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Expression of *Id2* in the developing limb is associated with zones of active BMP signaling and marks the regions of growth and differentiation of the developing digits

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ABSTRACT Here we report the pattern of expression of inhibitor of DNA binding/differentiation factor 2 (Id2) in the developing chicken limb. We show that prior to stage 25, *Id2* is expressed in the anterior and posterior mesoderm, the AER, and in the early skeletal chondrogenic aggregates. At more advanced stages of limb development *Id2* is expressed in the undifferentiated subectodermal and interdigital mesenchyme and exhibits specific domains of expression in the growing digits. These expression domains were closely coincident with zones of activation of BMP-signaling as deduced from the distribution of phosphorylated SMADs 1/5/8. In micromass cultures transcripts of *Id2* are associated with the nodules of chondrogenic differentiation. Expression of *Id2* both *in vivo* and *in vitro* was up-regulated in experiments of BMP-gain-of-function and down-regulated after treatments with BMP-antagonists. Interestingly, interdigital application of TGFβ2 transiently upregulates *Id2* in coincidence with the inhibition of interdigital cell death and the commitment of the interdigital mesenchyme to form an ectopic digit. These data suggest that Id2 is a molecular mediator of BMP signaling acting in concert with the TGFβ pathway during the formation of the digits.

KEY WORDS: *chondrogenesis, interdigital cell death, cell proliferation, p-Smad, tendon differentiation*

Introduction

The primordia of the vertebrate limbs develop in the lateral surface of the embryonic body by proliferation of the lateral plate mesoderm. These early growing buds are simple structures consisting of a core of mesodermal cells covered by an ectodermal jacket. In the course of development the bud undergoes intense outgrowth and the cartilaginous primordium of the skeletal components differentiates in the central region of the mesodermal core. At difference of these central cells destined to form cartilage, cells surrounding the chondrogenic aggregates are largely eliminated by apoptosis sculpturing the shape of the definitive limb. The opposite chondrogenic and apoptotic fates of the mesodermal cells are particularly relevant during the formation of the digits in the autopodial segment of the limb. In this region digits develop as diverging chondrogenic rays joined by intervening interdigital tissue, which will later be removed by massive apoptotic cell death in species with free digits (Zuzarte-Luis and Hurle, 2002).

Bone morphogenetic proteins (BMPs), Activins and transforming growth factor β2 (TGFβ2) are the main signaling molecules modulating digit morphogenesis. TGFβ2 and activins are expressed incipiently in the mesenchyme that will generate a digit in the developing autopod. Additionally, ectopic overexpression of any of them in the interdigital tissue inhibits its apoptotic fate and induces the formation of an additional extra-digit (Ganan *et al.*, 1996, Merino *et al.*, 1999a, Montero and Hurle, 2007). Bone morphogenetic proteins (BMP2, BMP4, BMP7 and BMP5) are expressed in the interdigital and peridigital tissue, rather than in the digit aggregates, but they are responsible for the outgrowth of the digit cartilages (Montero and Hurle, 2007). Accordingly, overexpression of BMPs in the digit mesenchyme generates a dramatic overgrowth of the digits while overexpression of BMP

Abbreviations used in this paper: AER, apical ectodermal ridge; BMP, bone morphogenetic protein; Id2, inhibitor of DNA binding/differentiation factor2; TGF, transforming growth factor.

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antagonists (i.e. noggin or gremlin) results in digit truncation (Merino *et al.*, 1998, Merino *et al.*, 1999b) A remarkable aspect of BMP signaling during limb development is that in addition to promotion of the growth of skeletal cartilages, they are also the triggering signals of programmed cell death in the undifferentiated mesoderm, including the interdigital mesenchyme (Ganan *et al.*, 1996, Macias *et al.*, 1997). Therefore unraveling the functional coordination between TGFβ/activin and BMPs in regulating growth and differentiation of the cartilaginous skeletal primordia and programmed cell death is a major question to understand digit morphogenesis. Hence, the identification of intermediate players of both signaling pathways can be particularly helpful for this purpose.

The Id (inhibitor of DNA binding/differentiation) proteins are a subfamily of the helix-loop-helix factors that contain an HLH dimerization motif but lack a basic DNA binding domain. The Id family contains four members (Id1, Id2, Id3 and Id4) and was initially discovered as inhibitors of cell differentiation functioning as antagonists of basic helix loop helix (bHLH) transcription factors (also called E-proteins). Thus, after heterodimerization with these type of transcription factors they impair its binding to the specific E-Box domains of the DNA due to the lack of DNA binding regions (Benezra *et al.*, 1990). It is now known that they can also inhibit the action of other transcription factors as Pax or Ets (Roberts *et al.*, 2001, Yates *et al.*, 1999). During development *Id1*, *Id2* and *Id3* possess overlapping patterns of expression showing only slight differences while *Id4* differs from the rest of the family members (Jen *et al.*, 1996).

