



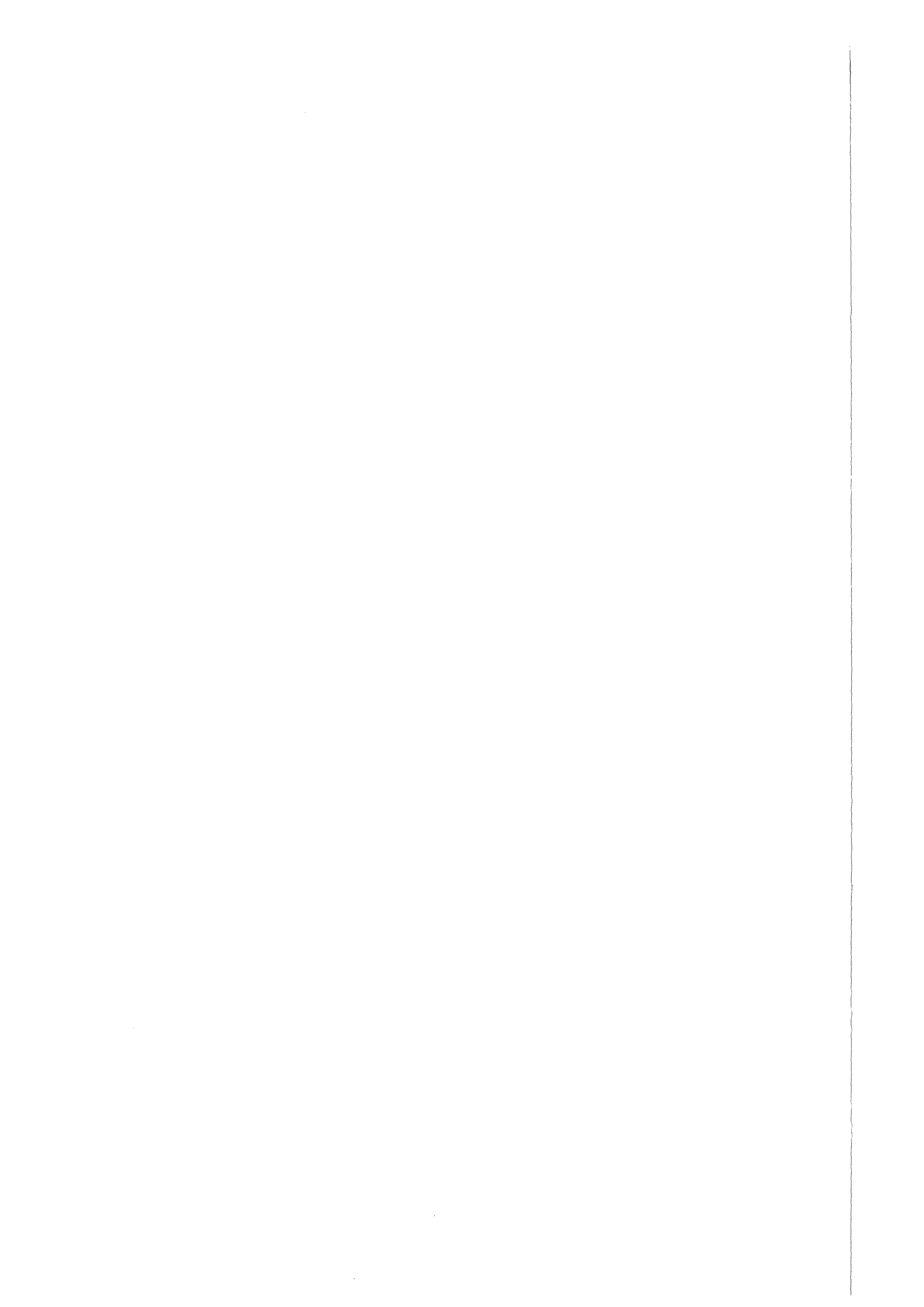
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Technik und Umwelt

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**The homeobox gene *gooseoid*:
Embryonic expression,
loss-of-function phenotype and
regulation by retinoic acid**

Changqi C. Zhu
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Abstract

The homeobox gene *gooseoid* is expressed in the Spemann organizer tissue of gastrulating vertebrate embryos, and in the craniofacial region and appendicular skeleton during organogenesis. The *gooseoid* knockout mutant mouse revealed defects related to the second phase of expression. In this study, new *gooseoid* expression sites in the developing trachea and external genitalia, and in the developing shoulder and hip joint with their associated ligaments and muscles were discovered. *gooseoid* null mutant mice were found to display abnormalities in the forming trachea, appendicular skeleton and genital region related to these sites of gene expression.

Some aspects of the *gooseoid* null phenotype, such as the malformations of the middle ear, bear striking similarities to defects caused by retinoic acid (RA) treatment of embryos, suggesting that *gooseoid* might mediate specific teratogenic RA effects. Such a relation was investigated at two time points in development. Following treatment of mouse embryos *in vivo* at embryonic day (E) 8 + 5 h, *gooseoid* was specifically affected in branchial arches I and II at E10.5. Expression was either reduced to background levels or restricted to the branchial cleft region. This change in *gooseoid* gene expression correlated with a loss of middle ear ossicles and a partial or complete deletion of the tympanic ring, supporting a role for *gooseoid* in executing the RA teratogenic effects.

Analysis at E10.5, a second RA-sensitive time window for mouse embryos, revealed that *gooseoid* was down-regulated 4 h after treatment. However, *gooseoid* expression returned to normal 24 h following treatment. BMP-4, a member of the transforming growth factor β (TGF- β) superfamily, was analysed because BMP-4 was shown to be an antagonist of *gooseoid* in frog gastrula embryos, and because BMP-4 plays a role in bone formation, the primary target of teratogenic RA action. Like *gooseoid*, BMP-4 was repressed by RA 4 h after treatment at E10.5, and expression was normal again after 24 h.

The mechanism of down-regulation of both *gooseoid* and BMP-4 was studied in mouse teratocarcinoma cells, using transient transfections of luciferase reporter gene constructs. A 4.8 kb *gooseoid* promoter fragment was down-regulated by RA in P19 cells. Repression was dependant on the co-transfection of the retinoid X receptor (RXR). RXR α was the strongest isoform in mediating this

effect, followed by RXR β and RXR γ . 9-cis RA was more potent as a ligand compared to all-trans (t) RA. Both RAR and RXR specific ligands were active in repression of *goosecoid*, with the RXR specific compound SR11237 being more efficient. BMP-4 was analyzed in F9 cells which express the gene endogenously. Both tRA and 9-cis RA treatment resulted in a down-regulation of BMP-4 mRNA 4 h after treatment of cell cultures. While tRA, 9-cis RA, and the RAR specific ligand Am80 were able to repress BMP-4 efficiently within 24 h of RA treatment, SR11237 had no effect, suggesting that down-regulation of BMP-4 by RA did not require activation of RXR homodimers.

