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Original Article

goosecoid expression represses *Brachyury* in embryonic stem cells and affects craniofacial development in chimeric mice

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ABSTRACT The homeobox gene *goosecoid,* originally identified in *Xenopus,* is expressed in the organizer or its equivalent during gastrulation in the frog, chick, zebrafish and mouse. To investigate the role of *goosecoid* in mouse development, we have generated embryonic stem cells that stably overexpress the murine homolog of *goosecoid.* These cells show a repression of the gastrulation-associated gene *Brachyury*. Interestingly, repression of *Brachyury* is conserved between *Xenopus* and mouse despite the lack of conservation of the *Brachyury* promoter. Further characterization of the *goosecoid*-overexpressing ES cells revealed that they maintain the expression of stage-specific embryonic antigen-1, and teratomas derived from *goosecoid*-overexpressing cells show the presence of cell types derived from all three germ layers. Some highly chimeric mice derived from *goosecoid*-overexpressing cells displayed skull defects. These observations suggest that *goosecoid* may play a role in specification of anterior mesendodermal fates and specifically in mouse craniofacial development.

KEY WORDS: *goosecoid, Brachyury, embryonic stem cells, chimeric mice, craniofacial development*

Introduction

Gastrulation is the process by which the three definitive germ layers are established during embryogenesis. In *Xenopus*, the dorsal lip of the blastopore functions as an organizer of mesodermal cell fate along the dorsoventral axis (Spemann and Mangold, 1924). The *goosecoid* gene was identified during a screen of a dorsal lip cDNA library for novel homeobox-containing genes (Blumberg *et al.*, 1991). *goosecoid* is initially expressed just before the onset of gastrulation in the region of the *Xenopus* embryo where the dorsal lip will form. Furthermore, in the chick and the mouse, *goosecoid* is first expressed at the site of primitive streak formation, where gastrulation is initiated in amniote embryos (Blum *et al.*, 1992; Izpisua-Belmonte *et al.*, 1993). The analogous early expression patterns at the onset of gastrulation in these species suggest that the function of *goosecoid* may have been conserved during evolution. Further indications that mechanisms of mesodermal induction have been conserved between the frog and the mouse come from experiments in which the distal tip of the gastrulating mouse embryo, containing a region (the node) that expresses the highest levels of murine *goosecoid*, was transplanted into early *Xenopus* embryos, resulting in the induction of a partial secondary body axis (Blum *et al.*, 1992).

In the mouse, *goosecoid* expression begins in the posterior epiblast, at the site where the primitive streak will form; during streak elongation, *goosecoid* expression becomes restricted to the node and is detected in the anterior-most mesoderm as it forms (Blum *et al.*, 1992). *goosecoid* is coexpressed with *HNF3*β from E6.5 through E9.5 in the anterior visceral endoderm and the anterior primitive streak, the head process, forebrain and oral epithelium (Belo *et al.*, 1997; Filosa *et al.*, 1997). *goosecoid* expression at 10.5 days of gestation is restricted to portions of the facial process, branchial arches, limbs and body wall (Gaunt *et al.*, 1993). Thus, during early embryogenesis, *goosecoid* expression

Abbreviations used in this paper: DMSO, dimethyl sulfoxide; ES cells, embryonic stem cells; *gscl*, *goosecoid*-like; RT-PCR, reverse transcription polymerase chain reaction; SSEA-1, stage-specific embryonic antigen 1.

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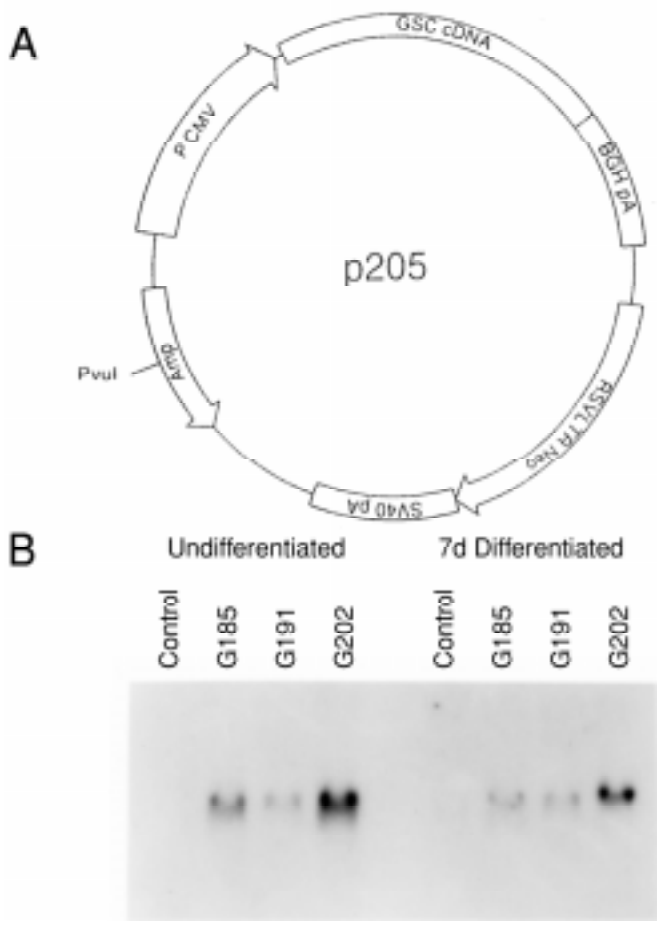

Fig. 1. Identification of ES cell lines overexpressing *goosecoid*. (A) *p205 vector used to overexpress goosecoid in ES cells. The murine cDNA was inserted into pRc/CMV in the sense orientation, and the vector was linearized at the unique PvuI site before electroporation into JM-1 ES cells.* **(B)** *Expression of goosecoid in selected cell lines as shown by RNA blot analysis. Cells were grown with LIF as embryoid bodies or differentiated as embryoid bodies without LIF for 7 days. RNA (20 μg per lane) was probed for goosecoid expression. The control cell line (C1) was derived from electroporation with the vector minus the goosecoid cDNA; G185, G191, and G202 goosecoid-expressing cell lines were derived from electroporation with the vector plus the goosecoid cDNA.*

begins in cells that will initiate the process of gastrulation by forming the primitive streak and then becomes restricted to a specific subpopulation of cells that will form the head process (Lawson *et al.*, 1991); during later embryogenesis, it is expressed in head and limb organ rudiments. This expression pattern along with studies in *Xenopus* suggest that *goosecoid* may play a dual role in formation of both the trunk and head organizers.