The double knock out mice for Id1 and Id3 (but not the single ones) display dramatic inhibition in neurogenesis and brain associated angiogenesis, and seem to be factors capable to modulate vascularization of tumors (Lyden *et al.*, 1999). In these mutants neuroblast precociously abandon cell cycle, and show stronger and extended expression of determination and differentiation neurogenic bHLH factors correlating with premature terminal differentiation (Lyden *et al.*, 1999). Additionally, Id1 and Id3 are expressed in the blood vessels of the central nervous system and the mutant forms aberrant endothelial cells with absence of branching and sprouting of the capillaries of the neuroectoderm displaying haemorrhages in the ventricular system. Interestingly mice defective in these factors are much less permissive to tumor growth due to defective angiogenesis within the invasive tissue (Lyden *et al.*, 1999).

Several defects are also associated to the lack of *Id2* in mice. *Id2* is expressed in the dendritic cells (DC), which are potent antigen-presenting cells with a pivotal role in antigen-specific immune responses. Id2-/- mice lack Langerhans cells (LCs) (the cutaneous contingent of DCs) and the splenic number of DC is severely reduced. During hematopoietic development expression of *Id2* is under the influence of TGFβ signalling and modulates the differentiation toward the DC lineage, repressing the B-cell fate (Hacker *et al.*, 2003). Similarly it has been

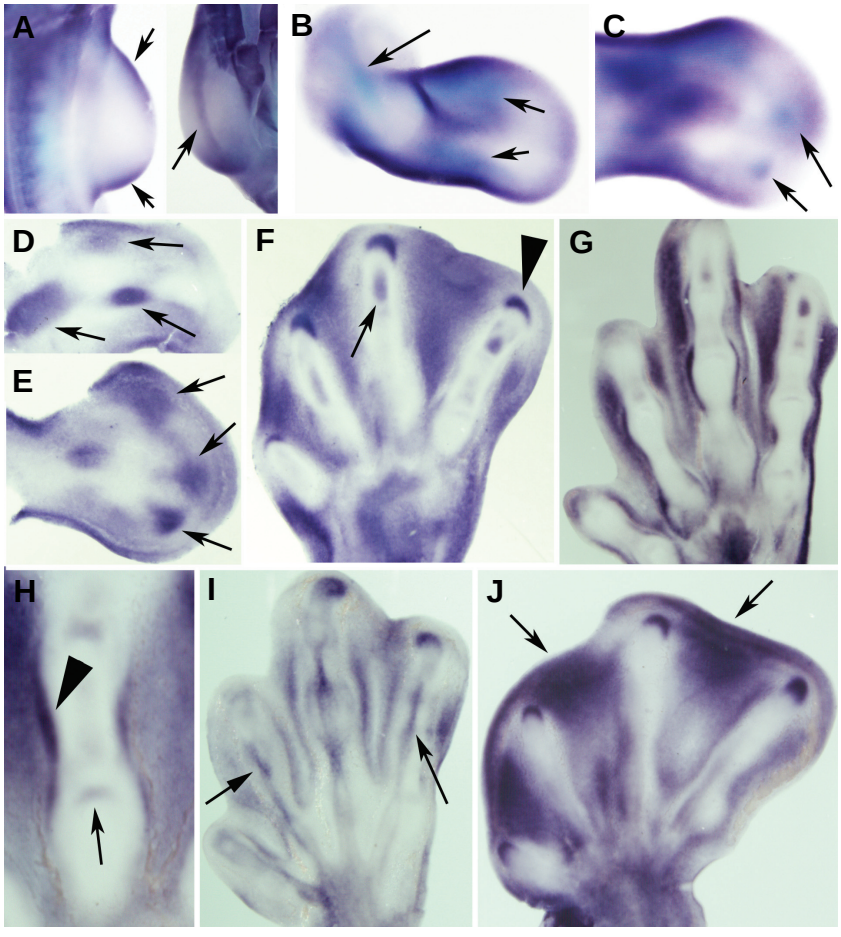

Fig. 1. *Id2* expression in the developing limb of chicken and duck embryos. (A-J) *In situ hybridization of chicken (A-I) and Duck (J) embryos in whole mount (A-C) and vibratome sections (D-J) specimens, showing the pattern of expression of* Id2. **(A)** *Image on the left of panel A is a lateral view of the limb bud at stage HH21 showing domains of* Id2 *in the anterior and posterior mesenchyme (arrows). Image on the right of panel A illustrates a frontal view showing a moderate labeling (arrow) of the apical ectodermal ridge (AER).* **(B)** *Wing bud of stage HH24 showing* Id2 *expression in the anterior and posterior mesenchyme with additional domains in the forming skeletal elements of the stylopod and zeugopod (arrows).* **(C)** *Expression of* Id2 *is recognizable at the level of the forming digits at stage HH25 (arrows).* **(D)** *Vibratome section of a HH24 limb where domains at the level of the skeletal elements are more easily recognizable (arrows).* **(E)** *Vibratome section of stage 26 limb showing the expression of* Id2 *in the prospective digit mesenchyme (arrows).* **(F)** Id2 *is expressed in the interdigital mesenchyme, the digit tip (arrowhead) and the developing cartilage of the distal phalanxes (arrow) at stage HH 30.* **(G-H)** *Image in (G) shows appearance of transcripts at the level of the developing perichondrium of the digits and the articular cartilage of HH 34 autopod. These domains are more easily recognizable in a detailed view of a digit in (H) (arrow and arrowhead).* **(I)** *This panel shows a superficial section of the HH34 autopod of the chicken embryo showing further domains of expression at the level of the developing tendons (arrows).* **(J)** Id2 *is preferentially expressed in the distal mesenchyme of the interdigits 2 and 3 (arrows) of the developing duck autopod at 9 days of incubation (equivalent to chicken stage HH31) in contrast to the widespread interdigital distribution in first interdigit and in the chick autopod (F).*