To further investigate the role of *goosecoid* and BMP-4 in mediating RA teratogenicity, gene expression was analyzed in RXR α null mutant mouse embryos following RA treatment. This mutant was chosen because it was shown to be resistant to RA teratogenic effects specifically in the limbs, and because *goosecoid* and BMP-4 both are expressed in developing limbs and play a role in limb development. While *goosecoid* was equally repressed in wildtype and RXR α mutant embryos, BMP-4 showed remarkable differences. Among three other homeobox genes analyzed in this part of the study, *Msx1* and *Hoxd-11*, like *goosecoid* were unaltered, whereas *Msx2* was not repressed in RXR α null mutant embryos.

In summary this work further establishes the role of *goosecoid* as an important regulator of mouse development during organogenesis stages, in particular in the developing limbs. The data presented show that *goosecoid* and BMP-4 both play a role in mediating the teratogenic effects of RA.

Das homeoboxgen *gooseoid*: Expression in der embryonalen entwicklung, loss-of-function phänotyp und seine regulation durch retinsäure

Zusammenfassung

Das Homeoboxgen *gooseoid* wird im Spemann Organisator während der Gastrulation, in der kraniofazialen Region sowie im appendikularen Skelett während der Organogenese des Wirbeltierembryos exprimiert. Die *gooseoid* knockout Mutante weist Defekte auf, die in Beziehung zur zweiten Phase der *gooseoid* Expression stehen. Diese Arbeit zeigt neue *gooseoid* Expressionsorte wie die sich entwickelnde Trachea, die äußeren Genitalien, die sich entwickelnden Schulter sowie das Hüftgelenk und die damit verbundenen Ligamente und Muskeln. Bei den *gooseoid* Nullmutanten-Mäusen konnten entsprechend den Expressionsorten Abnormalitäten in der sich bildenden Trachea, im appendikularen Skelett und in der Genitalregion aufgezeigt werden.

Einige Aspekte des *gooseoid* Nullphänotyps, wie die Mißbildung des Mittelohrs, zeigen auffallende Ähnlichkeiten mit Defekten, wie sie bei mit Retinsäure (RA) behandelten Embryos auftreten. Diese Tatsache deutet daraufhin, daß *gooseoid* bestimmte teratogene Effekte der Retinsäure vermittelt. Dieser Zusammenhang wurde an zwei Zeitpunkten in der Entwicklung untersucht.

Nach *in vivo* Behandlung von acht Tage und fünf Stunden alten Mausembryos war die *gooseoid* Expression spezifisch in den Kiemenbögen I und II am Tag 10,5 *p.c.* (*post coitum*) betroffen. Die Expression war entweder nicht mehr nachweisbar oder auf die Kiemenspaltenregion beschränkt. Die Änderung der *gooseoid* Expression korreliert mit dem Verlust der Mittelohrknöchelchen und dem teilweisen oder kompletten Verlust des tympanischen Ringes. Dies läßt darauf schließen daß *gooseoid* RA teratogene Effekte vermittelt.

Analysen von 10,5 Tage alten Embryos (E10,5), einem zweiten RA sensitiven Zeitfenster in der Mausembryogenese, zeigten, daß die *gooseoid* Expression vier Stunden nach RA-Behandlung herabreguliert wurde und 24 Stunden nach RA-Behandlung zum Normalwert zurückkehrte.

Weiterhin wurde BMP-4, ein Mitglied der transformierenden Wachstumsfaktoren β (TGF- β) Superfamilie, analysiert. In der Froschgastrula wurde für BMP-4 gezeigt, daß es antagonistisch zu *gooseoid* wirkt. Außerdem spielt BMP-4 eine Rolle bei der Knochenentwicklung, dem primären Ziel teratogener RA-Wirkung. Die Versuche zeigten, daß BMP-4 wie *gooseoid* vier Stunden nach RA-Behandlung am Tag 10,5 reprimiert wurde, nach 24 Stunden war die normale Expression wieder vorhanden. Zur Klärung des Mechanismus der Herunterregulierung von *gooseoid* und BMP-4 wurde mittels transienter Transfektionen von Luziferasereporter-Konstrukten in Mauseratokarzinomzellen untersucht. Ein 4,8 kb *gooseoid* Promotorfragment wurde durch RA in P19 Zellen herunterreguliert. Diese Repression war abhängig von der Kotransfektion von Retinoid-X-Rezeptor (RXR). Der Effekt wurde am stärksten durch die Isoform RXR α vermittelt, gefolgt von RXR β und RXR γ . Die 9-cis RA war der stärker aktivierende Ligand im Vergleich zum all-trans (t) Isomeren. Beide für RAR und RXR spezifische Liganden konnten *gooseoid* reprimieren, wobei die RXR spezifische Komponente SR11237 *gooseoid* effizienter reprimierte. BMP-4 wurde in F9-Zellen analysiert, welche das Gen endogen exprimieren. tRA und 9-cis RA Behandlung resultierte in Herunterregulierung von BMP-4 mRNA vier Stunden nach Behandlung der Zellen. Während tRA, 9-cis RA und der RA spezifische Ligand Am80 Bmp-4 effizient innerhalb 24 Stunden nach RA Behandlung reprimierten, hatte SR11237 keinen Effekt. Daraus läßt sich folgern, daß die Herunterregulierung von BMP-4 durch RA keine Aktivierung von RXR Homodimeren benötigt.

Zur weiteren Untersuchung der Rolle von *gooseoid* und BMP-4 bei der Vermittlung der RA Teratogenität wurde die Genexpression von RXR α Nullmutanten Mausembryos nach RA Behandlung analysiert. Diese Mutante wurde aus zwei Gründen gewählt, erstens ist sie resistent gegen RA vermittelte teratogene Effekte speziell in den Extremitäten und zweitens werden *gooseoid* und BMP-4 in den sich entwickelten Extremitäten exprimiert. Während *gooseoid* gleichermaßen im Wildtyp und in RXR α Mutanten Embryos reprimiert wurde, zeigte BMP-4 bemerkenswerte Unterschiede. Unter drei anderen analysierten Homeoboxgenen in diesem Teil der Arbeit war die Expression von *Msx1* und *Hoxd-11* wie die von *gooseoid* unverändert, wohingegen *Msx2* in RXR α Null mutanten Embryos nicht reprimiert wurde.

Die Arbeit bestätigt zusammenfassend die Rolle von *gooseoid* als wichtigen Regulator in der Mausentwicklung während der Organogenesestadien insbesondere in den sich entwickelnden Extremitäten. Die präsentierten Daten zeigen, daß *gooseoid* und BMP-4 eine Rolle bei der Vermittlung teratogener Retinsäureeffekte spielt.

