ELSEVIER

Mechanisms of Development 69 (1997) 31–37

M&D

Goosecoid misexpression alters the morphology and *Hox* gene expression of the developing chick limb bud

Tiffany A. Heanue[a], Randy L. Johnson[a,1], Juan-Carlos Izpisua-Belmonte[b,2], Claudio D. Stern[c], Eddy M. De Robertis[b], Clifford J. Tabin[a,*]

[a]*Department of Genetics, Harvard Medical School, Boston, MA 02115, USA*
[b]*Molecular Biology Institute, UCLA, Los Angeles, CA 90024, USA*
[c]*Department of Genetics and Development, Columbia University, New York, NY 10032, USA*

Received 12 May 1997; revised version received 15 August 1997; accepted 16 August 1997

Abstract

The homeobox-containing gene *goosecoid* (*gsc*) has been implicated in a variety of embryonic processes from gastrulation to rib patterning. We have analyzed the role it plays during chick limb development. Expression is initially observed at stage 20 in a proximal-anterior-ventral domain of the early limb bud which expands during subsequent stages. Later in limb development a second domain of expression appears distally which resolves to regions which surround the condensing cartilage. In order to understand the function of *gsc* in limb development, we have examined the effect of misexpressing *gsc* throughout the limb. Two striking phenotypes are observed. The first, evident at stage 24, is an alteration in the angle of femur outgrowth from the main body axis. The second, which can be detected at day 10 of development, is an overall decrease in the size of the limb with bones that are small, misshapen and bent. These phenotypes correlate with a decrease in levels of *Hox* gene expression in *gsc*-infected limb buds. From these results we suggest that *gsc* may normally function to regulate growth and patterning of the limb, perhaps through regulation of *Hox* gene expression. © 1997 Elsevier Science Ireland Ltd.

Keywords: goosecoid; Limb; *Hox*; Chick

1. Introduction

Goosecoid (*gsc*) is a homeobox-containing transcription factor that was first cloned from a *Xenopus* dorsal lip cDNA library in attempts to identify candidate molecules that regulate patterning during gastrulation or neural induction (Blumberg et al., 1991). The first clue to the importance of the dorsal blastopore lip in control of this process came from the experiments of Spemann et al. who discovered that when this tissue is transplanted to the ventral side of an equivalently staged embryo, a secondary embryonic axis is formed which is derived from the host (Spemann and Mangold, 1924). The tissue with this inductive ability is known as the Spemann's organizer. *gsc* is expressed in the dorsal blastopore lip and may be involved in some of the many functions of the organizer (Cho et al., 1991; Psychoyos and Stern, 1996). *gsc* is also expressed in the ventrolateral flank and limb regions of later embryos (Gaunt et al., 1993), indicating that it may also have important post-gastrulation activities.

Goosecoid homologues have been cloned in several vertebrate species, including mouse, chick and zebrafish (Blum et al., 1992; Izpisua-Belmonte et al., 1993; Stachel et al., 1993; Schulte-Merker et al., 1994). The conservation of *gsc* structure and expression pattern suggests that it may function equivalently in these organisms. For example, the chick homologue of *gsc* is expressed in Hensen's node (the functional equivalent of the *Xenopus* dorsal blastopore lip) as well as in the head and the limb regions (Izpisua-Belmonte et al., 1993).

The embryological functions of *gsc* have been explored by both loss of function and gain of function experiments. Mice homozygous for a targeted mutation in *gsc* primarily

* Corresponding author.
[1] Present address: Department of Biochemistry and Molecular Biology, M.D. Anderson Cancer Center, Houston, TX 77030, USA.
[2] Present address: Gene Expression Lab, Salk Institute, La Jolla, CA 92037, USA.

0925-4773/97/$17.00 © 1997 Elsevier Science Ireland Ltd. All rights reserved
PII S0925-4773(97)00149-4

exhibit craniofacial defects in the regions of the lower jaw and ear and also display fusion of the ribs (Rivera-Perez et al., 1995; Yamada et al., 1995). These defects are less severe than might have been expected for a gene required during gastrulation. The lack of a gastrulation phenotype may be attributable to functional redundancy with other closely related genes.

Misexpression experiments have provided additional information on the role of *gsc* during development. Most of these experiments have focused on its function during early development in *Xenopus*. *gsc* RNA injected into *Xenopus* embryos can induce a secondary axis, suggesting that *gsc* plays a role in organizing axial cell fates (Cho et al., 1991). Cell tracing analysis has indicated that *gsc* accomplishes this, at least in part, by regulating cell migration during gastrulation (Niehrs et al., 1993).