propose that Id2 favors natural killer (NK) differentiation versus T cell linage from a common NK/T progenitor, thus *Id2* null mice present a reduction of almost 90% of NK cells respect to wild type. Interestingly the number of precursors seems to be similar to wild type situation what indicates a clear constrain in cell fate decisions for these mice (Ikawa *et al.*, 2001). In sum, *Id2-null* mice have severe reduction of natural killer cells and lack several subclasses of dendritic cells.

Additionally, *Id2-null* mice show alterations in the reproductive system. Thus, female null mice show lactation defects associated with defective cell cycle progression and drastic reduced proliferation of the mammary epithelial cells, which is required during early pregnancy (Mori *et al.*, 2000). Male null mice present significantly reduced spermatogenesis with abnormal seminiferous tubules and dramatic reduction of sertoli cells consistent with the expression of *Id2* in these cells (Sablitzky *et al.*, 1998). It has been also shown that loss of *Id2* in the intestinal epithelium prevents exit from cell cycle and differentiation of enterocyte precursor cells during embryogenesis. The maintenance of this situation in the adult life causes neoplastic transformation in adults (Russell *et al.*, 2004). However, *Id2* has an important oncogenic potential since is a direct target of the Myc family of transciption factors and is overexpressed in tumors carriying mutations in its members (Lasorella *et al.*, 2002; Fernandez *et al.*, 2003). Furthermore, it has been shown that only Id2, and not the other members of the family, Id1 and Id3, is able to abrogate the antiproliferative effects of tumor suppressor proteins of the retinoblastoma family allowing cell-cycle progression by physically interactions with them, (Lasorella *et al.*, 2000).

Finally it is known that *Id4* has an essential role in the maturation of oligodendrocytes during development, thus progenitors lacking Id4 precociously maturate (Marin-Husstege *et al.*, 2006). In summary Id factors can work as important regulators of different biological processes and they are commonly associated to maintenance of undifferentiated state and the mitotic activity of cells, being often downregulated when cells differentiate (Lyden *et al.*, 1999, Shimizu-Nishikawa *et al.*, 1999; Ruzinova and Benezra, 2003, Sikder *et al.*, 2003; Iavarone and Lasorella, 2006).

SMAD-binding elements has been reported in the promoter of Id proteins (Hollnagel *et al.*, 1999). It has been also demonstrated that Id proteins contribute to coordinate proliferation and differentiation in cells subjected to BMP or TGFβ stimulation (Kowanetz *et al.*, 2004). In addition it is known that *Id2* is expressed in the early limb bud (Krishan *et al.*, 2005). Therefore we have decided to explore the expression and regulation of this transcription factor during limb skeletal morphogenesis.