Gain-of-function experiments in *Xenopus* have implicated *goosecoid* in the process of axis formation. Microinjection of *goosecoid* mRNA into the ventral side of *Xenopus* embryos led to the formation of partial secondary axes (Cho *et al.*, 1991). Cells injected with the *goosecoid* mRNA were able to recruit uninjected neighboring host cells into a secondary axis (Niehrs *et al.*, 1993). Injections of *goosecoid* mRNA into ventral marginal zone tissue of *Xenopus* gastrulae led to the dose-dependent formation of mesoderm of an increasingly more dorsal nature in explant cultures (Niehrs *et al.*, 1994). These experiments suggested a role of *goosecoid* in mesodermal cell fate determination.

Interestingly, a *goosecoid* knock-out in the mouse has not resulted in an obvious gastrulation phenotype (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995; Zhu *et al.*, 1998), but led to postnatal death due to craniofacial abnormalities related to *goosecoid* expression during organogenesis. This surprising result could be explained by functional complementation through a second, *goosecoid*-related gene. It has been proposed that the *goosecoid* gene family consists of at least three members in the mouse (Belo *et al.*, 1998; Zhu *et al.*, 1998). A full understanding of *goosecoid* function may therefore require the analysis of the combined action of all family members.

Brachyury, like *goosecoid*, is expressed in the newly forming primitive streak mesoderm from the beginning of gastrulation onwards (Wilkinson *et al.*, 1990; Blum *et al.*, 1992; Kispert and Herrmann, 1993). Both genes encode nuclear transcription factors, *goosecoid* a homeodomain protein (Blumberg *et al.*, 1991) and *Brachyury* a transcription factor of the T-box type (Kispert and Herrmann, 1994, Kispert *et al.*, 1995). Initially, when the first mesodermal cells arise (E6.4), both genes are expressed in the same population of cells (as has been shown for zebrafish embryos; Schulte-Merker *et al.*, 1994). When the streak lengthens and reaches the tip of the egg cylinder, *goosecoid* expression is maintained in the anterior-most mesendodermal cells (Blum *et al.*, 1992), which are fated to become the visible node at E7.5 (Sulik *et al.*, 1994), while *Brachyury* mRNA is localized throughout the primitive streak. At E7.5 *goosecoid* is expressed in the prechordal plate and the neuroectoderm overlying the notochordal plate, and at E8.5 in the prechordal plate and overlying the ventral diencephalon (Belo *et al.*, 1998). From E10.5 onwards *goosecoid* is found in derivatives of cranial neural crest, limb buds and external genitals (Gaunt *et al.*, 1993; Zhu *et al.*, 1998). By contrast, *Brachyury* expression persists in the streak, the notochord, and later in the tailbud for the entire period of axis formation and elongation (E6.5-E12.5; Kispert and Herrmann, 1994).

Numerous other studies in *Xenopus* have identified a series of genes that are able to induce or perturb the formation of mesoderm. In contrast, less is known about the specification of mesoderm identity in mammals, largely because of the difficulty of studying gastrulation, a time when the mammalian embryo has implanted and is relatively inaccessible. We address this question by using murine embryonic stem (ES) cells, pluripotent cells of the inner cell mass (Evans and Kaufman, 1981; Martin, 1981), as a model system.

In order to investigate the function of *goosecoid* in mesodermal cell fate determination in the mouse we have derived ES cell lines that stably overexpress the gene from a heterologous promoter. We have used these lines to form embryoid bodies *in vitro*, tumors in ectopic sites, and chimeras *in utero*. *Brachyury* expression in such clones displayed an inverse correlation with *goosecoid* transcript levels. A *Brachyury* reporter construct was repressed by *goosecoid* in ES cells, and mutating a homeobox-binding site removed this repression. Some chimeras generated with *goosecoid*-expressing ES cells have skull defects suggesting that *goosecoid* plays a role in craniofacial development. In summary, our results suggest that *goosecoid* was able to define discrete anterior mesendodermal cell fates.

Results

Production of ES cells that overexpress goosecoid

To derive cell lines that stably overexpress the murine *goosecoid* gene, the ES cell line JM-1 was electroporated with a plasmid containing the cytomegalovirus (CMV) promoter driving the expression of the entire coding region of the murine *goosecoid* cDNA, along with a neomycin-resistance gene driven by the Rous sarcoma virus (RSV) promoter (Fig. 1A). Colonies were selected in ES cell medium containing LIF and G418. As a control, JM-1 cells were transfected with the parent vector minus the *goosecoid* cDNA. RNA was prepared from individual colonies and screened by slot blot to detect clones overexpressing *goosecoid*. The expression of *goosecoid* in three representative cell lines is shown in Figure 1B. No endogenous *goosecoid* expression was detected in the control transfected cell lines when the cells were maintained in an undifferentiated state; however, when the cells were allowed to differentiate as embryoid bodies in the absence of LIF for 7 days, a faint signal derived from the endogenous *goosecoid* gene was detected in the control cells. Expression of the transfected *goosecoid* gene was unaffected by differentiation (Fig. 1B).

Inverse correlation between goosecoid and Brachyury transcript levels in transgenic ES cell clones

In order to test if *goosecoid* expression in ES clones was associated with the activity of specific marker genes of the primitive streak, *Brachyury* and *goosecoid* transcript levels were analyzed in four clones: a control clone transfected with the empty expression vector (C1) and three clones stably expressing *goosecoid* (G185, G191, G202). Semi-quantitative analysis by radioactive RT-PCR revealed that the control clone did not express *Brachyury*, while a faint *goosecoid* signal was detected (Fig. 2), probably due to the higher sensitivity of RT-PCR compared to the Northern analysis in Figure 1. Expression levels of *goosecoid* and *Brachyury* were similar in transgenic clones G185 and G191, while the third clone, G202, expressed 57 times higher *goosecoid* levels and *Brachyury* transcripts were barely detectable (Fig. 2). An inverse correlation of the activity of the two genes was confirmed in three mass cultures each representing at least 100 independent colonies (not shown). This result indicates that *goosecoid* expression in ES cells induces cell fates different from primitive streak mesoderm, which is characterized by *Brachyury* gene expression.

To further evaluate the cell fate of transgenic *goosecoid* clones, the expression of *lim1* and *HNF3β*, two anterior markers, which are both expressed in the primitive streak, prechordal plate and anterior visceral endoderm, was analyzed (Ang and Rossant, 1994; Shawlot and Behringer, 1995; Belo *et al.*, 1997). While *lim1* was not found in the *goosecoid* clones, *HNF3β* was highly expressed (not shown). This may be of significance in the light of the recent analysis of *goosecoid/HNF3β* double knockout mice which indicated a genetic interaction between these two genes (Filosa *et al.*, 1997). Taken together, our analysis of markers suggests an involvement of *goosecoid* in cell fate determination in the anterior midline.