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ACKNOWLEDGEMENT

1. Introduction

The development of an animal from the fertilized egg has been a source of wonder throughout human history (Gilbert, 1997). Many scientists strived to uncover the mechanisms behind embryonic development. In the last two decades, with the advent of molecular techniques, such as cloning of developmental master genes and their functional analysis by gene targeting or transgenic approaches, it became possible to unravel molecular mechanisms underlying development. Among these genes is a large family called homeobox genes. Individual genes are often conserved from invertebrates to vertebrates (from *Drosophila* to humans). Homeobox genes encode transcription factors which can regulate complex genetic programs. They bind to DNA through the homeodomain, a 60 amino acid helix-loop-helix motif.

1.1 The early development of vertebrate embryos: Spemann organizer and gastrulation

Early development has been particularly well studied in amphibian embryos. Fertilization initiates a cytoplasmic rotation. This so-called cortical rotation is believed to locally activate substances that are required for development on the prospective dorsal side of the embryo (Gerhart et al., 1989). Subsequently, the zygote undergoes a series of synchronous cleavage divisions leading to the formation of the blastula (Gilbert, 1997). The induction of mesoderm begins at about the 32-cell stage (Jones and Woodland, 1987). Prior to gastrulation, the embryo consists of two primary germ layers: the pigmented animal hemisphere composed of prospective ectoderm, and the vegetal hemisphere consisting of prospective endoderm (Christian and Moon, 1993). At the onset of gastrulation, the third germ layer, the mesoderm, differentiates from ectoderm cells which reside in the equatorial (or marginal zone) region of the embryo in response to inductive signals provided by underlying endodermal cells (Nieuwkoop, 1969). During gastrulation, the cells of the marginal zone invaginate into the embryo through a slit-like opening forming on the dorsal site: the so-called dorsal lip. Near the end of gastrulation, dorsal mesoderm cells induce the overlying ectoderm to form neural tissue.

Embryos then enter neuralation. The mechanisms underlying these inductive events were studied by tissue explant and grafting experiments in amphibian embryos. There are two well-defined centers in the early amphibian embryos: the Nieuwkoop center and the Spemann organizer. Signals emerging from the Nieuwkoop center induce formation of mesoderm tissue in the *Xenopus* dorsal lip, i.e. the induction of formation of the Spemann organizer. Transplantation of the dorsal lip, the region where gastrulation starts, into the ventral side of a host amphibian embryo induced the formation of a secondary body axis (Spemann, 1924). The transplanted tissue, whose normal fate was to become head mesoderm and notochord, was able to recruit cells from the ventral side of the embryo and organize them into axial structures such as somites and neural tube. Therefore, Spemann named the dorsal lip the organizer.

The Spemann organizer has three major properties: (1) its progeny forms the embryonic axial structures; (2) it recruits non-organizer cells to form axial or paraxial structures; and (3) it induces neural tissue.

Structures functionally equivalent to the amphibian organizer have been identified in other vertebrates on the basis of their ability to produce a secondary axis when heterotopically grafted in young gastrulae. The organizer structure in the mouse is the so-called node, found at the anterior tip of the primitive streak (Blum et al., 1992; Beddington, 1994). In chick, it is Hensen's node, located at the anterior end of the primitive streak of gastrulating embryos and partially derived from Koller's sickle (Izpisua-Belmonte et al., 1993). In fish, the shield, a dorsal marginal structure, resembles the dorsal blastopore lip of *Xenopus* (Shih and Fraser, 1996).

The past several years have witnessed a very productive search for molecules that are able to mimic Spemann organizer activity, and for molecules active in dorsal and ventral mesoderm specification. The secreted signaling molecule Bone Morphogenetic Protein 4 (BMP-4), a member of the transforming growth factor β (TGF β) superfamily, specifies ventral mesoderm in *Xenopus* embryos. The dorsal mesoderm is characterized by the molecules that antagonize BMP-4 activity, such as *chordin*, *noggin* and *follistatin*.

The secreted molecule *chordin* was cloned from a *Xenopus* dorsal lip library by differential screening (Sasai et al., 1994). This molecule is a potent dorsalizing factor that can induce twinned axis and can completely rescue axial development in embryos ventralized by UV radiation, a treatment that often abolishes the cortical

rotation. Its expression in the Spemann organizer can be activated by the organizer-specific homeobox gene *gooseoid*. Induction of *chordin* by activin, a TGF β -like signaling molecule active in mesoderm induction, requires *de novo* protein synthesis (Sasai et al., 1995).

Two other organizer-specific secreted molecules, *noggin* and *folliculin* also have very strong neuralizing activity (Lamb et al., 1993; Hemmati-Brivanlou et al., 1994). Each of these two secreted molecules can directly neuralize ectoderm explants in the absence of detectable mesoderm. *Noggin* protein itself mimics the Spemann organizer in dorsalizing *Xenopus* mesoderm (Smith et al., 1993), respecifying lateral mesoderm tissues from lateral (blood mesoderm) to more dorsal fates (muscle, heart, pronephros).

The dorsalizing and neuralizing activities of these three secreted molecules are obtained by antagonizing the ventral signaling molecule BMP-4 which instructs ectoderm to become epidermis and ventral mesoderm (Zimmerman et al., 1996; Sasai et al., 1995; Fainsod et al., 1997). *Chordin*, *noggin* and *folliculin* bind BMP-4 with high affinity and prevent BMP-4 from binding to its cognate cell-surface receptors (Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997). It appears that the function of the signaling molecules and transcription factors from Spemann organizer is to inhibit the ventral signal BMP-4. Ectoderm cells become epidermis other than neural tissue unless BMP-4 activities are inhibited by signaling molecules from the Spemann organizer.

The inhibition of BMP-4 activity on the dorsal side of the embryo is guaranteed through a genetic network. Activities emanating from the Nieuwkoop center induce the formation of the Spemann organizer. There the expression of *gooseoid* leads to the activation of *chordin*, which in turn antagonizes BMP-4, in concert with *folliculin* and *noggin*.

1.2 The homeobox gene *gooseoid*

The homeobox gene *gooseoid* was the first gene cloned from a cDNA library derived from the dorsal lip tissue of *Xenopus* embryos (Blumberg et al., 1991). It was found to be able to mimic Spemann organizer activity in inducing a secondary body axis when *gooseoid* mRNA was misexpressed in the ventral side of early *Xenopus* embryo (Cho et al., 1992). After the *Xenopus gooseoid* gene was

identified, homologous genes have been isolated from mouse (Blum et al., 1992), chick (Izpisua-Belmonte et al., 1993), zebrafish (Stachel et al., 1993 and Schulte-Merker et al., 1994) and human (Blum et al., 1994). Since its identification, this gene has been extensively studied, from expression pattern to gain- and loss-of-function experiments in different species, from early gastrulation to late organogenesis, the regulation by growth factors and the function of the protein itself in regulation of target genes.

The expression pattern in early gastrula embryos in all vertebrates suggested a role for *gooseoid* in gastrulation. Expression data in *Xenopus* showed that *gooseoid* mRNA was located in the deep layer of the upper lip of the dorsal blastopore (Cho et al., 1991). This expression persists in the prechordal plate, a derivative of organizer tissue (Steinbeisser and De Robertis, 1993; Artinger et al., 1997). The fate of these cells is to form mostly prechordal (head) mesoderm and notochord in later development (Keller, 1976; Slack, 1991).