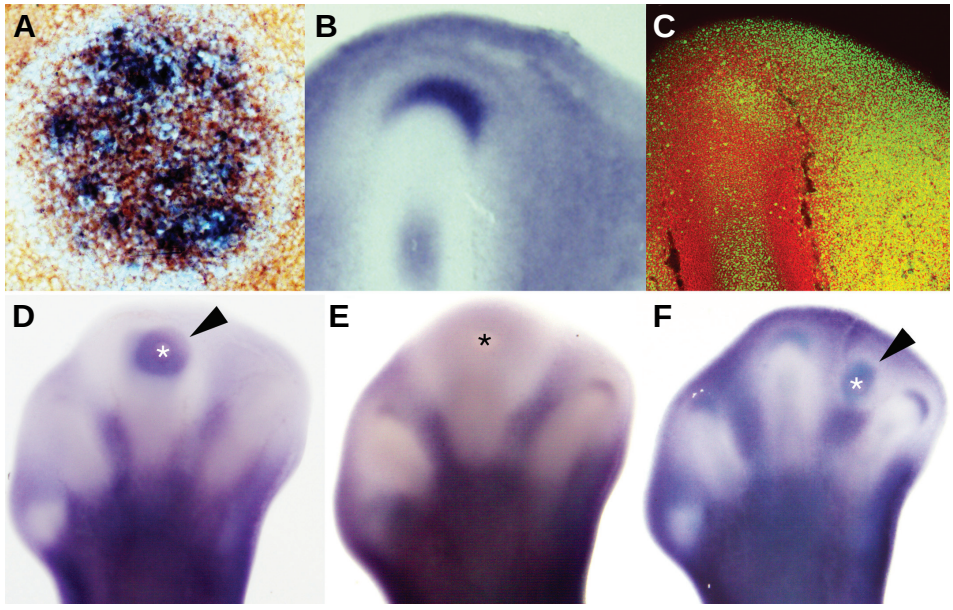

Fig. 2. *Id2* shares expression domains with BMP activity and is regulated by BMP and TGFβ signaling. (A) *Co-expression of* Id2 *(red) and* Smad8 *(blue) shown in a transversal section at the level of the distal phalanx of digit 3 of HH28 embryo.* **(B)** *Detailed view of digit III of a limb autopod from an embryo at stage HH28 showing the characteristic crescent domain of expression of* Id2 *present at the digit tip.* **(C)** *Equivalent view to that in (B) from a limb immunolabeled for phosphorylated SMAD1,5,8 (green) in order to detect regions of BMP activity. Counterstain in red corresponds to actin.* **(D)** *BMP7 treatments at the tip of the digit (asterisk in D) cause dramatic induction of* Id2 *expression (arrowhead in D).* **(E)** *Downregulation of Id2 expression 10 hr after application of a Noggin bead (asterisk) at the tip of the digit.* **(F)** *Application of a TGFβ1 bead (asterisk) in the interdigital tissue causes a transitory upregulation of* Id2 *expression preceding the formation of an ectopic digit (arrowhead).*

Results

Id2 *marks the limb regions regulated by BMP signalling*

We have examined the expression of *Id2* in the limb bud by *in situ* hybridization using whole mount specimens and vibratome sections. At stages 21-22 transcripts are observed in the anterior and posterior mesenchyme and in the AER (Fig. 1A). By stage 23-24 the anterior and posterior domains of expression increase in size and labeling intensity while transcripts are no longer observed in the AER. In addition, new domains of expression appear associated with the chondrogenic condensations of the stylopod and zeugopod (Fig. 1B and D). By stages 25, *Id2* become expressed in the mesenchymal condensations of digits (Fig. 1C and E). From stage 27 the undifferentiated subectodermal mesenchyme including the interdigital mesoderm show considerable levels of gene expression and the initial digit domains appear now divided into different regions, rather than maintaining an uniform labeling (Fig. 1F and Fig. 3D-F). As shown in Fig. 1F and 1I a crescent domain of expression is associated with the tip of all the growing digits until the stage in which the last phalanx is formed. A zone lacking expression distally surrounds this domain. More proximally in the digit ray, the peripheral perichondrial region is negative for this gene while intense labeling is found at the central region of the future diaphysis of the developing phalanxes (Fig. 1F). A more tenuous labeling is also observed in the developing