***goosecoid* acts as a repressor of *Brachyury* transcription in the mouse**

Two recent studies have shown that in *Xenopus*, *goosecoid* acts as a repressor of *Brachyury* transcription (Artinger *et al.*, 1997; Latinkic *et al.*, 1997). The inverse correlation of transcript levels

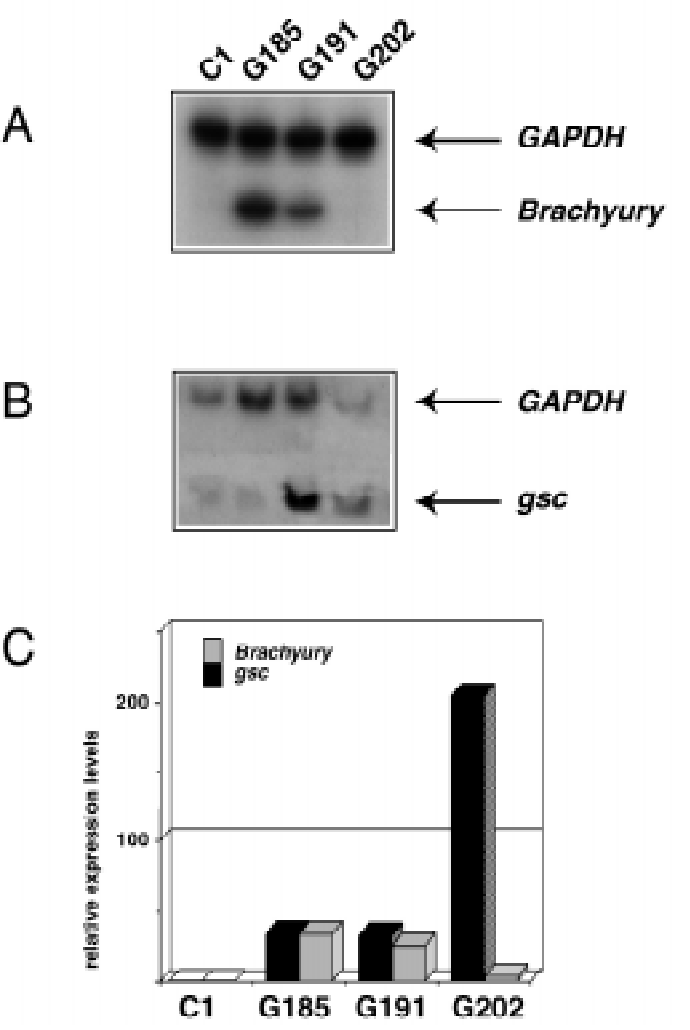

Fig. 2. Inverse correlation of *Brachyury* and *goosecoid* transcript levels in ES cell clones transfected with control (C1) or *goosecoid* expression plasmids (G185, G191, G202). *Transcript levels were determined by semi-quantitative RT-PCR for Brachyury* **(A)** *and goosecoid* **(B)**. *Values were normalized with respect to GAPDH -levels (***C***; relative expression level = 100). Note that in clone G202, which displays high goosecoid expression, Brachyury mRNA was barely detectable.*

shown above argues that such a mechanism might exist in the mouse as well. However, a comparison of the promoter regions of the *Brachyury* genes of frog and mouse did not reveal a great degree of conservation (Fig. 3). In particular, the *goosecoid*-binding sequence identified by Artinger *et al.* (1997), through which repression of *Brachyury* in *Xenopus* was mediated, was not conserved (Fig. 3, region V).

In order to test if *goosecoid* repressed *Brachyury* gene transcription despite this lack of conservation, a mouse *Brachyury* reporter construct comprising the 650bp primitive streak enhancer in front of a luciferase reporter gene (Clements *et al.*, 1996) was cloned. This construct was stably transfected into mouse embryonic stem cells (line E14.1, subclone KPA). A time course of reporter gene activity was determined in mass cultures, following the addition of 1% DMSO (v/v) in order to induce mesodermal differentiation. In agreement with published expression profiles for *Brachyury* and *goosecoid* (Johansson and Wiles, 1995), a peak of luciferase activity was found