In mouse gastrula embryos, In addition, *gooseoid* was found to be expressed in the early primitive streak mesoderm (Blum et al., 1992). *gooseoid* was expressed in the anterior mesoderm of gastrula embryos in a number of other vertebrate species (Izpisua-Belmonte et al., 1993; Stachel et al., 1993; Schulte-Merker et al., 1994; Filosa et al., 1997). Microdissected mouse *gooseoid*-expressing cells from gastrulation embryos could elicit a new body axis when transplanted to the blastocyst of *Xenopus* early gastrula embryos (Blum et al., 1992). The results from these gain-of-function studies suggest that the *gooseoid* homeodomain protein plays a central role in executing Spemann's organizer phenomenon.

However, *gooseoid* null mutant mice did not have a gastrulation phenotype (Rivera-Perez et al., 1995; Yamada et al., 1995). Instead, these mice had a craniofacial phenotype related to the second phase of *gooseoid* expression (Gaunt et al., 1993) at organogenesis stage. Those phenotypes include malformation of middle ear bones, loss of the tympanic ring and abnormalities of nasal cavity and tongue. Lack of a gastrulation phenotype could be explained by compensation through other closely related genes. Two additional *gooseoid* related genes have indeed been isolated recently, *GSX* in chick (Lemaire et al., 1997) and *gscl* in mouse (Galili et al., 1997; Schweickert and Blum, unpublished). It is not known yet if these two genes play any role in gastrulation. Recently, it was found that *gooseoid*

and another organizer-specific gene, the winged-helix gene HNF-3 β , genetically interact in regulation of mouse gastrulation (Filosa et al., 1997). Removal of one copy of *HNF-3 β* in a *gooseoid* null mutant background results in early phenotypes, severe ventralization of the brain including forebrain in day E8.75 embryos. These defects were accompanied by a loss or reduction of the expression of the midbrain organizer molecule fibroblast growth factor 8 (*FGF-8*), the signaling molecule *shh* important for notochord development; and the axon guidance molecule *netrin*. These phenotypes argue that *gooseoid* together with other genes is required for axial patterning during gastrulation.

1.3 BMP-4 in embryogenesis

In the early development of vertebrate embryos *gooseoid* and BMP-4 mark dorsal and ventral signaling centers respectively. Interestingly, they have been shown to antagonize each other in *Xenopus* early gastrula (Fainsod et al., 1994). As is the case for *gooseoid*, BMP-4 also plays important roles in the normal development of embryos at organogenesis stage (Hogan, 1996).

In mouse embryogenesis BMP-4 was demonstrated to be required for formation of mesoderm (Winnier et al., 1995). At organogenesis stage, BMP-4 is required for the normal development of a number of organs. Overexpression of BMP-4 in the chick limb bud leads to a dramatic increase in the volume of cartilage elements, altered cartilage shapes and joint fusions (Duprez et al., 1996). A knock-out of the BMP-4 antagonist *noggin* from mouse embryos results in cartilage hyperplasia of skeletons and joint fusions due to excess BMP activities (Brunet et al., 1998). BMP-4 is also an important signaling molecule required for epithelial-mesenchymal interactions and proper development of many organs, such as teeth (Vainio et al., 1993), hair follicles (Blessing et al., 1993), somites (McMahon et al., 1998) and pituitary (Treier et al., 1998; Ericson et al., 1998).

BMP-4 signaling is also required for interdigital apoptosis and scale formation (Zou et al., 1996; Ganan et al., 1996) and for apoptosis in the rhombencephalic neural crest (Graham et al., 1994).

BMP-4 signaling initiates from the cell surface by interacting with two distinct serine/threonine kinase receptors (Massague and Weis-Garcia 1996; ten Dijke et al., 1996). Ligand binding induces the formation of a complex in which the type II

receptor phosphorylates and activates the type I receptor; this protein then propagates the signal by phosphorylating a family of signal transducers, the Smad proteins (Massague et al. 1997). There are three types of Smad proteins. Smad1 and its closely related members Smad5 and Smad 9 mediate BMP signaling (Graff et al., 1996; Hoodless et al., 1996; Lechleider et al., 1996; Liu et al., 1996; Thomsen 1996; Yingling et al., 1996; Kretschmar et al., 1997; Suzuki et al., 1997; Watanabe et al., 1997). These Smads get phosphorylated on serine residues at their carboxy-terminal ends (SSXS motif) through the action of specific type I receptors (Macias-Silva et al., 1996; Kretschmar et al., 1997), and their phosphorylation leads to formation of a heteromeric complex with the second Smad class, which includes Smad4 in vertebrates (Lagna et al., 1996; Zhang et al., 1997). The tumor suppressor Smad4/DPC4 (Hahn et al., 1996) acts as a shared partner for both BMP-specific and TGF- β /activin-specific Smads (Lagna et al., 1996; Zhang et al., 1997) and plays an essential role as a transcriptional activator in the nucleus (Liu et al., 1997). Recently, a third class of Smads has been reported, whose members act as antagonists of these signaling pathways (Hayashi et al., 1997; Imamura et al., 1997; Nakao et al., 1997; Topper et al., 1997; Tsuneizumi et al., 1997).

1.4 Retinoic acid: a morphogen in development

RA, a derivative of vitamin A, plays a pivotal role in homeostasis and vertebrate embryogenesis. Pharmacological doses of both RA and synthetic derivatives of RA have been used for the treatment of several diseases of the skin (Wieder and Lowe, 1995) and a variety of cancers, including promyelocytic leukemia, breast cancer, carcinomas of the respiratory tract, and ovarian cancer (Hill and Grubbs, 1992; Hong and Itri, 1994). However, exogenous RA is teratogenic to developing embryos.

1.4.1 Retinoic acid and development

Accumulating evidence suggests that RA is a natural morphogen in vertebrate embryonic development. RA in the chick limb was directly measured (Thaller and Eichele, 1987). Highly sensitive reporter genes controlled by RA-inducible promoters have been used to assay indirectly for active retinoids. Mice

transgenic for these reporter genes reveal the presence of retinoids in the nervous system and limbs during embryogenesis (Hogan et al., 1992; Balkan et al., 1992; Rossant et al., 1991). *In vitro* assays have found active retinoids in early mouse embryos, in the posterior of the chick embryo (Chen et al., 1992) and in explants from the chick nervous system (Wagner et al., 1992).

More than forty years ago it was noted that fetuses from rat mothers reared on vitamin A-deficient (VAD) diets exhibit severe congenital malformations known as the fetal VAD syndrome (Wilson and Warkany, 1948, 1949; Warkany et al., 1948; Wilson et al., 1953). These malformations include abnormalities of the eyes, respiratory tract, heart and great vessels, urogenital system and diaphragm, and cleft palate (Hale, 1933). Addition of retinol to the diet of VAD dams during pregnancy reversed nearly all of those malformations, a clear demonstration that vitamin A is critical for normal development.