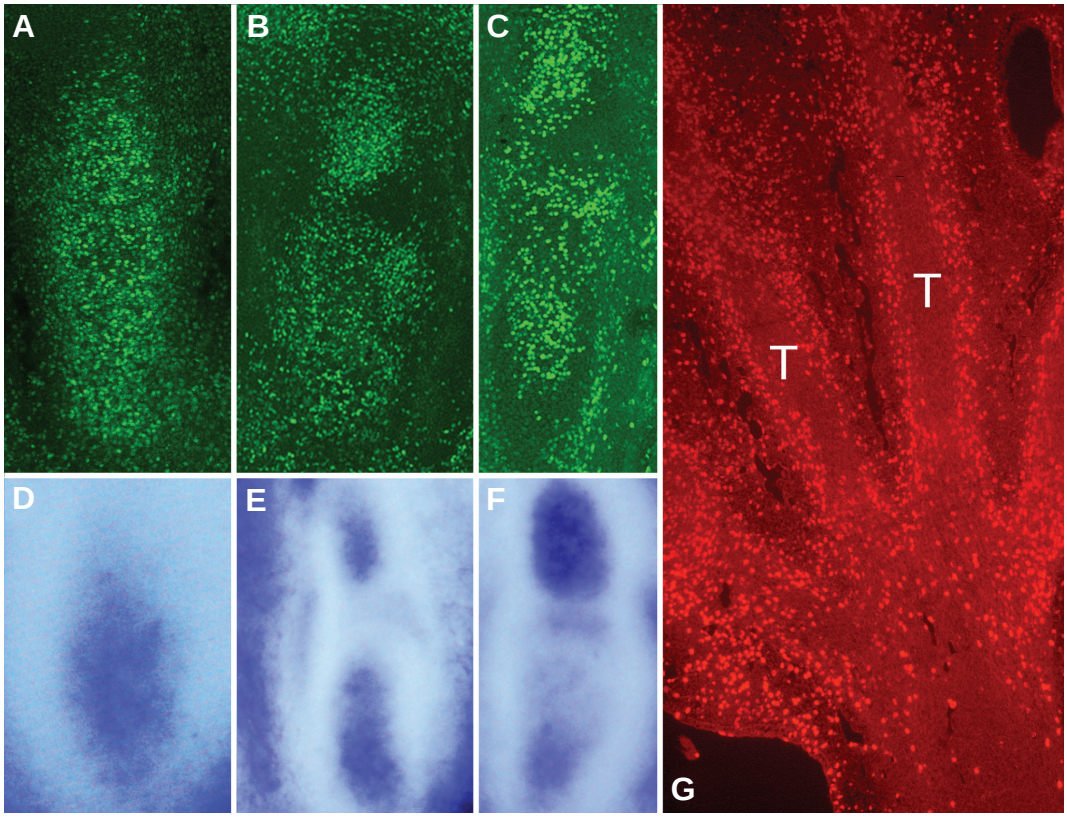

Fig. 3. The pattern of cell proliferation in the developing digits and tendons correlates with the expression of *Id2* within the maturating cartilage. (A-F) *Different stages of digit development showing immunolabeling against BrdU to compare the pattern of cell proliferation* **(A-C)** *with the expression of* Id2 **(D-F)** *in digits of equivalent stages. Note the close correlation between BrdU incorporation and the expression of* Id2 *in digits at initial* **(A,D)** *intermediate* **(B,E)** *and advanced stages of segmentation into phalanxes* **(C,F)**. **(G)** *Superficial longitudinal section through the developing autopod showing the incorporation of BrdU in the contour of the maturing dorsal tendon blastemas (T).*

epiphyses. In the neighboring regions of the developing joints, cartilage labeling is very tenuous although it can be recognized at the boundaries of the articular surfaces (Fig. 1H). From HH34 the expression in the differentiating cartilages becomes reduced, maintaining recognizable domains in the prospective articular cartilage of the developing joints. In addition at these stages tendinous and perichondrial domains are visible (Fig. 1G-H).

To check whether the interdigital domains of *Id2* save correlation with apoptotic cell death we have studied its expression in the developing autopod of the duck embryo, where apoptotic domains are restricted to the distal-most mesenchyme of the webbed interdigits (Zuzarte-Luis and Hurle, 2002). As shown in Figure 1J *Id2* expression in duck is highly similar to that of the chick with the exception of the interdigital domains 2 and 3, where transcripts are considerably more abundant at the level of the distal tissue.

Id2 *is a BMP response gene during digit development*

With some exceptions in the growing digits, most domains of expression of *Id2* (see Fig. 1) correspond with regions in which members of the BMP family are expressed, suggesting that *Id2* might be a BMP-response gene. However the perichondrial surfaces of the digits and the developing joints are initially negative for *Id2* in spite of the expression of BMP2, BMP7 and GDF5 in the perichondrium and developing joints. Furthermore previous reports have shown that the tip of the developing digits lack transcripts of BMP genes (see Montero and Hurle, 2007). In addition, it must be taken into account that domains of BMP expression are not necessarily associated with BMP signaling since they are secreted cytokines and several BMP antagonists, including Gremlin, Noggin, Ventroptin and Bambi are expressed in the developing autopod and may inhibit BMP signaling (Chimal-Monroy *et al.*, 2003, Grotewold *et al.*, 2001, Merino *et al.*, 1998, Merino *et al.*, 1999b). Hence to clarify whether *Id2* expression correlates with regions of active BMP signaling we examined by double *in situ* hybridization the possible coexpression with *Smad8*, a BMP intracellular mediator, of which expression resembles that of *Id2* (see Zuzarte *et al.*, 2004). Indeed we found that both genes were coexpressed in all domains of expression, including the digital domains as shown in Fig. 2A. Furthermore, by immunohistochemistry we analyzed the distribution of phosphorylated SMADs 1/5/8, transcription factors activated in regions of BMP activity (Massague *et al.*, 2005), and we found that *Id2* expression finely reproduced the pattern of distribution of p-SMAD1,5,8 in the developing limb autopod, including the digit domains (Fig. 2C, compare to Fig. 2B). These strong correlations suggest that *Id2* expression is associated with BMP signaling. In support of this interpretation, BMP signaling regulates the expression of Id proteins in different cell types (Lopez-Rovira *et al.*, 2002, Nakashima *et al.*, 2001).

To further investigate the control of *Id2* expression in digit morphogenesis, we have performed gain and loss of function experiments for BMP signaling. As mentioned BMPs are major modulators of digit chondrogenesis and its expression in the interdigital tissue is promoting interdigital apoptosis. Ectopic application of BMP proteins in the tip of the digit potentiates chondrogenesis inducing a dramatically enlarged phalanx (Macias *et al.*, 1997, Zuzarte-Luis *et al.*, 2004). We have found that this phenotype is preceded by upregulation of *Id2* in a fashion that prefigures the ectopic outgrowth of such cartilage (Fig. 2D). By contrary, identical treatments with Noggin, a BMP

antagonist, causes digit growth truncation (Merino *et al.*, 1998). Accordingly, Noggin beads applied at the distal tip of the digit completely abolish the expression of *Id2* (Fig. 2E).