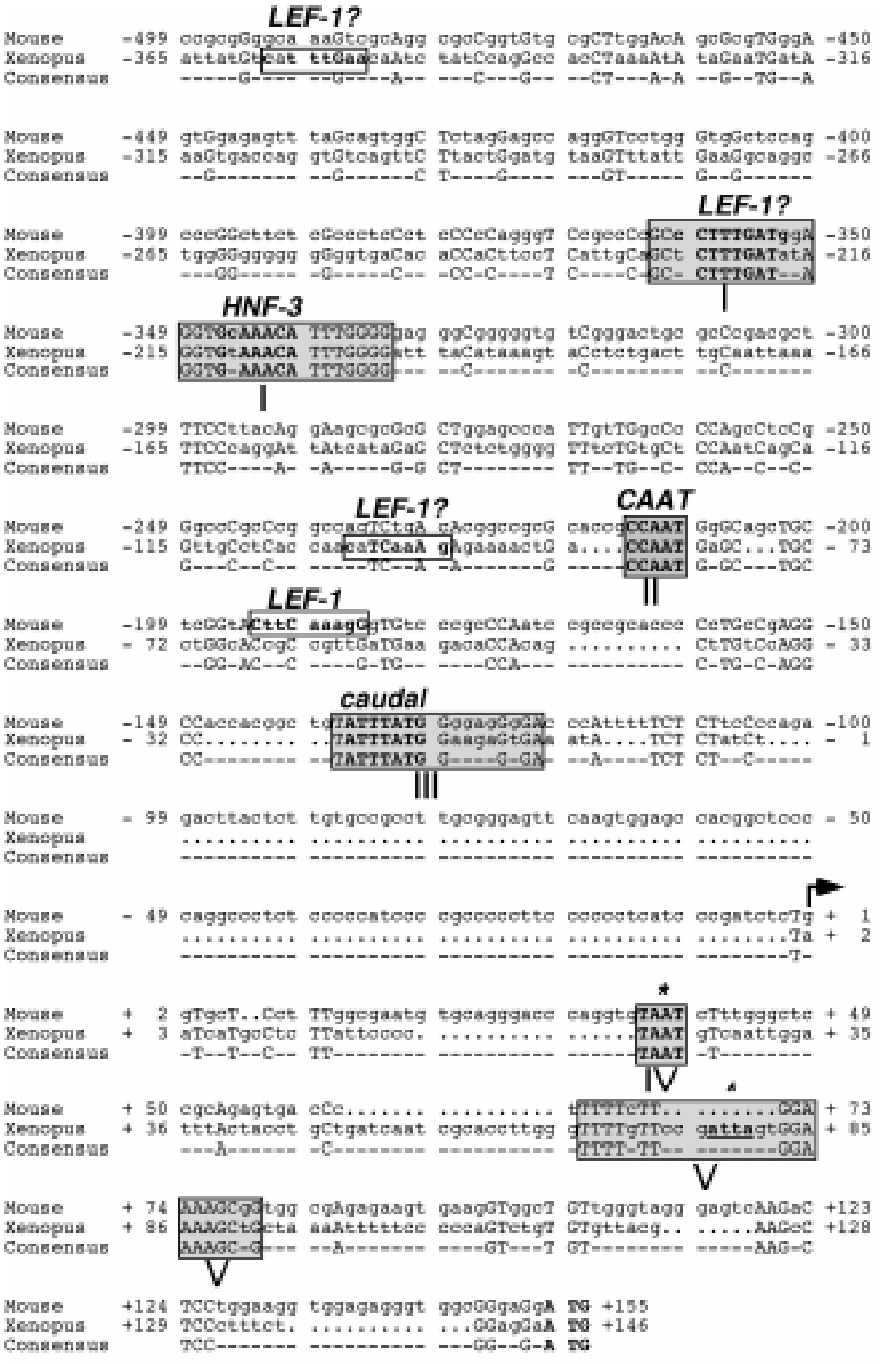

Fig. 3. Alignment of *Brachyury* promoter sequences. *Mouse and Xenopus sequences (database accession numbers U61531 and AF007123, respectively) were aligned and numbered with respect to the transcriptional start site (arrow). Nucleotides conserved between sequences are shown in capital letters. Conservation was restricted to five regions (I-V) indicated by shaded boxes. Consensus binding sites for transcription factors are indicated and highlighted by bold letters. Asterisks mark consensus homeobox binding sites (TAAT and ATTA). Putative LEF-1 sites that are not conserved between mouse and frog are boxed. Sequences are shown up to the start codon (bold letters).*

between the second and third day after the addition of DMSO (not shown).

The effect of *goosecoid* on *Brachyury* reporter activity was investigated by transient transfection of a *goosecoid* expression construct into mass cultures representing at least 100 clones and subsequent DMSO treatment. As shown in Figure 4, a repression of about 50 percent was observed. This experiment was repeated several times with mass cultures and single clones. Thus, despite overall poor conservation of the promoter sequences in *Xenopus* and mouse, the same repression effect of *goosecoid* on *Brachyury* transcription was observed.

A careful analysis of the mouse *T* promoter sequence revealed the presence of one potential homeobox-binding sequence TAAT, in contrast to five such sites found in the *Xenopus Brachyury* promoter (Fig. 3). This element was conserved between mouse and frog (region IV; boxed and marked with asterisks in Fig. 3). This element was mutated into ACTG in the context of the 650bp promoter fragment and the effect of coexpression of *goosecoid* in ES cell cultures stably containing the mutated reporter gene was analyzed. As shown in Figure 4, the mutant construct was no longer repressed by *goosecoid*. Thus this single homeobox-binding site was necessary and sufficient for *goosecoid*-mediated repression of *Brachyury* transcription. In addition, we found that the reporter activity was markedly enhanced both in the absence and presence of *goosecoid* as compared to the wild-type construct (Fig. 4).

goosecoid-expressing cells maintain SSEA-1 expression

Since *Brachyury* was repressed by high levels of *goosecoid* expression, we asked if other differentiation markers were altered in our transgenic ES lines. Stage-specific embryonic antigen-1 (SSEA-1) is first expressed at the 8-cell stage of murine embryonic development (Solter and Knowles, 1978). Undifferentiated murine teratocarcinoma cells and ES cells express SSEA-1, whereas their differentiated derivatives do not (Solter and Knowles, 1978; Fox *et al.*, 1981). In the embryonic region of 6- and 7-day egg cylinder-stage embryos, SSEA-1 is expressed by embryonic ectoderm but not by mesoderm (Fox *et al.*, 1981). Immunofluorescence assays indicated that both control and G202 cells remained positive for SSEA-1 expression when maintained as undifferentiated cells in the presence of LIF (Fig. 5A,B). Thus, *goosecoid*-expressing cell lines maintained the SSEA-1 expression pattern of non-mesodermal cells.

In vivo differentiation of *goosecoid*-expressing cells

To test the *in vivo* developmental potential of the *goosecoid*-overexpressing cells, we injected control and G202 cells subcutaneously into nude mice. Both control and G202 cell lines gave rise to teratomas. RNA blot analysis of RNA isolated from the teratomas demonstrated that tumors derived from G202 cells maintained the expression of the transfected *goosecoid* gene (data not shown), indicating that *goosecoid* overexpression did not diminish tumorigenicity.

Histological analysis suggested that both control- and G202-derived tumors contained ectoderm-, endoderm-, and mesoderm-derived cell types (Fig. 5C,D).

In addition, analysis of 42 E18.5-19.5 chimeras derived from the injection of G202 cells into normal blastocysts showed that G202 cells were able to contribute to brain, heart and liver (data not shown). Thus, *goosecoid* expression is compatible with differentiation into cells derived from all three germ layers. While chimeras derived from the injection of G202 cells into normal blastocysts were born, they were eaten by their mothers within 72 h of birth. Analysis of 20 G202 chimeras delivered by caesarian section revealed 3 cases of craniofacial abnormalities, including cleft palate, and shortening of the squamosal bone, whereas 66 non-chimeric littermates were normal (Fig. 6). All 3 of these chimeras had at least 50% contribution from the G202 ES line as determined by GPI analysis (data not shown).

Discussion

We have generated *goosecoid*-expressing ES cell lines to determine the role of *goosecoid* in mouse development. Three *goosecoid*-expressing ES lines, G185, 191, and G202, and one control ES line, c1, were identified and used in this study. Expression of downstream genes was examined in all 3 transgenic clones and the control clone. *Brachyury* expression was repressed in G202, the line with the highest *goosecoid* expression. *HNF3β* was induced, while there was no change in *lim1* expression. Mutation of a potential homeobox-binding site in the *Brachyury* 5' leader sequence abolished the repression of *Brachyury* through *goosecoid*. Clone G202 maintains expression of SSEA-1 and these ES cells can give rise to all germ layer derivatives in teratomas formed in nude mice. Some highly chimeric mice made with the G202 line exhibited craniofacial defects.

The organizer transplantation experiments of Spemann and Mangold (1924) revealed not only that the dorsal lip of the blastopore was capable of organizing cells to form a new axis, but also suggested the existence of separate head and trunk organizers. Recent evidence has shown that the vertebrate head organizer, originally postulated to be the prechordal mesoderm, may be the anterior visceral endoderm (Thomas and Beddington, 1996; Varlet *et al.*, 1997; reviewed in Belo *et al.*, 1997; Bouwmeester and Leyns, 1997). The organizer genes, *goosecoid*, *lim1* and *HNF3β*, are all expressed in the anterior visceral endoderm as well as in the trunk organizer suggesting that these genes may have dual organizer roles in development.

