–H) infected with a control RCAS retrovirus (A,C; E,G) or a *Has2* RCAS retrovirus (B,D; F,H). The RCAS control cultures form numerous precartilage condensations of closely packed rounded cells on day 2 (A) or day 3 (E), which subsequently enlarge and differentiate into cartilage nodules which deposit a refractile matrix (C,G). In contrast, only a few very small condensations form in cultures infected with the *Has2* RCAS

vector (B,F), and there a striking decease in the number of refractile cartilage nodules that differentiate (D,H).

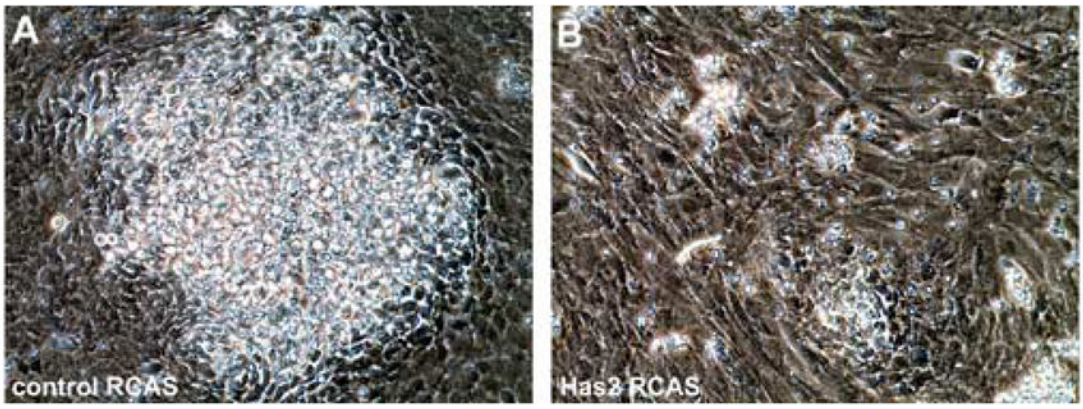

Fig. 5.

(A,B) Higher magnification phase contrast microscopy images of day 4 limb bud mesenchymal cell micromass cultures established at 2.5×10^4 cells/10 μl and infected for 3 h at the onset of culture with a control RCAS (A) or *Has2* RCAS (B) retroviral vector. Control cultures (A) form numerous precartilage condensations or nodules of closely packed rounded cells, whereas most of the cells of *Has2* infected cultures (B) remain large, flattened, and spatially separated from one another.

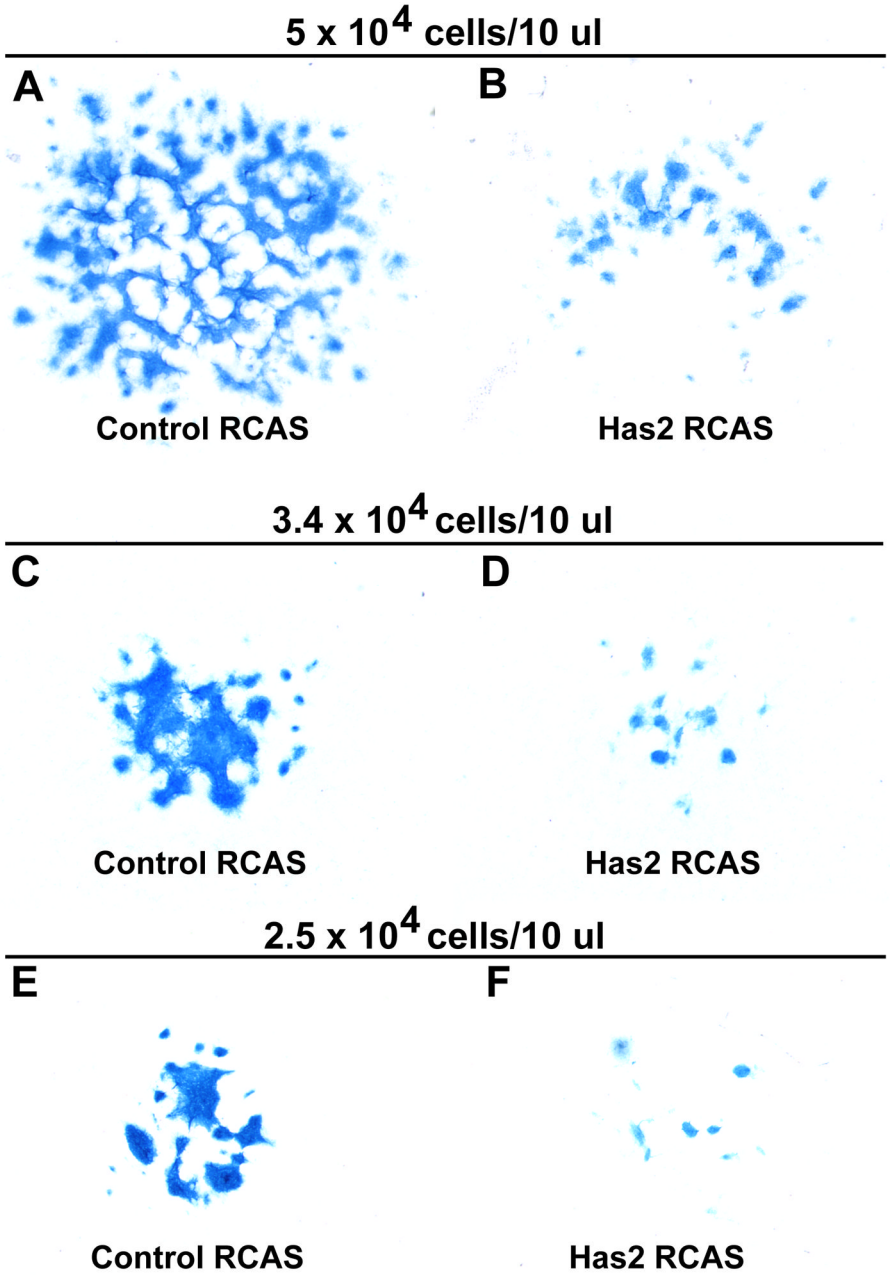

Fig. 6.