To determine whether the expression of *gsc* in the limb bud is related to some function of this gene which is not obvious from the targeted mutations in mice, we chose a gain of function approach, studying the consequences of misexpressing this gene in the developing limb. We have taken advantage of the chick embryo, in which the limb region can be specifically manipulated using retroviral vectors without disrupting the rest of the embryo's developmental program. Our results demonstrate that ectopic expression of *gsc* during limb development has significant consequences on the overall pattern of the limb, suggesting that *gsc* does indeed play a role in regulating specific aspects of limb morphogenesis. We find that ubiquitous expression of *gsc* in the limb causes two dramatic phenotypes. The first is an alteration in the angle of femur outgrowth from the main body axis, with *gsc*-infected femurs assuming an acute angle relative to the main body axis and not possessing the characteristic bend of the limb at the juncture of the femur and tibia/fibula. The second, later phenotype observed is the overall decrease in the size of *gsc*-infected limbs, with cartilage elements being small, misshapen and bent. Further studies have revealed that *Hox* gene expression is downregulated in *gsc*-infected limbs. Thus, *gsc* appears to play a role in the growth and patterning of the limb, possibly by regulating *Hox* gene expression.

2. Results

2.1. gsc expression in normal chick limbs

Many aspects of chick limb development and the signaling centers which control it have been well described. To get an indication of which steps of limb development may be regulated by *gsc*, we carefully examined its expression in the limb at various stages by in situ hybridization in whole-mount and in section. Low levels of *gsc* expression within the limb are first observed in stage 20 fore- and hind-limb buds in a proximal-anterior-ventral (p-a-v) region (Fig. 1A), consistent with previous descriptions in the mouse limb bud (Gaunt et al., 1993). By stage 22, expression has intensified and remains localized to the p-a-v quadrant (Fig. 1B). At this stage the limb bud is morphologically uniform, consisting solely of undifferentiated mesenchyme. However, patterning signals are already present in the limb bud and the cells destined to give rise to the most proximal elements have already been specified. Fate mapping experiments have demonstrated that the p-a-v domain which expresses *gsc* is destined to give rise to shoulder/pelvic elements and the proximal region of the humerus/femur (Lewis, 1977). By stage 23 the cartilaginous condensations for these proximal skeletal elements have started to form. At this stage the *gsc* expression domain has expanded, but maintains a p-a-v bias (Fig. 1C). By stage 25 a second domain of expression appears distally in the region of condensing cartilage (Fig. 1D). By stage 27–28, the early expression domain begins to fade and the distal domain of expression resolves to regions surrounding the condensing cartilage of the outgrowing limb bud (Fig. 1E,F and data not shown). In particular the expression is most intense at the tips of the tibia/fibula and radius/ulna in the regions fated to give rise to the elbow/knee and wrist/ankle joints. Analysis of limbs in section of older embryos reveals that this later *gsc* expression domain is most likely staining the forming tendons and in particular the attachment points of the tendons at the joint regions (Fig. 1G). These expression results suggest that *gsc* might play a role in regulating morphogenesis of the shoulder/pelvic region and later in the formation of tendons which connect at the elbow/knee and wrist/ankle joints. Its expression does not, however, correlate with any of the classically defined signaling centers of the early limb bud.

2.2. gsc misexpression results in alterations in limb outgrowth

To examine the function of *gsc* in the developing limb we inserted the full coding sequence into a replication competent retroviral vector. Infection of the prospective right hind-limb region of a stage 10 embryo with a retroviral vector containing the *gsc* gene results in complete infection of the right hind-limb at the earliest limb stages, as assayed by whole-mount in situ hybridization with an RCAS probe on a stage 24 embryo (Fig. 2). The left limb serves as an internal control for the uninfected condition. Earlier limb buds appear normal in both infected and wild-type limbs. The earliest gross morphological difference between infected and contralateral limbs was observed 2.5 days post-infection, at stage 24. Normally the limbs grow out from the body wall in a downward direction relative to the main body axis. In *gsc*-injected embryos, the limb bud projects from the flank at close to a perpendicular angle (Fig. 2). In addition there is a delay in the ventral rotation of the limbs, a process critical for establishing proper proximal articulation of the femur and humerus with the pelvic and shoulder girdles, respectively. These limbs were stained with alcian blue and cleared to visualize cartilage condensa-