Analysis of genes that may interact with *goosecoid* revealed that overexpressing *goosecoid* in ES cells results in the repression of *Brachyury* and induces expression of *HNF3β*. The repression of *Brachyury* that is seen in the ES line with the highest *goosecoid* expression is consistent with studies in *Xenopus* which revealed that *goosecoid* represses *Brachyury* by directly binding to consensus sites within the *Brachyury* promoter (Latinkic *et al.*, 1997; Artinger *et al.*, 1997). Both *goosecoid* and *Brachyury* are initially expressed in the same population of cells in the mouse embryo. *goosecoid* expression is maintained in the anterior-most part of the primitive streak (Blum *et al.*, 1992) and is later found in the prechordal plate and in derivatives of the neural crest in the head, while *Brachyury* expression is maintained throughout the streak, and is found in the notochord and later in the tailbud. *goosecoid* may play a role in gastrulation and axis formation, as it does in *Xenopus*, when it is co-expressed with *Brachyury* in the node and primitive streak. *goosecoid* also has another role in craniofacial development, when *goosecoid* expression is found in the anterior visceral endoderm, prechordal plate and derivatives of the cranial neural crest. In these tissues, *Brachyury* is not expressed, perhaps as the result of repression by *goosecoid*. The level of *goosecoid* present may be a factor in the ability of *goosecoid* to repress *Brachyury* expression. Only our highest *goosecoid*-expressing clone was capable of repressing expression of *Brachyury*.

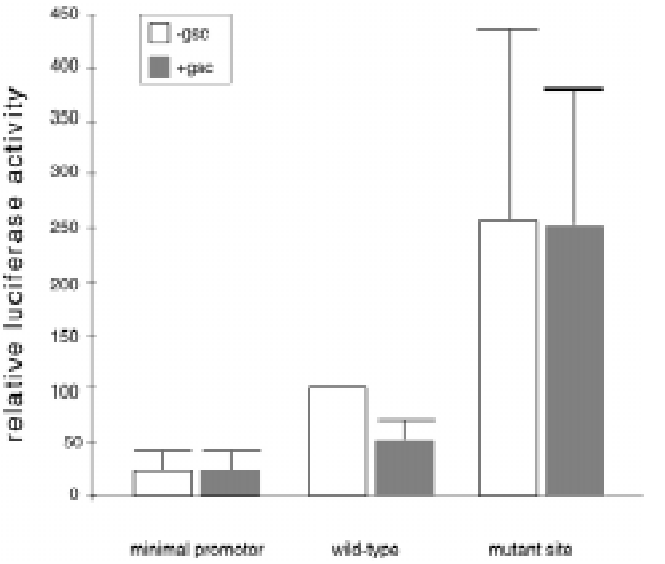

Fig. 4. Repression of *Brachyury* transcription by *goosecoid*. *Transient transfection of a goosecoid expression plasmid into ES mass cultures containing a stably integrated 650bp wild-type or mutant Brachyury-promoter luciferase reporter construct. Bars represent the luciferase activity relative to the wild-type construct in the absence of goosecoid (average of 4 experiments). Maximal values are indicated. Note that the mutated construct was no longer repressed by goosecoid, and that the reporter activity was markedly enhanced in comparison with the wild-type construct.*

Both *goosecoid* and *HNF3β* are coexpressed in several regions of the early mouse embryo including the anterior primitive streak, prechordal plate and anterior visceral endoderm. The induction of *HNF3β* seen in the *goosecoid*-expressing clones is consistent with the observation that double mutant embryos that are *goosecoid* null and are heterozygous for *HNF3β* exhibit a loss of *HNF3β* expression in E8.75 embryos. *goosecoid/HNF3β* double mutant embryos in the most severe cases lost expression of *HNF3β* in axial mesoderm (Filosa *et al.*, 1997). In these double mutants a new phenotype arises in which the forebrain was reduced in size and there were abnormal branchial arches and heart looping. The loss of *HNF3β* expression and the new phenotype in the *goosecoid/HNF3β* double mutant embryos suggests a genetic interaction between *HNF3β* and *goosecoid* (Filosa *et al.*, 1997).

To further examine the interaction between *goosecoid* and *Brachyury*, we compared the murine and *Xenopus Brachyury* promoter sequences. Surprisingly, the degree of conservation was rather low and restricted to five small regions within the 500 (frog) and 650 (mouse) bp upstream of the start ATG. In particular, the binding site through which *goosecoid* mediates repression of *Brachyury* in *Xenopus* was not conserved (Artinger *et al.*, 1997).

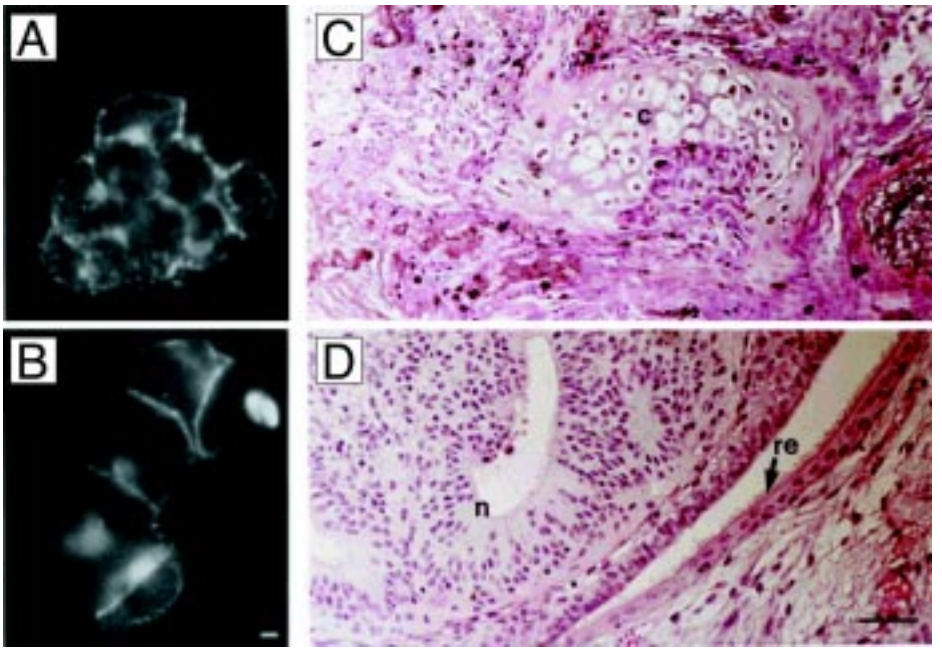

Fig. 5. Characterization of *goosecoid*-expressing cell lines. (A *and* **B)** *goosecoid-expressing cell lines maintain SSEA-1 expression. Monolayer cultures of C1 (A) and G202 (B) cells were fixed and stained with antisera specific for SSEA-1. Bar, 10 mm.* **(C** *and* **D)** *goosecoid-expressing cells form teratomas in nude mice. C1 and G202 cells were injected subcutaneously into the flanks of nude mice. After 3.5 weeks, tumors were harvested, fixed, embedded in plastic, and sectioned. Hematoxylin-and-eosin staining of sections had derivatives of all three germ layers: (C) c, cartilage (mesoderm derived). (D) n, neural epithelium (ectoderm derived); re, ciliated respiratory epithelium (endoderm derived) (arrow indicates ciliae). C and D are from G202 cell injections into nude mice. Bar, 10 mm.*