TGFβs upregulate Id2 *in the initial digit aggregates*

A significant feature of this study is the precocious expression of *Id2* in the autopod showing this factor as one of the earliest marker of the digital territory. However, ectopic overexpression of BMPs can only promote the growth of preexisting cartilage in the developing autopod but not the formation of an ectopic one (Macias *et al.*, 1997). In this regard interdigital application of TGFβs/Activins, induces the formation of an ectopic digit (Chimal-Monroy *et al.*, 2003, Ganan *et al.*, 1996). Thus, to evaluate the possible implication of Id2 in digit formation, we have explored its regulation when a bead bearing TGFβ1 was implanted in the interdigital mesenchyme. We have detected a transient induction of *Id2* expression 3 hours after the interdigital implantation of a TGFβ-bead (Fig. 2F) (identical results were found after application of an Activin soaked bead; not shown). This ectopic expression is transient and after 12 hours is substituted by the characteristic domains present at the tip of developing digits. These finding suggest a role for Id2 under the control of TGFβs/activins in the initial steps of digit formation.

Chondrocyte proliferation in the digit primordia and expression of Id2

An interesting aspect of Id2 in limb development is its appearance in chondrocytes of the developing cartilage rudiments. To check whether the digital *Id2* expression may reflect zones of proliferation within the developing cartilages we compared the pattern of BdrU incorporation into the digit blastemas (Fig. 3A-C) with the digit domains of I*d2* (Fig. D-F). As shown in Fig. 3A BrdU digit labeling is uniform at stages 26-27. In subsequent stages labeling of BdrU incorporation becomes progressively divided into domains located in the central core of the phalanxes with higher proliferation in the prospective diaphysis and lower epiphyseal labeling around the zones of joint differentiation (Fig. 3B-C). In sum this pattern resembles the expression of *Id2* indicating a possible role for this factor in cartilage proliferation. In concordance with this finding incorporation of BrdU in the developing tendons was also correlated with the late expression of this gene in the maturing tendons (Fig. 3G).

Expression of *Id2* during *in vitro* chondrogenesis

To further evaluate the involvement of Id2 in chondrogenesis and its regulation by BMPs, we have studied its expression in the *in vitro* model system of micromass cultures. As shown in figure 4 (A) transcripts of *Id2* were abundant in the incipient chondrogenic nodes at day 2 of culture. By day 4 of culture expression was maintained in the mature cartilage nodes (Fig. 4B). Furthermore, in consistency with our *in vivo* observations, expression of *Id2* was upregulated, thought at moderate levels, when BMP7 was added to the culture medium (Fig. 4C).

Discussion

Here we report the pattern of expression and regulation of the *inhibitor of DNA binding/differentiation 2* (Id2), an helix-loop-helix factor that sequesters bHLH transcription factors by forming heterodimers that lack the ability to bind DNA. We show that expression of *Id2* in the developing limb corresponds closely with the zones of active BMP signaling. In the early limb bud, *Id2* exhibits anterior and posterior mesodermal domains corresponding quite closely with the domains of expression of BMP4, BMP7 and BMP2 (Montero *et al.*, 2001). In addition *Id2* is also expressed at low levels in the AER. This structure is a key-signaling center of the limb bud regulated by a complex signaling network, which includes BMPs (Pizette *et al.*, 2001). The expression of *Id2* in the AER and the anterior and posterior mesoderm might be related with the demonstrated function of this transcription factor in maintaining cells in an undifferentiated state. Although we cannot provide a reasonable explanation for the transitory expression of *Id2* in the AER, since BMPs are active in this structure until the final stages of limb morphogenesis. Expression of *Id2* in the undifferentiated subectodermal mesoderm at advanced stages of development is also consistent with the function of Id2 in maintaining cells undifferentiated. *Id2* has been also found in this study in the areas of interdigital cell death outlining the prospective apoptotic territories both in chick and in duck embryos. It is well known that the interdigital tissue requires to be maintained in an undifferentiated state in order to be sensible to cell death-triggering signals. This aspect is manifested by the expression in this tissue of markers of undifferentiation including *Msx*2 or *Snail* genes (Ganan *et al.*, 1998, Montero *et al.*, 2001). Taking into account that Id2 appears to be a regulator of undifferentiation (Lyden *et al.*, 1999, Shimizu-Nishikawa *et al.*, 1999) we hypothesized that *Id2* expression in the prospective apoptotic tissue might favor the maintenance of the undifferentiated state of the interdigital mesenchyme. However Id2 has been described as an proapototic factor in neurons (Gleichmann *et al.*, 2002). In addition Zhao *et al.* (2007) have provided some evidences for a possible positive implication of Id2 in apoptotic interdigital cell death in the quail embryo.

A remarkable finding of this study is that *Id2* exhibits characteristic expression domains in differentiating chondrocytes during the formation of the limb skeleton. The chondrogenic aggregates in early limb buds and chondrogenic nodules in micromass cultures are positive for this gene. *Id2* is also a precocious marker