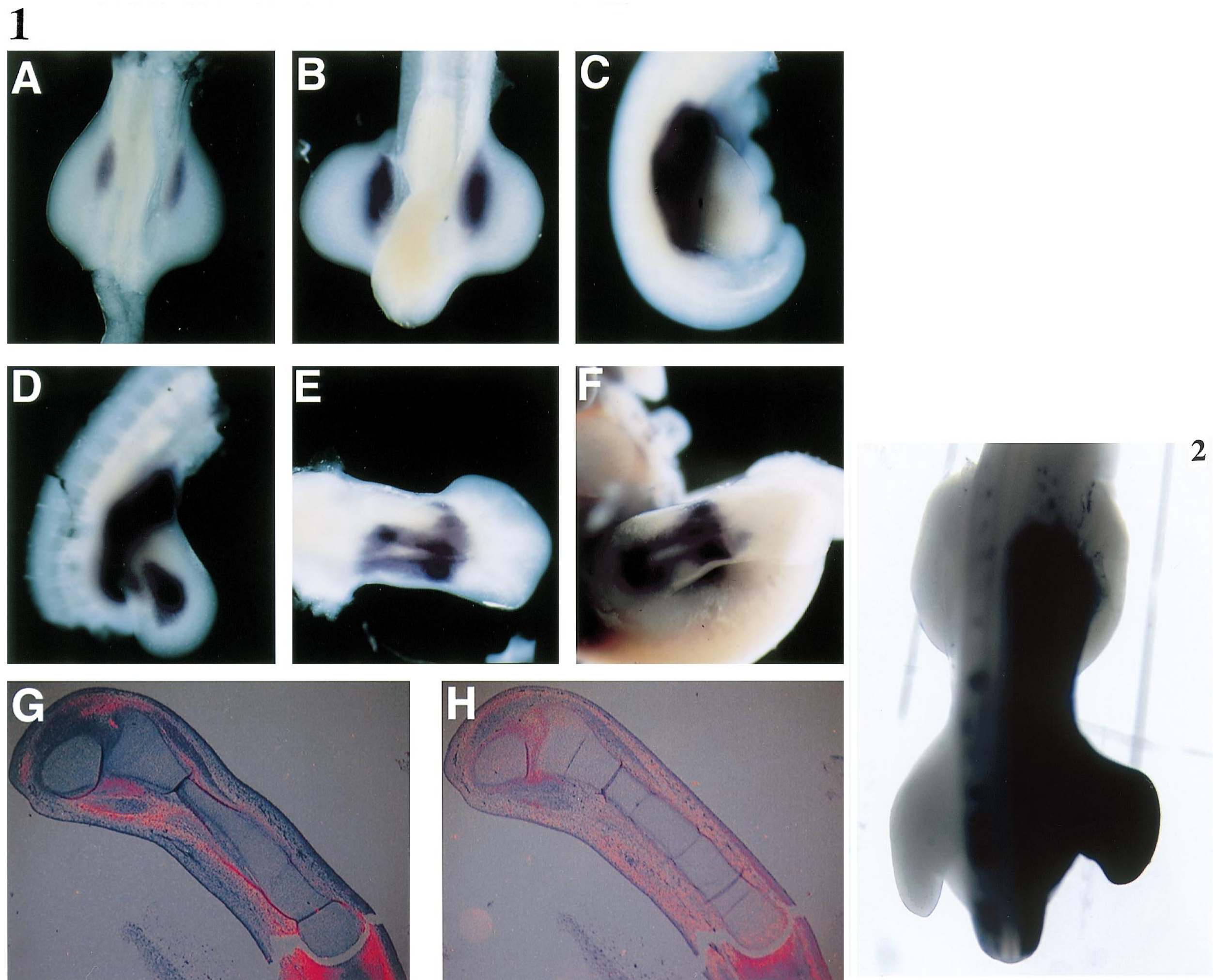

Fig. 1. *gsc* is expressed in two temporal-spatial domains in the developing chick hind-limb. Whole-mount in situ hybridization was used to detect *gsc* messenger RNA at various stages of limb development. (A) Stage 20 hind-limb shows *gsc* expression in a faint proximal-anterior-ventral (p-a-v) region. (B) In stage 22 hind-limbs expression has intensified but remains restricted to the proximal region. (C) By stage 23 the *gsc* expression domain has expanded but remains restricted to the p-a-v region. (D) A second more distal domain of *gsc* expression appears in stage 25 hind-limbs in the region of condensing cartilage. (E,F) By stage 27 the p-a-v domain of expression has faded and the distal domain of expression resolves to perichondrial regions surrounding condensing cartilage, especially in the knee and ankle regions. Analysis of later stage embryos by radioactive in situ hybridization on limb sections with a *gsc* probe reveals a signal (visualized in red) which most likely represents expression in the developing tendons (G). Expression of *Hoxd-11* on adjacent sections shows a broader domain of expression which encompasses most of the soft tissue of the limb, including the developing tendons. A,B, ventral views; C–F, dorsal views.

Fig. 2. *gsc* misexpression at stage 10 results in complete infection of the hind-limb and a morphological phenotype. *gsc* misexpression in the right hind-limb field of a stage 10 embryo results in complete infection of the right hind-limb as assayed by whole-mount in situ hybridization with an RCAS probe on a stage 24 embryo. At this stage a morphological phenotype is first evident when examining the angle of outgrowth of the infected right hind-limb. The infected limb protrudes from the flank at close to a perpendicular angle.