This element, located in region V (Fig. 3), is missing in the corresponding mouse sequence, although sequences upstream and downstream are conserved. Nevertheless, a *Brachyury* reporter construct that contained the primitive streak enhancer was down-regulated following transient cotransfection of a *goosecoid* expression plasmid, in agreement with the observed inverse correlation of expression of these two genes in ES cell clones (Fig. 2), and in mass cultures of ES and P19 teratocarcinoma cells stably expressing *goosecoid* (data not shown). Mutation of a potential homeobox-binding site in the *Brachyury* 5' leader sequence abolished repression of *Brachyury* through *goosecoid*. Interestingly, the promoter activity of the mutant reporter gene was markedly higher compared with the wild-type construct. This result is compatible with the proposed role of *goosecoid*, namely that *goosecoid* functions as a repressor of *Brachyury* in the anterior of the primitive streak resulting in distinct mesodermal characteristics. As endogenous *goosecoid* becomes up-regulated in ES cultures following DMSO treatment as well (Johansson and Wiles, 1995; our unpublished results), a mutant construct should not be repressed and so should display higher activity than a wild-type one.

A recent paper by Papin and Smith (2000) claimed that down-regulation of *Xbra*, the *Brachyury* homolog in *Xenopus*, was not directly mediated through *goosecoid*. In their experiments, a chimeric dominant-negative *goosecoid* gene construct consisting of two VP16 transactivation domains fused to the *goosecoid* coding region was unable to interfere with activin-induced *Xbra* repression. Such a chimeric protein should bind to the same target sequences as *goosecoid,* but instead of acting as a repressor it should activate transcription. As we have shown previously, *goosecoid* acts in a negative feedback loop to repress its own transcription (Danilov *et al.*, 1998). The VP16 fusion protein thus should act as a strong inducer of the endogenous *goosecoid* gene, which would explain the observed unaltered repression effect. Endogenous *goosecoid* transcription was not analyzed in the experiments of Papin and Smith (2000).

Conserved sequence elements between the mouse and frog *Brachyury* promoter contain potential binding sites for two other transcription factors that are important regulators of early vertebrate development. LEF-1 binding sites are present in both the mouse and frog promoter (Love *et al.*, 1995; Giese *et al.*, 1997). One of these is located in conserved region I (Fig. 3). The presence of these sites indicates that *Brachyury* may be a direct target of the Wnt signaling pathway active in early vertebrate embryogenesis. This pathway results in the activation of the architectural transcription complex LEF-1/β-catenin and leads to epithelial-mesenchymal transitions concomitant with primitive streak formation (Huber *et al.*, 1996; Schneider *et al.*, 1996). Conserved region III comprises a perfect binding site for the homeobox transcription factor caudal (Margalit *et al.*, 1993). As the expression of *caudal* genes overlaps with that of *Brachyury* in mouse, chick and frog (Meyer and Gruss, 1993; Epstein *et al.*, 1997; Pillemer *et al.*, 1998), they may be positive modulators of *Brachyury* expression in the forming mesoderm.

The appearance of craniofacial defects in some *goosecoid* chimeras is consistent with the defects seen in *goosecoid* null mutants (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995). The *goosecoid* null mutants have a reduction in the palatine bone, whereas one chimera, generated from the G202 *goosecoid*-overexpressing cell line, had a severe cleft palate. Two *goosecoid* chimeras have a shortened squamosal bone, while this bone appears to be normal in *goosecoid* null mutants. None of the G202 *goosecoid*-expressing chimeras had the tympanic ring bone defect characteristic of the null mutants. The similarities in phenotypes between the *goosecoid* null and chimeric embryos has a precedent in the similar phenotype resulting from ectopic expression of *Hox-3.1* and the targeted disruption of *Hox-3.1*, which display similar vertebral transformations (Le Mouellic *et al.*, 1992; Pollock *et al.*, 1992). It has been proposed that the *Hox-3.1* gene is regulated antipodally; that is, overexpression of the gene and the loss of expression both result in similar phenotypes (Pollock *et al.*, 1992).

Thus, the level of *goosecoid* overexpression may affect the severity of the phenotype seen. It is therefore possible that higher levels of ectopic *goosecoid* expression would result in more of the severe phenotypes seen in the null mutant. The degree of chimerism and the regions of the embryo to which the ES cells contribute will also affect the phenotype that is seen.

The observation that mice homozygous for a null mutation of *goosecoid* undergo apparently normal gastrulation (Rivera-Pérez *et al.*, 1995; Yamada *et al.*, 1995) suggests the existence of other *goosecoid*-related genes (De Robertis, 1995). A second *goosecoid* gene, *gscl*, has been identified in the mouse and is expressed at the time of gastrulation (Galili *et al.*, 1997; Wakamiya *et al.*, 1997). It has been argued that the *goosecoid* gene family consists of at least three genes (Belo *et al.*, 1998; Zhu *et al.*, 1998), and a full understanding of *goosecoid* function will require the analysis of the whole family.

Our observation that *goosecoid* overexpression leads to the repression of *Brachyury* and induces *HNF3*β expression demonstrates that these cell lines are a potentially valuable resource for the identification of other genes, such as *chordin* (Sasai *et al.*, 1994), whose expression is regulated by *goosecoid*. Studies are under way to determine targets of *goosecoid* regulation in the mouse.