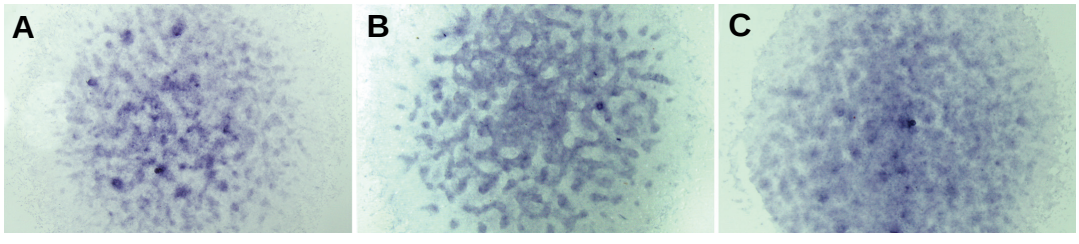

Fig. 4. *Id2* expression in chondrogenic micromass cultures. (A-B) Id2 in situ *hybridization in micromass cultures after 2* **(A)** *and 4* **(B)** *days of incubation. Note initial labeling in a spotted pattern (A), which is expanded as cartilage differentiates (B).* **(C)** Id2 *expression is moderately upregulated respect to B upon 2 days of treatment with 50 ng/ml BMP7 protein.*

of the digit forming territory and is highly expressed in the tip of the growing digits. In addition, as morphogenesis progresses, *Id2* displays well-defined domains in the chondrocytes of the developing phalanxes. Together these findings are consistent with a function of Id2 in chondrogenesis. However, no chondrogenic phenotype was reported in mice deficient for *Id2* (Lasorella *et al.*, 2000, Yokota *et al.*, 1999). Taking into account that Id1 and ID3 are expressed in similar patterns in the developing limb (Jen *et al.*, 1996) it is likely that lack of skeletal phenotypes in the single mutants for these genes are due to functional redundancy between members of this family. In this regard it has been shown *in vitro* that BMP induces several Id genes when promoting chondroblast differentiation (Liu *et al.,* 2007).

The dynamic domains of *Id2* expression, accompanying digit formation might bear different functional significance. The pattern of expression of *Id2* in the diaphysis and epiphysis of the developing phalanxes is rather coincident with zones of higher BrdU incorporation in the digit blastemas prior to hypertrophic differentiation. This pattern of expression is consistent with the role of Id proteins promoting cell proliferation in other systems (Barone *et al.*, 1994, Hara *et al.*, 1994, Lyden *et al.*, 1999, Mori *et al.*, 2000, Rothschild *et al.*, 2006), but not with the inhibition of cell differentiation proposed for this transcription factor. It must be taken into account that, at the stages covered by this study, proliferating chondrocytes of the diaphysis are in course of transformation into prehypertrophic chondrocytes. These findings indicate that the role of Id2 in the developing cartilages is associated with growth rather than maintaining cells undifferentiated. In this regard, it is also significant that *Id2* transcripts are absent from the developing joints in spite of being formed by a local dedifferentiation of the digit cartilage (Craig *et al.*, 1987).

The early expression of *Id2* in the prospective digit territories as well as the maintained expression in the mesenchyme of the tip of the growing digits may reflect a specific function in cartilage/tendon differentiation. As the incipient digit mesenchyme of the early bud, the tip of growing digits is a complex region undergoing cell aggregation (Montero *et al.*, 2007). However upon cell aggregation, the distal mesoderm of the autopod is also able to develop into the precursors of the digit tendons that are formed in close relation with cartilaginous aggregates (Hurle *et al.*, 1990, Schweitzer *et al.*, 2001). At molecular level TGFβs/Activins and BMP signaling are confluent at these places. Tgfβs are highly expressed in the tendinous condensation, however interdigital application of TGFβs induces a cartilaginous aggregate, which is preceded by a transient domain of *Id2* expression. In turn, local application of BMPs or Noggin stimulates or inhibits respectively both chondrogenesis and the digit domain of *Id2*. It is then likely that the regulation of *Id2* by these signals is related with the specification of the chondrogenic versus the tendinous fates of the undiferentiated cells. Indeed, *Id2* has been involved in the control of cell fate decision between two different cell types in other model systems as the terminal differentiation of different lineages of haematopoietic cells from a common progenitor (Ikawa *et al.*, 2001). Tendons develop in close association with cartilages, and *scleraxis*, an helix-loop-helix transcription factor promotes the specification of mesenchyme towards the tendon lineage (Asou *et al.*, 2002, Brent *et al.*, 2003). At least in the axial skeleton tendon and cartilaginous fates are alternative and cartilage differentiation is required to actively repress tendon development (Brent *et al.*, 2005). Therefore it is conceivable that stimulation of *Id2* at the tip of the digits promotes chondrogenesis and avoids tendon differentiation by inhibiton of the basic helix-loop-helix transcription factor *scleraxis*. In this regard, the transient induction of *Id2* following interdigital application of TGFβs may repress the effect of TGFβs in the promotion of tendinous fate (Liu *et al.*, 1996, Salingcarnboriboon *et al.*, 2003). Furthermore, in consistency with their dramatic effects on the expression of *Id2*, BMP signaling inhibits *scleraxis* while noggin expands its expression (Schweitzer *et al.*, 2001).

The expression of Id2, in the maturing tendons is not contradictory with our interpretation. Maturing tendons express both *Id2* and *BMPs* suggesting that at these stages Id2 might be promoting the growth of the already differentiated tendon blastemas in a similar fashion to the findings observed here for the developing phalanxes. Maturing tendons express both *Id2* and *BMPs* and expression of *Id2* correlates with the pattern of BrdU incorporation in the differentiating tendon blastemas in a similar fashion to the findings observed here for the developing phalanxes.