The cloning and study of retinoic acid receptors (RAR) and retinoic X receptors (RXR) greatly facilitated our understanding of the biological action of RA (Chambon, 1994; Mangelsdorf et al., 1994). RAR and RXR belong to the nuclear hormone receptor superfamily. These receptors act as ligand dependent transcription factors by binding to RA response elements (RAREs) on DNA. tRA binds to only RARs, whereas 9-cis RA can bind both RAR and RXR (Leid et al., 1992; Mangelsdorf et al., 1994). RAR and RXR form heterodimers on a RARE, which is an AGGTCA direct repeat spaced by two to three base pairs (Mangelsdorf and Evans, 1995). On direct repeats spaced by one base pair (DR1), RXR can bind as both a homodimer and as heterodimer with RAR. RXR is also an auxillary partner for other nuclear receptors, such as the thyroid hormone and vitamin D receptors (Glass, 1994).

The highly diverse effects of RA signaling would be accounted for by the multiplicity of functionally distinct receptors (Leid et al., 1992; Chambon, 1994). This possibility has been tested *in vivo* by generating null mouse mutants for the various receptors (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Mendelsohn et al., 1994). Single receptor mutant mice did not recapitulate the fetal VAD syndrome, suggesting a high degree of functional redundancy amongst the various RARs, but compound RAR mutant mice exhibited a large number of malformations (Lohnes et al., 1994), including abnormalities characteristic of the VAD syndrome. These evidences demonstrated that RA is the retinoid signaling molecule used during

development and that RARs are indeed transducers of this signal *in vivo*. A synergistic effect was observed in compound mutant mice bearing one null allele of RXR and one null allele of RAR, suggesting that RXR/RAR heterodimers are the functional units transducing the retinoid signal for a large number of RA-dependent processes. The same study indicated that RXR α is the main RXR implicated in the developmental functions of RARs (Kastner et al., 1997).

1.4.2 Retinoic acid in anterior-posterior axis patterning

After the initiation of gastrulation, the subdivision of the embryo along the anterior-posterior axis begins. Along the anterior-posterior body axis in early development, the homeobox gene *otx2* defines the most anterior domain of the body, i.e. forebrain, and Hox genes pattern the body axis posterior to the domain of *otx2*. As with the homologous *Drosophila* genes, the vertebrate Hox genes are expressed in a manner which is colinear with the organization of these genes in chromosomal clusters. The closer to the 3' of the cluster the genes are localized, the earlier and more anterior they are expressed along the anterior-posterior body axis. Loss-of-function mutations of Hox genes are often associated with anterior transformations of vertebrae, i.e., one of the posterior vertebra adopts the identity of a more anterior one. Surprisingly, the alterations to the axial skeletons of RAR mutant mice are homeotic and are similar to certain Hox gene loss-of-function phenotypes (Lohnes et al, 1994). The axial skeletons of RAR γ homozygotes and all RAR double-mutant combinations reported ($\alpha1\gamma$, $\alpha1\gamma\alpha2+/-$, $\alpha\gamma$, $\beta2\gamma$ and $\alpha\beta2$) exhibit homeotic transformations. The transformations are almost exclusively anterior transformations and are restricted to the cervical region. In contrast, overexpression of Hox genes led to transformations to posterior structures (Krumlauf, 1993). Treatment of embryos with excess RA also resulted in posterior transformation, i.e., one of the anterior vertebra adopts the feature of a more posterior one. All of these data are consistent with the notion that embryonic RA positively regulates the expression of Hox genes in the posterior of the embryo.

The first direct link between RA and the regulation of Hox genes came from investigations of the differentiation of teratocarcinoma cells (Colberg-Poley et al., 1985; Breier et al., 1986). Studies with the human teratocarcinoma cell line NT2/D1 demonstrated that many genes in all four Hox clusters were RA-inducible (Simeone

et al., 1990; Simeone et al., 1991) and that genes near the 3' end of the cluster are induced extremely rapidly by low concentrations of RA. With increasing distance from the 3' end of the cluster, the time and concentration of RA required for equivalent induction of a gene increases. Regulation of some Hox genes is direct because no new protein synthesis is required (Simeone et al., 1991). In addition, RAREs were found downstream of the murine *Hoxa-1* and human *HoxA-1* genes and the murine and chick *Hoxb-1* genes (Langston and Gudas, 1992) and *Hoxd-4* (Moroni et al., 1993; Pöpperl and Featherstone, 1993). Using lacZ reporter genes in transgenic mice, mutational analysis of specific RAREs 3' of both *Hoxa-1* and *hoxb-1* has suggested that these elements are involved in regulating aspects of early neural expression. Germline mutations in the *Hoxa-1* 3' RARE resulted in lower levels of *Hoxa-1* expression and a temporal delay in establishing its normal anterior boundary (Studer et al., 1998; Gavalas et al., 1998), strengthening the hypothesis that RA plays an important role in controlling early Hox expression *in vivo* and that it is involved in the patterning of the anterior and posterior body axes.

1.4.3 RA and limb patterning

A role for RA in normal limb development has been well documented. RA has been implicated in limb patterning by experiments in which local application of RA leads to pattern duplications in the limb (Helms, 1994). The zone of polarizing activity (ZPA) in the posterior limb bud is able to pattern the posterior to anterior axis of the limb. Grafting of a ZPA to the anterior of another limb bud can result in duplicated digits. Application of RA can mimic ZPA activity. Recently, *shh* was found to be the morphogen in the ZPA. Application of RA can induce the expression of *shh* (Helms, 1994). Direct evidence for a function of RA in limb patterning came from $RAR_{\alpha\gamma}$ double-mutant mice which showed defects in forelimb development, the majority of which involved the loss of anterior pattern elements, particularly the first digit, prepollex and radius (Mendelsohn et al., 1994).

1.4.4 Teratogenic effect of RA on embryonic development

Excess RA is detrimental to the development of vertebrate embryos. Embryos exposed to excess RA throughout the period of embryogenesis (i.e. from early

postimplantation stages to the end of organogenesis) show abnormalities of many organ systems (Shenefelt, 1972). The teratogenic effect of RA on embryos is stage and dosage dependent. This reflects the effect of RA on different tissues and different developmental genes at different developmental stages of embryogenesis.

Application of exogenous RA to embryos at early developmental stage down-regulates *otx2* in forebrain and midbrain, causing a truncation of the head (Simeone et al., 1995), and it shifts Hox gene expression to a more anterior boundary causing lack of identity of hindbrain and posterior transformation of cervical vertebra.

One of the main targets of RA teratogenesis is bone development. For example, RA affects the development of the craniofacial skeleton, axial skeleton and limb long bones.

At the cellular level, excess RA either inhibits neural crest cell migration or alters its migration pathway, depending on the embryonic stage when excess RA is applied (Thorogood et al., 1982; Webster et al., 1986; Pratt et al., 1987; Lee et al., 1995; Gale et al., 1996; Mallo, 1997).