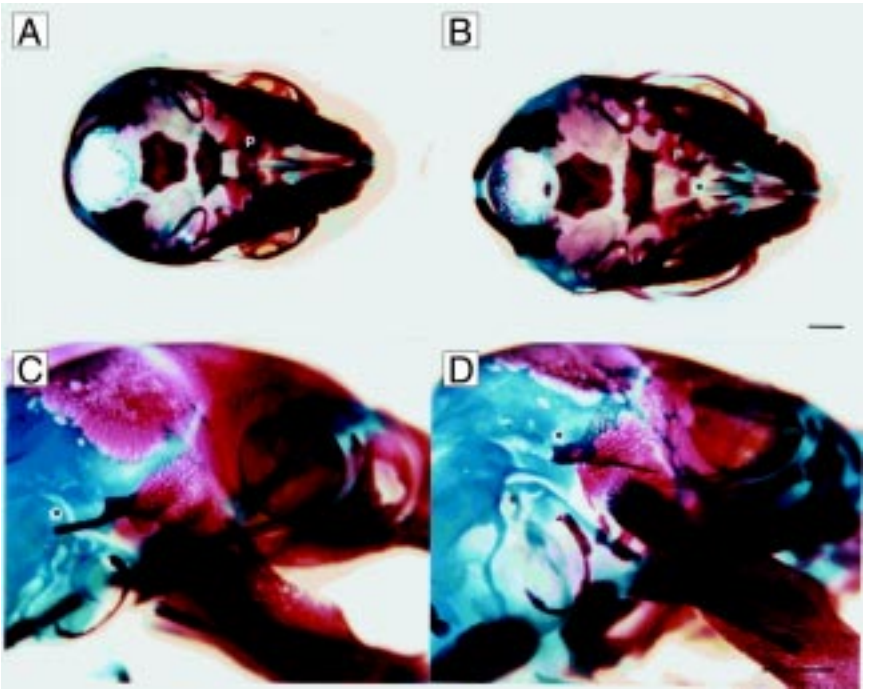

Fig. 6. ***goosecoid*** **chimeras with craniofacial defects.** *E18.5 embryos were stained with alizarin red and alcian blue 8SG. The chimeras in B and D had approximately 50% G202 ES cell contribution based on GPI analysis.* **(A)** *Skull from a non-chimeric littermate; p, palatine.* **(B)** *Skull from a goosecoid chimera with cleft palate (asterisk); p, palatine.* **(C)** *Side view of a skull from a non-chimeric littermate with a normal squamosal bone (asterisk).* **(D)** *Side view of a goosecoid chimera with a shortened squamosal bone (asterisk). Bar, 1 mm.*

Materials and Methods

Cloning of p205

mRNA was isolated from 500 E6.5 mouse gastrulae, and a cDNA library was prepared in lambda MOSSlox (Amersham) (Danilov *et al.*, 1998). Two full-length *goosecoid* cDNA clones were isolated by using the genomic probe P2 (Blum *et al.*, 1992). The *goosecoid* cDNA was inserted into the expression vector pRc/CMV (Invitrogen) and designated p205.

Production of *goosecoid*-expressing cell lines

The ES cell line JM-1 (Miettinen *et al.*, 1995; Meneses, J.J. and Pedersen, R.A., unpublished observations) was electroporated with 10 μg of *Pvu*I-digested p205. Colonies were selected in medium containing 150 μg/ml G418 and transferred to duplicate 24-well plates containing mitomycin-treated G418-resistant STO feeder cells in high-glucose Dulbecco's modified Eagle's medium. The medium was supplemented as described by Robertson (1987) and by the addition of 5-10% conditioned medium from recombinant leukemia inhibitory factor (LIF)-producing CHO cells (a gift of Genetics Institute, Cambridge, MA). One plate was used to freeze cells; colonies in the duplicate plate were grown until confluent. Individual clones were characterized by slot-blotting RNA isolated from each well and probing the filters sequentially for *goosecoid* and cytoplasmic β-actin.

RNA isolation and RNA blot analysis

RNA was prepared by using Ultraspec (Biotecx) according to the manufacturer's instructions. For RNA blot analysis, 20 μg of total RNA was dissolved in 3 μl of water. To each sample, 3.3 μl of sample buffer containing 65% deionized formamide, 8.1% formaldehyde, 1.3xMOPS buffer (10xMOPS buffer=0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 7.0) were added. Each sample was heated at 65°C for 5 min, then cooled on ice. The RNA was fractionated by electrophoresis on 1% agarose gels containing 6.2% formaldehyde and 1xMOPS buffer. RNA was transferred to nitrocellulose filters (Schleicher and Schuell) by capillary action overnight in 20xSSC (1xSSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Filters were baked at 80°C under vacuum for 2 h, wet with 2xSSC, and prehybridized overnight at 42°C in 50% formamide, 6.6xSSC, 5xDenhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 0.1 μg/ml denatured salmon sperm DNA, and 10% dextran sulfate. Filters were hybridized with ^{32}P-labeled DNA probes (Random Priming Kit, Boehringer Mannheim) in the same solution at 42°C overnight. Filters were washed for 30 min in a solution of 2xSSC, 0.1% SDS at ambient temperature, followed by 2 washes at 65°C in a pre-heated solution of 0.2xSSC, 0.1% SDS. Filters were exposed to Kodak X-Omat film at -70°C.

Semiquantitative RT-PCR

Total RNA was isolated from cultured cells by the guanidinium thiocyanate/phenol method using the PeqGOLD TriPure^{TM} kit according to the manufacturer's instruction (peqlab Biotechnologie GmbH, Erlangen, Germany). Total RNA was subjected to DNaseI treatment prior to RT reactions. Ten μg of RNA, 10xDNase-buffer (500 mM Tris HCl , pH 7.5 50 mM $MgCl_2$), 40 U RNAsin (Pharmacia, Freiburg Germany), 10 U DNaseI (RNase free, Boehringer Mannheim, Germany) and 10 μg yeast t-RNA were incubated in a total volume of 50 μl for 10 min at 37°C. Following the addition of 40 μl 4M LiCl and 325 μl H_2O, RNA was extracted and resuspended in 25 μl DEPC- H_2O.

For cDNA synthesis, 800 ng DNaseI-treated total RNA was incubated in a mixture of 5xRT buffer, 13 μM dT20, 400 μM dNTPs (Pharmacia, Freiburg Germany), 10 μM DTT, 10 U RNAsin, 60 U Superscript II reverse transcriptase, at 42°C for 45 min.

Amplifications were performed with Taq-Polymerase (Pharmacia) and radioactively-labeled α[^{32}P]dCTP (mCi; Amersham-Buchler GmbH, Braunschweig) with gene specific primers. GAPDH, 5': 5'-TGTTCC-AGTATGATTCTACCC-3', 3': 5'-CCATCCAC-AGTCTTCTGAG-3', amplification of 440bp; *Brachyury*, 5': 5'-TGCTGCCTGTGAGTCATAAC-

3', 3': 5'-TGCTGCCTGTGAGTCATAAC-3', amplification of 343bp; *goosecoid*, 5': 5'-GTTCTGTAC-TGGTGTCTCCG-3', 3': 5'-TCAGCTGTC-CGAGTCCAAAT-3', amplification of 280bp.