tions. By this stage femur, tibia and ankle and digit condensations are apparent. In the uninfected left limb, the femur, tibia and fibula all have a posterior bias in their location relative to the midline of the limb bud and the femur condensation assumes close to a 90° angle from the main body axis (Fig. 3A,C, angle **x**). Furthermore, the limb bud condensations have a characteristic bend at the femur and tibia/fibula junction which approximates a 120° angle (denoted by the angle **y**, Fig. 3C). In the *gsc*-infected limb there is no significant difference in the sizes of the skeletal elements at this stage. However, the relative positions of the proximal elements are shifted within the limb bud so that the femur condensation forms an acute angle relative to the main body axis (Fig. 3D, angle **x′**). Additionally, the femur, tibia and fibula condensations are situated medially within the limb bud so that the angle formed at the femur and tibia/fibula condensation junction is approximately 180° (Fig. 3D, angle **y′**). This early misexpression phenotype suggests that the normal expression of *gsc* localized in the anterior aspect of the limb bud may contribute to the normal positioning of the femur both within the limb and in the angle which it assumes relative to the body wall.

Embryos allowed to develop until day 10 (8.5 days post-infection) exhibit a second striking phenotype which affects the morphogenesis of the skeletal elements themselves. The *gsc*-infected right hind-limbs are significantly smaller than the uninfected left hind-limbs. When stained with alcian blue and cleared, it is evident that all skeletal elements (femur, tibia, fibula, ankle and digits) are present in the infected right limb (Fig. 3F), though they are markedly smaller in size than in the uninfected left limb (Fig. 3E). This shortening is not simply a consequence of alteration in the shapes of the bones. For example, in Fig. 3F the tarsometatarsals are reduced to remnants and the femur is clearly smaller in total size than the contralateral control. In addition the cartilage elements are misshapen, being both thickened and dramatically bent. This latter aspect could be a secondary consequence of alterations to the tendons where *gsc* is expressed. Thus, the late overexpression phenotype suggests that *gsc* normally plays roles both in regulating the growth of cartilage elements and in regulating tendon growth.

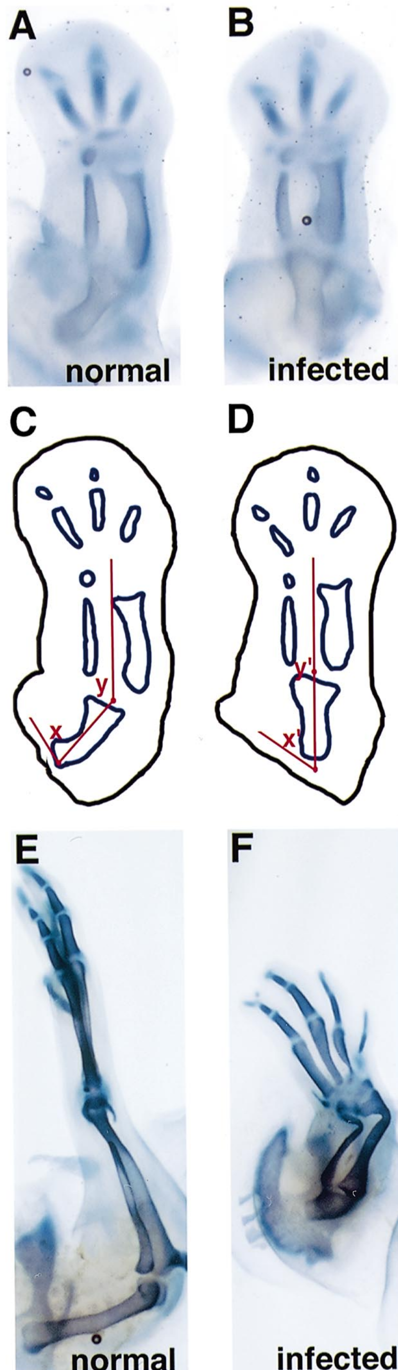

Fig. 3. *gsc* misexpression results in alterations in limb outgrowth. A retroviral vector driving *gsc* expression was infected into presumptive stage 10 hind-limbs results in a phenotype which is first evident at stage 24. Normal (A) and *gsc*-infected (B) limbs were stained with alcian blue and cleared with glycerol to visualize cartilage condensations. In *gsc*-infected hind-limbs the position of the cartilage elements are shifted within the limb bud. A schematic representation of these limb buds (C,D) highlights the differences between normal and *gsc*-infected limbs in the angles that the condensing cartilage elements assume relative to the main body axis (angles **x** and **x′**) and the angle at the femur and tibia/fibula junction (angles **y** and **y′**). When embryos are allowed to develop to day 10 they exhibit a second phenotype which affects the shape and size of the skeletal elements of infected limbs (F) relative to contralateral uninjected controls (E). The *gsc*-infected hind-limbs are significantly smaller and the skeletal elements are misshapen, being both thickened and bent. In this example the effects are most severe in the reduction of the tarsometatarsals and femur and the twisting of the tibia and fibula. Other specimens display defects in all the bones of the limb.