Excess RA can also trigger apoptosis, i.e., programmed cell death (Piedrafita and Pfahl, 1997; Li et al., 1998). Under normal conditions, apoptosis is a physiological phenomenon, whereas inhibition of apoptosis will lead to tumor formation, and enhancement of apoptosis will result in teratogenesis in embryos. The apoptotic effect of RA may explain some of the teratogenic effects resulting from excess RA application.

The primary effect of excess RA is alteration of normal expression of a number of transcription factors and matrix proteins. Four known mechanisms have been described to explain how excess RA can alter the expression of genes.

First, as discussed before, excess RA can ectopically up-regulate target genes which contain RAREs in their regulatory sequence, like *Hoxa-1* and *Hoxb-1*.

Second, excess RA down-regulates a number of genes by squelching AP-1 transcription factor binding to AP-1 response elements. Those genes include stromelysin (Nicholson et al., 1990) and collagenase (Schüle et al., 1991). The transcription factor AP-1 is composed of Fos and Jun proteins (Bohmann et al., 1987; Angel et al., 1988; Bos et al., 1988). Jun and Fos form heterodimers and bind to an AP1 binding site (TGAGTCA) to positively regulate expression of genes involved in cell division and proliferation (Curran and Franza, 1988; Hart et al.,

1989; Vogt and Bos, 1989). This mechanism may explain why RA can limit cell growth, and possibly, malignant progression (Schüle et al., 1991).

Third, excess RA in certain circumstances can lead to degradation of the general transcription factor Sp1 (Piedrafita and Pfahl, 1997). The RA-induced degradation of Sp1 may have profound effects on the basal transcription of many genes. The promoter occupancy of Oct4 by Sp1 was lost after treatment of cells with RA, and the loss of the promoter occupancy by Sp1 correlates with the down-regulation of Oct4 by RA (Minucci et al., 1996).

Fourth, repression of Oct3/4 by RA is one of the examples that involves the up-regulation of the trans-repressors COUP-TFI and COUP-TFII. These two orphan nuclear receptors can compete with RAR and RXR for the binding to RARE with high affinity (Ben-Shushan et al., 1995).

1.5 Aim of the study

The main goal of this work was to investigate the possible correlation between the *gooseoid* gene function and RA teratogenicity because the *gooseoid* null mutant mice bear some resemblance to RA teratogenic effect.

As a starting point, *gooseoid* expression at organogenesis stage was re-investigated in detail between E10.5 and E16.5. In the course of this analysis, new expression sites were discovered in the developing trachea, shoulder and hip joints and adductor muscles, and genitalia. Re-evaluation of the *gooseoid* null mutant mouse led to the discovery of phenotypes related to the expression sites in trachea, proximal limb and adductor muscles.

The possibility of *gooseoid* as a mediator of RA teratogenicity was analysed by three types of experiments. First, mouse embryos were treated at E8 + 5 h with tRA. A correlation of alteration of *gooseoid* gene expression and loss of middle ear bones were found. Second, the immediate early effect of RA on *gooseoid* and BMP-4 expression in mouse embryos was studied at another RA sensitive time window, E10.5. *gooseoid* was down-regulated 4 h after RA treatment of mouse embryos. This down-regulation was transient since the expression pattern returned to normal 24 h following RA treatment. The mechanism of the down-regulation of *gooseoid* by RA was studied in P19 mouse teratocarcinoma cells with reporter gene assay. A 4.8 kb *gooseoid* promoter-luciferase reporter gene was down-

regulated by RA if RXR was co-transfected. Third, alteration of *goosecoid* expression was investigated in $RXR\alpha^{+/-}$ and $RXR\alpha^{-/-}$ mouse embryos because $RXR\alpha^{-/-}$ mice were resistant to RA teratogenic effect on developing limbs compared with $RXR\alpha^{+/+}$ and $RXR\alpha^{+/-}$ mouse embryos.

Since *goosecoid* and BMP-4 antagonize each other in early gastrula frog embryo, and because of the role of BMP-4 in bone formation, alteration of BMP-4 expression by RA was analysed in both E10.5 mouse embryos and F9 mouse teratocarcinoma cells. BMP-4 was down-regulated 4 h following tRA treatment of E10.5 mouse embryos. 24 h after tRA treatment BMP-4 expression was normal again. The mechanisms behind the down-regulation was studied in F9 cells with RAR and RXR specific ligands. The effect of RA on the expression of BMP-4 together with other three homeobox genes *Msx1*, *Msx2* and *hoxd-11* was also analysed in $RXR\alpha^{+/-}$ and $RXR\alpha^{-/-}$ mouse embryos.

2. Results

2.1 Part I. The homeobox gene *gooseoid*: descriptive and functional analysis during mouse organogenesis

In order to unravel the role of *gooseoid* in murine organogenesis, the expression pattern of *gooseoid* was re-evaluated during mid embryogenesis. Two new *gooseoid* expression sites were identified in the developing trachea and external genitalia. In addition, the expression pattern in the appendicular skeleton was studied in detail. *gooseoid* expression was found in the shoulder and hip joint region, and in several ligaments and adductor muscles. In collaboration with Gen Yamada (Kurume University, Japan), additional phenotypic abnormalities associated with this expression profile were found in *gooseoid* homozygous mutant mice (Zhu et al., 1998).

2.1.1 Expression of *gooseoid* in the region of the developing larynx and trachea

Fig. 1 shows the expression of *gooseoid* at E15.5 in the region of the larynx and the trachea by radioactive *in situ* hybridization. A clear signal was found around the thyroid, cricoid and tracheal cartilages. *gooseoid* mRNA was confined to the mesenchyme surrounding the condensing cartilages, and the signal was notably stronger on the ventral side. The larynx and the trachea are derived from the hypobranchial groove, i.e. the inner branchial arch grooves. In human embryos, the laryngeal cartilages are derived from the fourth and sixth branchial arch mesoderm (McLachlan, 1994). Expression of *gooseoid* at E10.5 to E12.5 is confined to arch I and II (Gaunt et al., 1993). In the mouse arch IV is very small, and arch VI cannot be distinguished. It is therefore not clear if the pattern at E15.5, depicted in Fig. 1, represents a continuation of an earlier expression of *gooseoid*, as is the case for arch I and II derived structures such as the tongue, mandible and malleus (Gaunt et al., 1993).