Materials and Methods

Animal models

In this work we have employed Rhode Island chick embryos ranging from stages HH20 to HH32 (Hamburger and Hamilton, 1951) and Royal Pekin duck embryos at days 8 and 9 of incubation.

Processing of the limbs

The fixation of the limbs was performed by overnight immersion in 4% paraformaldehyde solution, in 0.12 M phosphate buffer, pH 7.4, at 4°C. Fixed limbs and were rinsed twice in phosphate-buffered saline/Tween 0.1%. At this point, 100μm thick sections were obtained in a vibrotome. Alternatively tissue was cryoprotected in 10% sucrose solution (in phosphate buffer), embedded in 7.5% gelatine (Sigma type A) 10% sucrose solution and sectioned at 12μm in a cryostat.

Probes and in situ *hybridization*

The chicken coding cDNA fragment of the *Id2* sequence was obtained by RT-PCR from RNA extracts of HH25 autopods. The following primers were designed:

5´ primer, 5´- CCTGCTGTACAACATGAACG-3´

3´ primer, 5´- CACTCGCCATTAGTTCTGAGG-3´.

PCR product was cloned into pGEM-T (Promega) and the authenticity of the fragment was confirmed by dideoxy sequencing. The PCR conditions were 94°C, 4 min and then 35 cycles of 94°C, 20 s; 60°C, 30 s; 72°C, 60 s; and final extension at 72°C, 10min. PCR products were subcloned into pGEM T-easy (Promega). *Id2* digoxygenin-labeled sense and antisense RNA probes were generated for *in situ* hybridization analysis. For whole mount or 100μm vibratome sections *in situ* hybridization, samples were treated with 10 mg/ml of proteinase K for 20-30 minutes at 20°C. Hybridization with digoxigenin-labeled antisense RNA probes was performed at 68°C. Reactions were developed with BCIP/NBT substrate or with purple AP substrate (Roche). For double labeling, fluorescein- and digoxigenin-labeled probes were hybridised together and detected one after another. Alkaline phosphatase reaction from the first antibody was avoided after labeling by heating at 65°C and treatment with 0.1M glycine-HCl ph2,2 treatments. The fluorescein probe was revealed first using an alkaline phosphatase conjugated anti-fluorescein antiserum (Roche), diluted 1:2000, and Fast Red as the chromogene.

Experimental manipulation of the limb

The regulation of the *Id2* gene by BMPs and TGFβ was studied by analyzing the effects of local administration of recombinant proteins

including 0.5μg/μl BMP7 (a gift of Creative Biomolecules, Hopkinton, MA), 0.5μg/μl Noggin (generously donated by Regeneron Pharmaceuticals Inc., Tarrytown) or 10μg/ml TGFβ1 (R&D) into the limb mesoderm, using heparin acrylic beads as carriers (Sigma). After the period of incubation, samples were processed for *in situ* hybridization. In all the experiments, PBS soaked beads were also implanted as a control.

Immunostaining and confocal microscopy

For pSMAD1,5,8 (Cell Signaling) immunostaining limbs were dissected and fixed in 4% PFA and frontal 100μm vibratome sections were taken and bleached in Den´t bleach (50% Methanol, 25% H_20, 15% H_2O_2, 10%DMSO) before incubation O/N in primary antibody (dilution 1:100) at 4°C, washed in TBS and incubated O/N in secondary antibody. Samples were examined with a laser confocal microscope (LEICA LSM 510) by using a Plan-Neofluar 10X, 20X or Plan-Apochromat 63X objectives, and argon ion laser (488 nm) to excite FITC fluorescence and an HeNe laser (543 nm) to excite Texas Red.

Cell proliferation

Cell proliferation was analyzed by anti-bromodeoxyuridine immunolabeling. For this purpose 100 ml of bromodeoxyuridine (BrdU) solution (100 mg/ml) was injected on the amniotic sac. After 30 minutes of further incubation, the embryos were fixed in 4% paraformaldehyde. The autopod was then dissected free and sectioned in the vibrotome. Immunostaining to detect BrdU incorporation was carried out in tissue sections according to the instructions of the manufacturer (Becton Dickinson) using anti-BrdU and FITC-conjugated secondary antibody.

Micromass cultures

Micromass cultures, BMP7 treatments and *in situ* hybridization were performed as previously described (Chimal-Monroy *et al.*, 2002).

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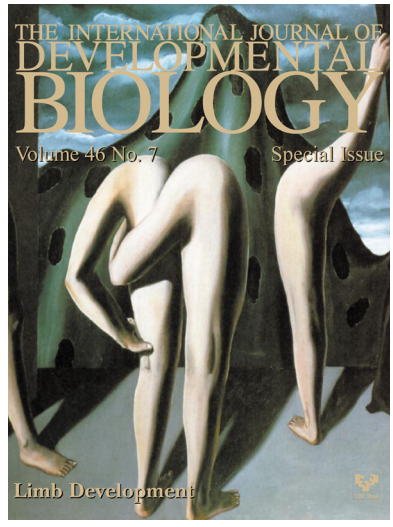

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