2.3. Hoxa and Hoxd genes are downregulated in gsc-infected limbs

It has recently been suggested that *Hox* genes affect patterning of skeletal elements in the embryo by modulating the local rates of proliferation of the condensing cartilage (Duboule, 1995; Goff and Tabin, 1997). To examine whether *gsc* might act to affect the growth of the skeletal elements by interacting with the *Hox* genes, we examined *Hoxd-11*, *Hoxd-12*, *Hoxd-13*, *Hoxa-11* and *Hoxa-13* expression in *gsc*-infected and uninfected limbs by in situ hybridization. *gsc*-infected limbs show decreased levels of expression of all these members of the *Hoxa* and *Hoxd* clusters when assayed at stages 22–30 (Fig. 4A–D and data not shown). Several other genes known to play a role in the growth and patterning of the vertebrate limb (*Shh*, *BMP-2* and *BMP-4*) were also assayed and found to be unaffected by *gsc* misexpression (Fig. 4F and data not shown). These data suggest that the late *gsc* effect on skeletal growth may be mediated specifically by the *Hox* genes. None of the *Hoxa* and *Hoxd* cluster genes are expressed in the early p-a-v region where *gsc* is first detected in the limb bud. In contrast, *Hoxc* genes are expressed in this region (Oliver et al., 1988). Therefore, we examined *Hoxc-6*, *Hoxc-8* and *Hoxc-9* in early limb buds following *gsc* infection. Expression of these members of the *Hoxc* cluster was unaffected in *gsc*-infected limbs (Fig. 4E and data not

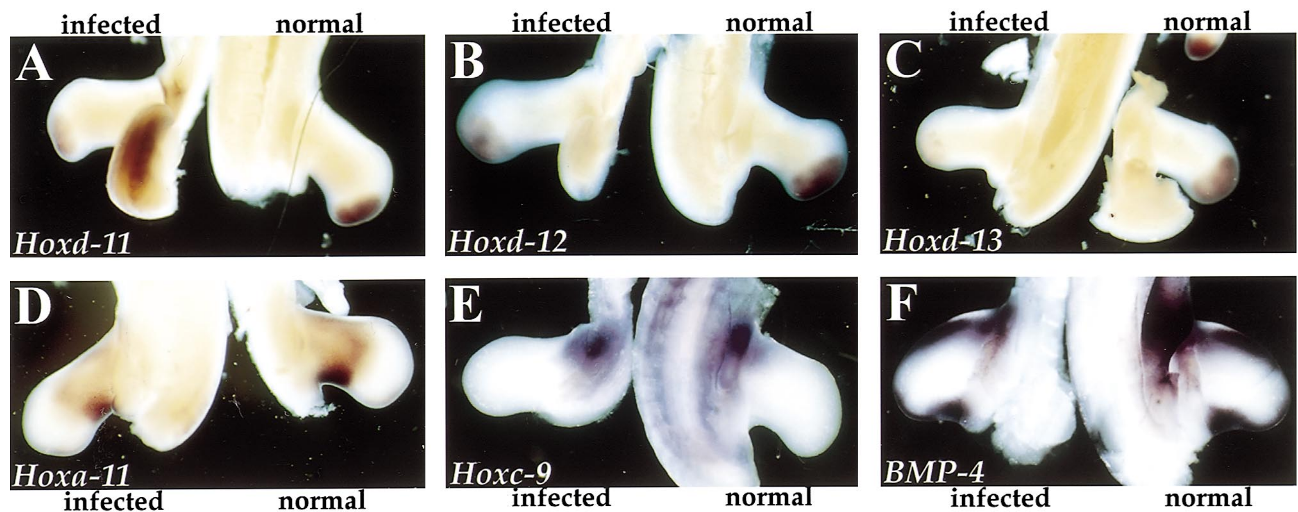

Fig. 4. *Hox* genes of the *a* and *d* clusters are downregulated in *gsc*-infected limbs. Embryos were infected with *gsc* at stage 10 and allowed to develop until stage 24. Embryos were then analyzed for *Hox* gene expression by whole-mount in situ hybridization using probes for *Hoxd-11* (A), *Hoxd-12* (B), *Hoxd-13* (C), *Hoxa-11* (D) and *Hoxc-9* (E). Embryos were cut in half and photographed in the ventral view and thus unaffected left limbs are on the right and *gsc*-injected right limbs are on the left. In addition, embryos were probed with *BMP-4* (F). Expression of the *Hoxa* and *Hoxd* cluster genes is downregulated in *gsc*-infected limbs (A–E), while genes of the *Hoxc* cluster and *BMP-4* are unaffected (E,F).

shown), demonstrating specificity in *gsc Hox* targets. Later in limb development genes of the *Hoxd* cluster, which are regulated by *gsc* misexpression, are known to be expressed in a pattern that overlaps with the *gsc* expression in the developing tendons (Fig. 1H). Therefore, it is possible that *gsc* has a role in regulating *Hox* genes involved in the formation of the soft tissues of the limb and in particular the tendons.