GAPDH and *Brachyury* primers were incubated in the same reaction (10''/94°C, 10''/55°C, 10''/72°C, 27 cycles, followed by a 2' extention at 72°C). To detect GAPDH in combination with *goosecoid*, GAPDH reactions were run for 8 cycles, then fresh PCR-mix was added which contained *goosecoid* primers and PCR was performed for 22 additional cycles (30''/94°C, 30''/55°C, 30''/72°C).

RT-PCR reactions were analyzed on a 6% polyacrylamide gel. The gel was dried and autoradiographed on X-ray film (Hyperfilm). Bands were quantified on a phosphoimager (Fuji Co. Phosphoimager, MacBAS software package).

Cloning of wild-type and mutant Brachyury promoter luciferase constructs

Point mutations in *Brachyury* reporter constructs were introduced by site-directed mutagenesis using the PCR-overlap extension protocol (Dieffenbach *et al.*, 1995). At position +35 the putative homeobox binding site TAAT was changed to ACTG. Primers were designed to add *Bam*H I and *Hin*d III restriction sites at the 5'- and 3'- ends of the promoter, respectively. The following primer pairs were used:

sense: MS-1: 5'- GAGAGGATCCGCGGGGCAAAGT -3',
anti-sense: MS-2: 5'- AAAGCAGTCACCTGGGTCCCTGCACA -3', and
sense: MS-3: 5'- TGTGCAGGGACCCAGGTGACTGCTTT -3'
anti-sense: MS-4: 5'- GAGAAAGCTTCCAGGAGTCTTGACTCC-3'

The final PCR product was digested with *Bam*H I and *Hin*d III, gel purified, and inserted into pT81-LUC-vector (Nordeen, 1988), digested with *Bam*H I/*Hin*d III. The wild-type promoter fragment was obtained from a PCR reaction containing primers MS-1 and MS-4. The promoter constructs were verified by sequencing.

Transfections and luciferase reporter gene assay

ES cells (E14.1 subclone KPA) were cultured on gelatinized dishes in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum in the presence of LIF (500 U/ml). ES cells ($6x10^6$) were cotransfected with 25 μg pT81-LUC plasmid (empty vector, wild-type or mutant *Brachyury* promoter) and 4 μg of a plasmid containing the neomycin phosphotransferase gene (PGK-neo) by electroporation in PBS (Mg^{2+}/Ca^{2+}-free), 10 mM Hepes, pH 7.05 with a Biorad gene pulser (250 μF, 0.4V, 1 pulse, time constant 5-7). DNA for transfection was linearized, phenol/chloroform-extracted and dissolved in sterile water. Transfected cells were grown in the presence of LIF (500 U/ml) and selected for stable neo-plasmid incorporation from day 2 after transfection with G418 (250 μg/ml).

Mass cultures representing at least 100 clones were transiently transfected with a *goosecoid* expression plasmid (pcDNA3.1-*goosecoid*-cDNA-E) using the Superfect-transfection reagent (Qiagen, Hilden Germany). Double-transfected cells were grown in suspension in ES medium in the absence of LIF, with 1% DMSO (v/v), to promote aggregation and differentiation. Luciferase activity was measured in cell lysates (lysis buffer 0.1 M Tris-acetate pH7.5, 2 mM EDTA, 1% Triton X-100) on day 2 or 3 after *goosecoid* transient transfection, using a Lumat LB 9501 luminometer and luciferin at 0.2 mM. Total protein concentrations as indicated by OD_{595} measurements were used for normalization of luciferase activity.

SSEA-1 staining

Monolayer cultures of control and G202 cells were plated onto gelatinized chamber slides and cultured overnight in medium containing LIF. Monolayers were rinsed in phosphate-buffered saline (PBS) and fixed for 15 min in freshly prepared 4% paraformaldehyde in PBS (Mg^{2+}/Ca^{2+}-free). After fixation, cells were rinsed in PBS and then made permeable with PBS plus 3% bovine serum albumin (BSA), 0.2% Triton X-100 for 10 min. They were rinsed twice and blocked for 10 min with PBS plus 3% BSA (PBA), then incubated with MC-480 supernatant (Developmental Studies Hybridoma Bank, Iowa City, IA), mouse IgM (20 μg/ml), or PBA (no primary control) overnight at 4°C. Monolayers were washed with PBA twice and once with PBA plus 1% Triton X-100, then twice more with PBA for 5 min each. Monolayers were incubated with a biotinylated goat anti-mouse IgM (Vector Laboratories) at a dilution of 1:200 in PBA for 45 min at ambient temperature. Two 5-min washes in PBA were performed, and monolayers were incubated for 30 min at ambient temperature in streptavidin conjugated to FITC (Amersham) at a dilution of 1:150 in PBA. Two 5 min washes in PBA were done, followed by one wash in PBS. Samples were mounted with 70% glycerol in PBS with p-phenylenediamine added to decrease quenching of fluorescence.

ES cell differentiation in vivo

Control and G202 cells (10^6 cells in 0.1 ml PBS) were injected subcutaneously into male nude mice (BALB/c nu/nu). Mice were killed when tumors were 1-2 cm in size (3.5 weeks after injection) and fixed for 6 h in Bouin's fixative and dehydrated through ethanol. Tumors were embedded in plastic, sectioned, and stained with hematoxylin-and-eosin for histological analysis.

Chimeras were generated by injecting G202 or C1 cells into C57BL/6J or (C57BL/6JxCBAJ)F_1 blastocysts. Blastocysts were transferred to CD1 surrogates.

Generation of goosecoid chimeras

C1, G202, G185 or G191 ES cell lines were injected into C57Bl/6 or CD-1 blastocysts and transferred into the uteri of pseudopregnant CD-1 female mice. Embryos were harvested at E14.5 and E18.5.

Glucose phosphate isomerase assay

Tail, brain, heart and liver tissue was removed from embryos delivered by caesarian section (Hogan *et al.*, 1994) at E18.5-19.5, placed in 100 μl of water and freeze-thawed 4 times (dry ice/ethanol bath to warm water). The lysate (1 μl) was loaded onto an equilibrated cellulose acetate plate (Helena) and run for 30-40 min at 180 V in running buffer (50 mM Tris, 375 mM glycine, pH 8.5). Gels were stained with 1 ml of staining solution (0.1 M Tris pH 8, 20 mM $MgCl_2$, 15 mg fructose 6-phosphate, 1 mg methylthiazolium tetrazolium, 0.2 mg phenazine methylsulfate, 1 mg NADP and 10 U glucose-6-phosphate dehydrogenase) mixed with 1 ml warm 1% low melt agarose. The color was developed for 15-30 min in the dark.

Skeletal preparations of chimeras and non-chimeric littermates

Embryos were delivered by caesarian section at E18.5-19.5 (Hogan *et al.*, 1994). Fetuses were fixed in 95% ethanol and stained with alizarin red and alcian blue 8SG according to the method of McLeod (1980).

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