Retroviral overexpression of *Has2* inhibits the accumulation of Alcian blue staining cartilage matrix by micromass cultures of limb bud mesenchymal cells established at 5×10^4 cells/10 μl (A,B), 3.4×10^4 cells/10 μl (C,D), or 2.5×10^4 cells/10 μl (E,F). (A–E) Alcian blue-stained day 3 (A,B), day 4 (C,D), and day 6 (E,F) cultures infected with a control RCAS retrovirus (A,C,E) or with a *Has2* RCAS retrovirus (B,D,F). There is a striking decrease in the extent of Alcian blue staining cartilage matrix in the *Has2* infected cultures (B,D,F) compared to corresponding controls (A,C,E).

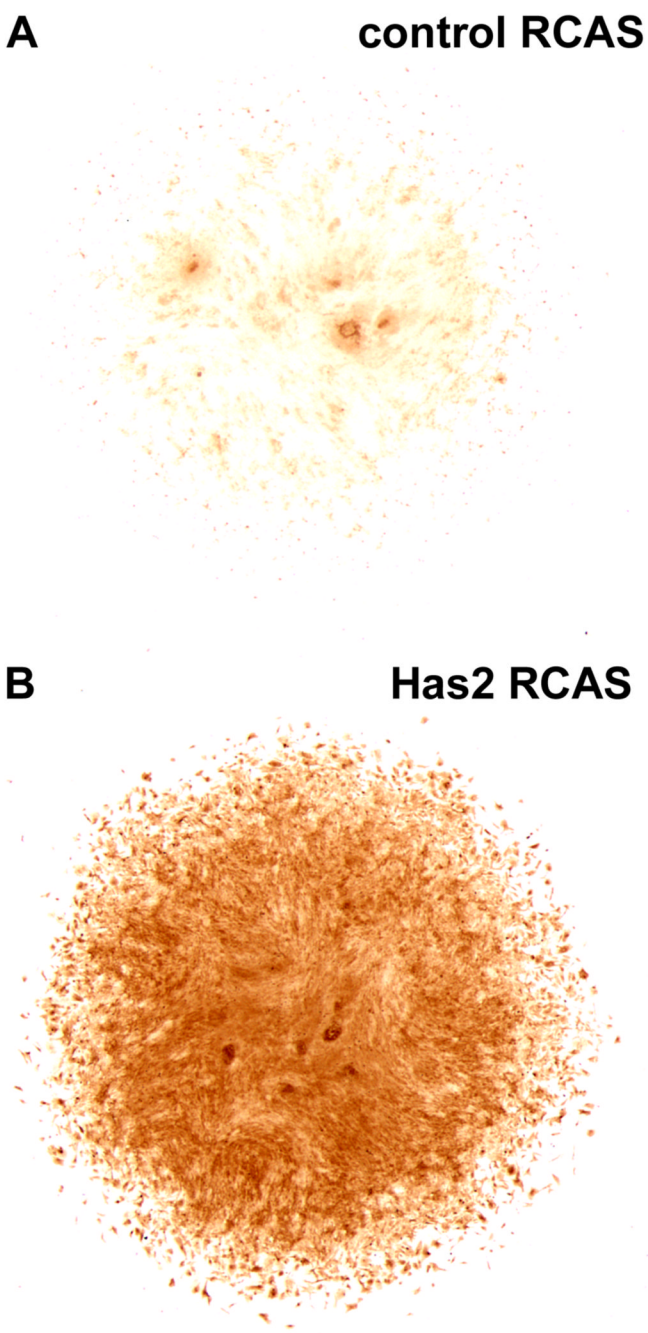

Fig. 7.

(A, B) Distribution of hyaluronan (HA) in RCAS control (A) and *Has2* RCAS (B) infected limb bud mesenchymal cell micromass cultures established at 2.5×10^4 cells/10 μl as assayed by histochemical staining with a biotinylated HA binding protein (HABP). Relatively little HABP staining is detectable in day 5 RCAS control cultures (A), whereas widespread HABP staining is present throughout the *Has2* infected cultures (B).

Table 1

Retroviral overexpression of *Has2* inhibits the accumulation of Alcian blue staining cartilage matrix by micromass cultures of limb mesenchymal cells established at various cell densities

Cell density (per 10 µl)	Treatment	Area of culture stained with Alcian blue	
		pixels^2 (× 10^3)	Treated/control
5 × 10^4	control RCAS	137 ± 11	-
	Has2 RCAS	54 ± 9	0.39
3.4 × 10^4	control RCAS	42 ± 7	-
	Has2 RCAS	14 ± 6	0.33
2.5 × 10^4	control RCAS	28 ± 5	-
	Has2 RCAS	8 ± 2	0.29

Values are the means of 8 (5 × 10^4 control RCAS), 5 (5 × 10^4 *Has2* RCAS), and 4 (3.4 × 10^4 control RCAS, 3.4 × 10^4 *Has2* RCAS; 2.5 × 10^4 *Has2* RCAS) determinations ± SEM, or 2 determinations ± range (2.5 × 10^4 control RCAS). Determinations are from day 3 (5 × 10^4) or day 6 (3.4 × 10^4 and 2.5 × 10^4) cultures.