3. Discussion

The expression of *goosecoid* in the chick limb has two distinct temporal and spatial domains. In the early limb bud *gsc* is expressed in a proximal-anterior-ventral (p-a-v) region. This region does not correspond to any known signaling centers, but occupies a region fated to give rise to shoulder/pelvic elements as well as the proximal humerus/femur. The second domain of expression occurs in the region surrounding the condensing cartilage of the developing limb, which is likely to represent expression in the developing tendons. These patterns of expression suggest that *gsc* may normally play a role early in the formation of proximal limb elements and later in tendon development.

In order to understand the function of *gsc* in the developing limb, misexpression studies were undertaken. Misexpression of *gsc* in the prospective hind-limb field at the time when *gsc* is normally expressed (stages 20–26) resulted in alteration of early limb outgrowth. By stage 24, when alcian blue can first be used to detect cartilage condensations, the articulation of the skeletal elements is altered relative to the uninfected limb. Normally, the femur condensation assumes a right angle relative to the main body axis. In *gsc*-infected limb, the femur condensation projects at an acute angle relative to the body axis. This alteration suggests that one of the functions of *gsc* in the early limb may be to determine the articulation of the limb. This effect could be mediated by shifting the location within the limb bud where the proximal end of the femur begins the condensation process, or alternatively it could, in principle, be explained by alterations in the relative growth of soft tissues surrounding the forming skeletal elements.

We observed a later effect of *gsc* misexpression on the growth of the bones. In limbs where *gsc* is misexpressed and analyzed at day 10, the limbs are significantly smaller and the cartilage elements are small, misshapen and bent. Since the size of the initial condensation is normal in infected limbs (see Fig. 3A,B), this phenotype suggests that *gsc* expression plays a role in regulating the growth of the forming cartilage elements. If *gsc* does play a role in regulating the growth of cartilage, it must act non-cell autonomously, regulating the expression of other signaling molecules which then influence the proliferation and elongation of cartilage. We have examined the expression of members of the BMP family and found their expression to be unaffected by ectopic *gsc*, indicating that other signaling molecules are controlled by *gsc* expression. In addition, *gsc* may play a role in tendon development at later stages, perhaps by regulating growth of tendons. Such a role could explain the bends in the forming cartilage elements. If the tendons, once attached to the ends of bones, fail to grow at the same rate as the cartilage elements they could pull on these elements, thus causing the observed bends.

Further insight into the role of *gsc* came from examining the effect of *gsc* misexpression on *Hox* gene expression. The *Hoxc* genes, which overlap in their expression with the early

p-a-v *gsc* domain, are not affected by *gsc* misexpression. In contrast, the *Hoxa* and *Hoxd* genes, which normally are not expressed in the p-a-v quadrant of the early limb bud (Nelson et al., 1996), are downregulated in *gsc*-infected hindlimbs. Therefore, the p-a-v expression of *gsc* may contribute to the absence of *Hoxd* gene expression in that region and thereby affect the early patterning of the limb and in particular the limb articulation. The *gsc* effect on *Hox* gene expression and the later effect on skeletal proliferation in the limb is interesting with regards to current views on *Hox* genes. It has recently been suggested that skeletal alterations seen in mice that are homozygous nulls for particular *Hox* genes may be the result of changing the rate of cell growth or cell recruitment for the prechondrogenic condensation of the elements (Duboule, 1995). According to this model, particular combinations of *Hox* genes translate into a particular rate of proliferation, each positively or negatively controlling the rate. The ability of *Hox* genes to influence mitotic rates of the forming skeletal elements has been verified in the case of *Hoxd-13*. Misexpression of *Hoxd-13* in the limb bud results in a shortening of the femur and tibia. Thymidine incorporation studies demonstrated that proliferation is indeed decreased in the cartilage of these elements (Goff and Tabin, 1997). The results of *gsc* misexpression in the limb provide an additional connection between *Hox* genes and proliferation. When *gsc* is ectopically expressed throughout the limb bud, *Hox* genes are downregulated. The late phenotype is a marked decrease in the size of the limb, probably resulting from a decrease in cell proliferation. One possible explanation for these results is that *gsc* normally functions to repress cell proliferation in the limb by regulating *Hox* gene expression. Consistent with the idea that *gsc* acts as a negative regulator of gene expression, recent work has identified a transcriptional repressor domain within the *gsc* protein (Smith and Jaynes, 1996). Thus, it is possible that *gsc* normally functions to repress *Hox* gene expression in the developing limb.