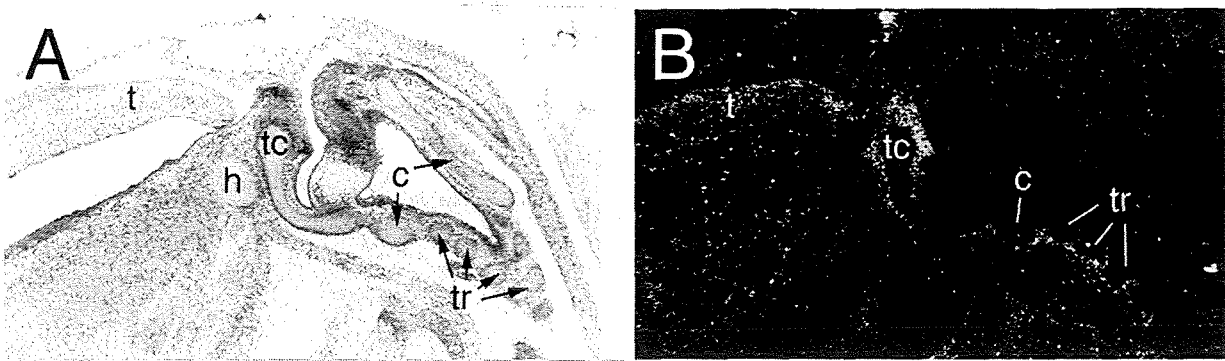


Fig. 1. *gooseoid* is expressed in the area of the developing thyroid, cricoid, and tracheal cartilage. Sagittal section of a E10.5 mouse embryo. **A**, brightfield; **B**, darkfield. c: cricoid cartilage; h: hyoid cartilage; t: tongue; tc: thyroid cartilage; tr: tracheal cartilage.

2.1.2 The submucosal layer of the tunica mucosa is deleted in the trachea of *gooseoid* mutant mice

In order to investigate if a lack of *gooseoid* expression in the developing larynx and trachea in knockout mice resulted in developmental defects of these structures, I performed a skeletal analysis of tracheal cartilage from both wild-type and *gooseoid* null mutant mouse embryos. Genotyped newborn mice were stained with alcian blue and alizarin red in order to visualize bone and cartilage, respectively, and stained tracheae were dissected. The trachea, from larynx to branchi, was analysed for developmental defects in *gooseoid* null mutant embryos. The trachea consists of a framework of incomplete rings of hyaline cartilage. As shown in Fig. 2, the thyroid, cricoid and trachea cartilages from *gooseoid* null mutant E17.5 mouse embryos (Fig. 2 B) were present, and they displayed normal morphology of tracheal cartilage rings compared with wildtype embryos (Fig. 2 A). The bifurcation of trachea cartilage seen in the mutant cartilage (Fig. 2 B) could also be observed in some wild-type tracheae (not shown), and can therefore not be attributed to the *gooseoid* mutation. A notable difference between the tracheal cartilages of wildtype and *gooseoid* null mutant mice was that tracheal cartilages from *gooseoid* null mutant mice were thinner than those of wild-type embryos (Fig. 2).

Histologic analysis of tracheae was carried out in collaboration with Gen Yamada. The normal tracheal cartilages are united by fibrous tissue and smooth muscle, and lined by mucosa on the luminal side. The tunica mucosa, which is

continuous with the larynx above and the interpulmonary bronchi below, is a layer of pseudostratified columnar epithelium interspersed with goblet cells (Fig. 3). Longitudinal elastic fibres separate the epithelium from the underlying submucosa of loose connective tissue, containing blood vessels, nerves, lymphoid nodules and the tracheal seromucous glands. External to the submucosa are the perichondrium and cartilage (Williams, 1995). In *gooseoid* null mutant tracheae, the submucous layer was not present (Zhu et al., 1998). The epithelium, which was thinner than the epithelium of wild-type embryos, was directly bordering the hyaline cartilage. Since *gooseoid* was not expressed at the site of the developing submucosa during organogenesis, the absence of this layer in mutant mice should represent a non-cell autonomous effect of *gooseoid* gene function (s. Discussion).

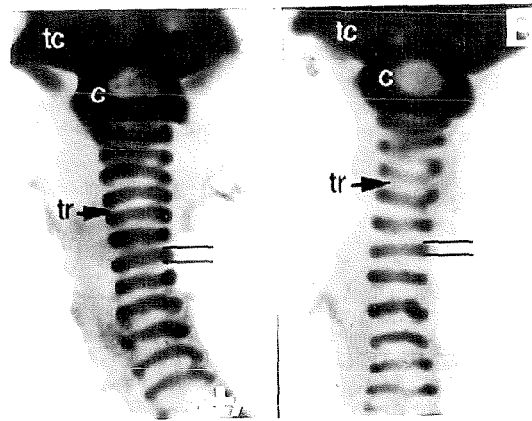


Fig. 2. Thyroid, cricoid, and tracheal cartilages of wild-type and *gooseoid* mutant mouse embryos. A: cartilage from wild-type E17.5 mouse embryo; B: cartilage from E17.5 *gooseoid* null mutant mouse embryo. th: thyroid cartilage; c: cricoid cartilage; tr: tracheal cartilage.

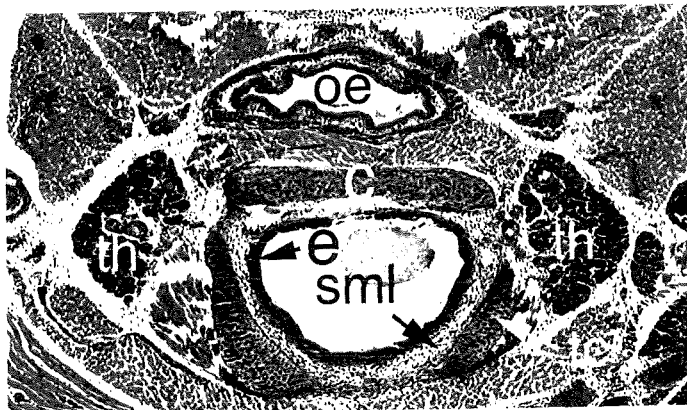


Fig. 3. Normal structure of trachea from wild-type new-born mouse (transverse section). e: ciliated columnar epithelium of tunica mucosa; oe: oesophagus; sml: submucous layer of tunia mucosa; tc: tracheal cartilage; th: thyroid gland.

2.1.3 Expression of *gooseoid* in the shoulder and hip joint and in the appendicular skeleton

In the initial report of the *gooseoid* null mutant mouse, no phenotype related to the expression in the proximal limb bud at E10.5 and E12.5 was described (Yamada et al., 1995; Rivera-Perez et al., 1995). In order to be able to analyze the mutant more specifically I re-investigated the expression pattern of *gooseoid* in wild type mouse embryos from E13.5 to E15.5. Fig. 4 A, B shows expression at E13.5 in the shoulder joint and in the ligament attached to the humerus (asteriks in panel A). At E14.5 *gooseoid* was expressed in the hip joint region between the head of the femur and the cup-shaped acetabulum of the innominate bone (Fig. 4 C, D), as well as in the perichondrium surrounding the cartilage primordia of the iliac bone (Fig. 4 C, D), pubic bone and - less pronounced - the ischial bone (Fig. 4 E, F). *gooseoid* mRNA was abundant in ligaments attached to the femur (asteriks in Fig. 4 E, F) and in a forming adductor muscle initiating at the pubic bone (Fig. 4 E, F). Within the developing hindlimb, *gooseoid* was expressed in the perichondrium and mesenchyme surrounding the tibia and fibula at E13.5 and E14.5 (Fig. 4 E-H). The discrete expression sites in the pelvic region are most likely the continuation and segregation of the early expression of *gooseoid* in the proximal limb bud. The expression pattern of *gooseoid* in those ligaments and adductor muscles suggests that *gooseoid* may play a role in the patterning and formation of those tissues.