Recently, several labs have described the phenotype of mice which are homozygous for mutations in *gsc* (Rivera-Perez et al., 1995; Yamada et al., 1995). These embryos survive until birth, but die shortly afterwards. As these embryos gastrulate normally, it appears that *gsc* is not necessary for organizer function. It is possible that other molecules share a redundant function in this process with *gsc*. Abnormalities were observed in the *gsc* knockout mice in the craniofacial region and the ribs, yet no limb phenotype was reported. However, recent re-examination of these knockout mice identified a limb phenotype (J.A. Belo and E.M.D.R., unpublished data). At the shoulder and hip joints, bone abnormalities result in an alteration of the articulation at these joints. At the shoulder, the insertion of the clavicle is affected by a shortened acromion process and articulation at the humerus region is affected by an altered coracoid process and head of the humerus, leading to an altered angle between the humerus and the main body axis. In the hip region, a deletion of a large region of the acetabulum and an increase in the size of the head of the femur result in altered articulation at this joint. These phenotypes are consistent with the alteration in hip articulation seen in early limb buds following viral misexpression in the chick. Moreover, the defects in bone size and shape observed in the *gsc* knockout mouse are consistent with a role for *gsc* in regulating the proliferation of cartilage elements. In light of the late expression pattern and aspects of the late phenotypes in *gsc* misexpression, formation of tendons might be worth re-examining in the *gsc* mutant embryos. It is also possible that *gsc* plays a redundant role in these regions and we have here revealed a role for *gsc* in limb patterning that is not obvious from the loss of function experiments. In particular, a *gsc-2* gene has been identified in the chick which partially overlaps the expression of *gsc* (or *gsc-1*) during gastrulation (Lemaire et al., 1997). The role of this and potentially other members of the *gsc* family is worth evaluating during limb development.

The combination of these results suggest that *gsc* plays a role in patterning cartilage elements in the developing limb, in particular affecting the articulation of the hip and growth of the femur. It is unlikely that these phenotypes can be completely explained by the proposed mechanism for *gsc* function in the organizer, i.e. that of influencing cell migration (Niehrs et al., 1993). Instead, the results reported here indicate that in the limb region *gsc* seems to function upstream of *Hox* genes to regulate their expression. In turn the *Hox* genes may function to regulate the proliferation of the condensing cartilage, or to regulate the condensation process itself to allow the normal patterning of the limb to occur.

4. Experimental procedures

4.1. Embryos

Chick embryos were obtained by incubation of fertilized White Leghorn eggs provided by SPAFAS (Norwich, CT) at 37.5°C and staged according to Hamburger and Hamilton (1951).

4.2. In situ hybridization

Plasmid p*gsc*-L (Izpisua-Belmonte et al., 1993) was linearized with *Nco*I and transcribed with T7 RNA polymerase (Boehringer) using digoxigenin-UTP (Boehringer) to create an RNA probe. DNA templates used to generate RNA probes for RCAS, *Hox* genes and BMP-4 are described elsewhere (Johnson et al., 1994; Burke et al., 1995). Wholemount in situ hybridization was performed using the method described in Riddle et al. (1993). Radioactive in situ hybridization on limb sections followed the protocol outlined in Vortkamp et al. (1996) with the modification that counterstaining and photography of sections followed the protocol described in Nelson et al. (1996).

4.3. Retroviral vector construction and viral misexpression

The replication-competent retroviral vector RCAS-A (Hughes et al., 1987) was engineered to express a full length cDNA of chicken *gsc* (Izpisua-Belmonte et al., 1993). The complete *gsc* open reading frame was ligated into the *Nco*I-*Sal*I site of the *Cla12*-*Nco*I adapter (Hughes et al., 1987) and then cloned into the *Cla*I site of RCAS-A. The *gsc* virus was injected into stage 10 chicken embryos targeting the prospective hind-limb field (Morgan et al., 1992). Injected embryos were allowed to develop for a further 2–8 days, when morphological phenotypes were evident, and collected for analysis. Neither of the phenotypes observed with *gsc* misexpression are seen following injection of virus containing alkaline-phosphatase in the same vector (data not shown).

4.4. Cartilage staining

Whole-mount alcian blue staining was used to examine cartilage. Embryos were cleared with glycerol/KOH to visualize the skeletal elements (Goff and Tabin, 1997).

Acknowledgements

We wish to thank Craig Nelson and Andrea Vortkamp for providing assistance and members of the Tabin lab for technical advice. This work was supported by a grant to C.J.T. from the NIH. We also acknowledge the support of the Human Frontiers Science Program (E.M.D.R. and C.D.S.).

References

Blum, M., Gaunt, S.J., Cho, K.W., Steinbeisser, H., Blumberg, B., Bittner, D., De Robertis, E.M., 1992. Gastrulation in the mouse: the role of the homeobox gene goosecoid. Cell 69, 1097–1106.

Blumberg, B., Wright, C.V., De Robertis, E.M., Cho, K.W., 1991. Organizer-specific homeobox genes in Xenopus laevis embryos. Science 253, 194–196.

Burke, A.C., Nelson, C.E., Morgan, B.A., Tabin, C., 1995. Hox genes and the evolution of vertebrate axial morphology. Development 121, 333–346.

Cho, K.W., Blumberg, B., Steinbeisser, H., De Robertis, E.M., 1991. Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. Cell 67, 1111–1120.

Duboule, D., 1995. Vertebrate Hox genes and proliferation: an alternative pathway to homeosis? Curr. Opin. Genet. Dev. 5, 525–528.

Gaunt, S.J., Blum, M., De Robertis, E.M., 1993. Expression of the mouse goosecoid gene during mid-embryogenesis may mark mesenchymal cell lineages in the developing head, limbs and body wall. Development 117, 769–778.

Goff, D.E., Tabin, C.J., 1997. Analysis of *Hoxd-13* and *Hoxd-1i* misexpression in chick limb buds reveals that *Hox* genes affect both bone condensation and growth. Development 124, 627–636.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. J. Exp. Morph. 88, 49–92.

Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J., Sutrave, P., 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. In: Ede, D.A., Hinchliffe, J.R., Balls, M. (Eds.), Vertebrate Limb and Somite Morphogenesis, Cambridge University Press, Cambridge.

Izpisua-Belmonte, J.C., De Robertis, E.M., Storey, K.G., Stern, C.D., 1993. The homeobox gene goosecoid and the origin of organizer cells in the early chick blastoderm. Cell 74, 645–659.

Johnson, R.L., Laufer, E., Riddle, R.D., Tabin, C., 1994. Ectopic expression of *sonic hedgehog* alters dorsal-ventral patterning of somites. Cell 79, 1165–1173.

Lemaire, L. et al., 1997. Segregating expression domains of two goosecoid genes during the transition from gastrulation to neurulation in chick embryos. Development 124, 1443–1452.

Lewis, J., 1977. Growth and determination. In: Ede, D.A., Hinchliffe, J.R., Balls, M. (Eds.), Vertebrate Limb and Somite Morphogenesis. Cambridge University Press, Cambridge, pp. 236–239.

Morgan, B.A., Izpisua-Belmonte, J.C., Duboule, D., Tabin, C.J., 1992. Targeted misexpression of Hox-4.6 in the avian limb bud causes apparent homeotic transformations. Nature 358 (1), 3004–3012.

Nelson, C.E., Morgan, B.A., Burke, A.C., Laufer, E., DiMambro, E., Murtaugh, L.C., Gonzales, E., Tessarollo, L., Parada, L.F., Tabin, C., 1996. Analysis of *Hox* gene expression in the chick limb bud. Development 122, 1449–1466.

Niehrs, C., Keller, R., Cho, K.W., De Robertis, E.M., 1993. The homeobox gene goosecoid controls cell migration in Xenopus embryos. Cell 72, 491–503.

Oliver, G., Wright, C.V.E., Hardwicke, J., De Robertis, E.M., 1988. A gradient of homeodomain proteins in developing forelimbs of Xenopus and mouse embryos. Cell 55, 1017–1024.

Psychoyos, D., Stern, C.D., 1996. Restoration of the organizer after radical ablation of Hensen's node and the anterior primitive streak in the chick embryo. Development 122, 3263–3273.

Riddle, R.E., Johnson, R.L., Laufer, E., Tabin, C., 1993. *Sonic hedgehog* mediates the polarizing activity of the ZPA. Cell 75, 1401–1416.

Rivera-Perez, J.A., Mallo, M., Gendron-Maguire, M., Gridley, T., Behringer, R.R., 1995. Goosecoid is not an essential component of the mouse gastrula organizer but is required for craniofacial and rib development. Development 121, 3005–3012.

Schulte-Merker, S., Hammerschmidt, M., Beuchle, D., Cho, K.W., De Robertis, E.M., Nusslein-Volhard, C., 1994. Expression of zebrafish goosecoid and no tail gene products in wild-type and mutant no tail embryos. Development 120, 843–852.

Smith, S.T., Jaynes, J.B., 1996. A conserved region of engrailed, shared among all *en*-, *gsc*-, *Nk1*-, *Nk2*- and active *msh*-class homeoproteins, mediates active transcriptional repression in vivo. Development 122, 3141–3150.

Spemann, H., Mangold, H., 1924. Überinduktion von embryonanlagen durch implantation artfremder organisatoren. Wilhelm Roux's Arch. Dev. Biol. 100, 599–638.

Stachel, S.E., Grunwald, D.J., Myers, P.Z., 1993. Lithium perturbation and goosecoid expression identify a dorsal specification pathway in the pregastrula zebrafish. Development 117, 1261–1274.

Vortkamp, A., Lee, K., Lanske, B., Segre, G.V., Kronenberg, H.M., Tabin, C.J., 1996. Regulation of rate of cartilage differentiation by *indian hedgehog* and *PTH-Related Protein*. Science 273, 613–622.

Yamada, G., Mansouri, A., Torres, M., Stuart, E.T., Blum, M., Schultz, M., De Robertis, E.M., Gruss, P., 1995. Targeted mutation of the murine goosecoid gene results in craniofacial defects and neonatal death. Development 121, 2917–2922.