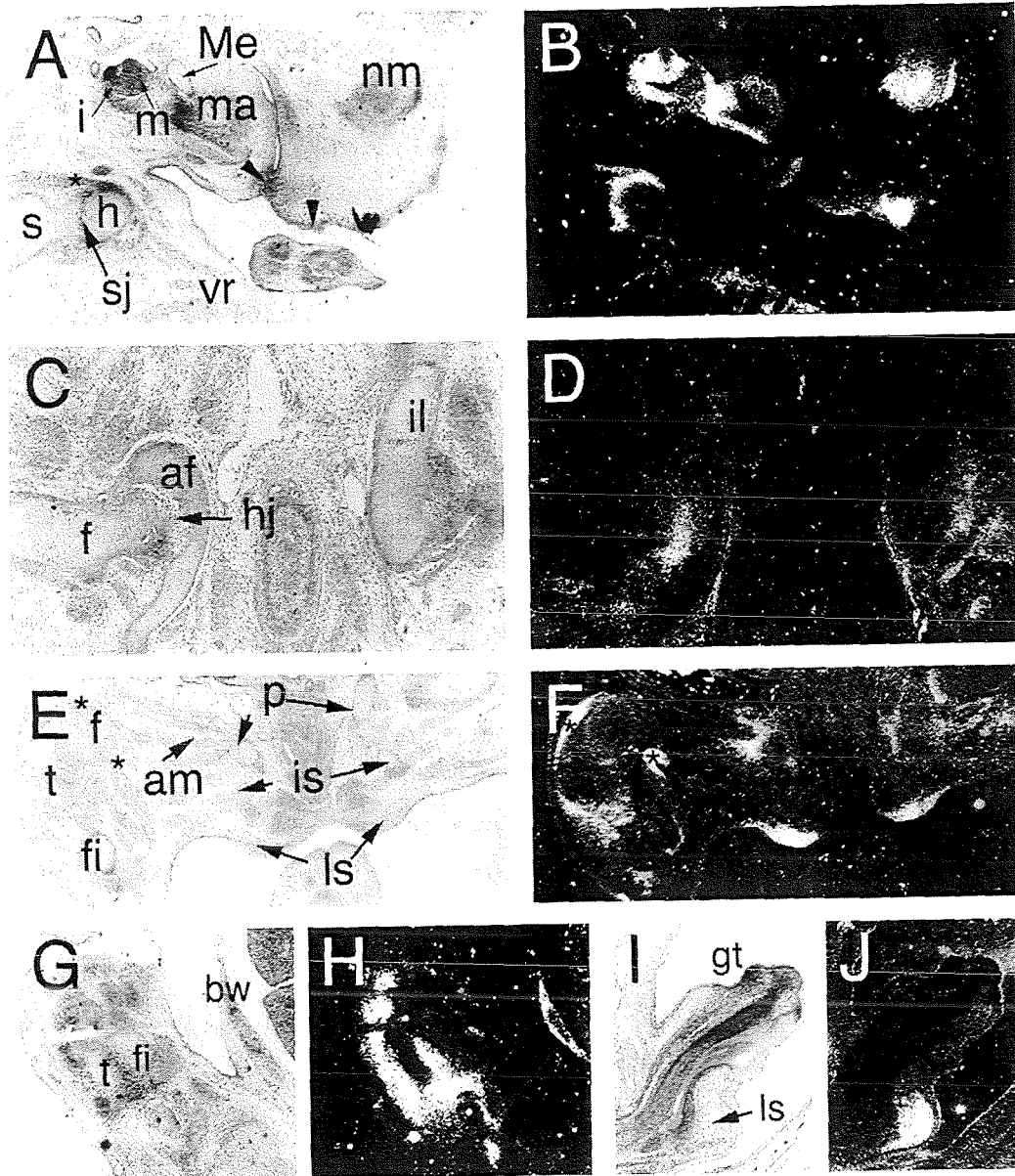


Fig. 4. Expression pattern of *goosecoid* in the appendicular skeleton and labioscrotal folds at E13.5-E15.5. A-D: *goosecoid* is expressed in the shoulder joint and hip joint region. A, B: E13.5, sagittal section of shoulder joint, nasal and middle ear region. C, D: E14.5, transverse section through the developing hip joint and iliac bone. E, F: Expression of *goosecoid* surrounding the developing ischial and pubic bone, and in the labioscrotal swellings at E14.5 (transverse section). G, H: Expression of *goosecoid* in the hind limb at E13.5 (transverse section). I, J: Expression of *goosecoid* in the mesenchyme of the labioscrotal swellings at E15.5 (sagittal section). A, C, E, G, I: bright field; B, D, F, H, J: dark field. af: acetabular fossa; am: adductor muscle; bw: body wall; f: femur; fi: fibula; gt: genital tubercle; h: humerus; hj: hip joint; il: iliac bone; is: ischial bone; ls: labioscrotal swellings; m: malleus; ma: mandible; Me: Meckel's cartilage; nm: nasal mesenchyme; p: pubic bone; s: scapula; sj: shoulder joint; t: tibia; vr: ventral rib. The asterisks in 'A', 'E' and 'F' indicate ligments initiating at the humerus and femur. Small arrowheads in 'A' mark sites of *goosecoid* expression in the upper and lower lip.

2.1.4 Malformation of innominate bone and hip joint in *gooseoid* mutant mice

Newborn wild-type and *gooseoid* mutant animals were analyzed for abnormalities in the pelvic region by skeletal analysis and histological serial sections (Zhu et al., 1998). Bone and cartilage staining did not reveal gross alterations of the hip joint, while a dramatic hyperproliferation of the cartilage of the ischial bone was apparent in the mutants when compared to wild-type littermates. The ischial bone was significantly shortened in the mutant, and the pubic and iliac bone showed slight reductions in size as well. While the phenotype of the ischial bone clearly represents a specific result of the *gooseoid* mutation, the effect on pubic and iliac bone may be a consequence of the previously noted general reduction in size of mutant mice. The pubic symphysis formed normally in mutant animals.

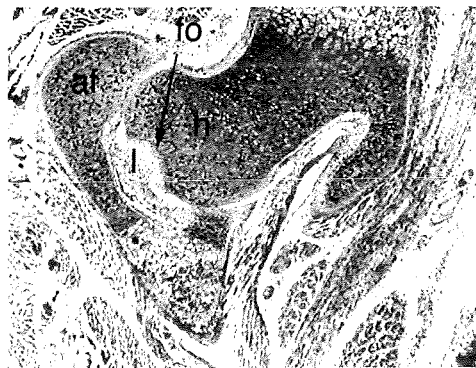


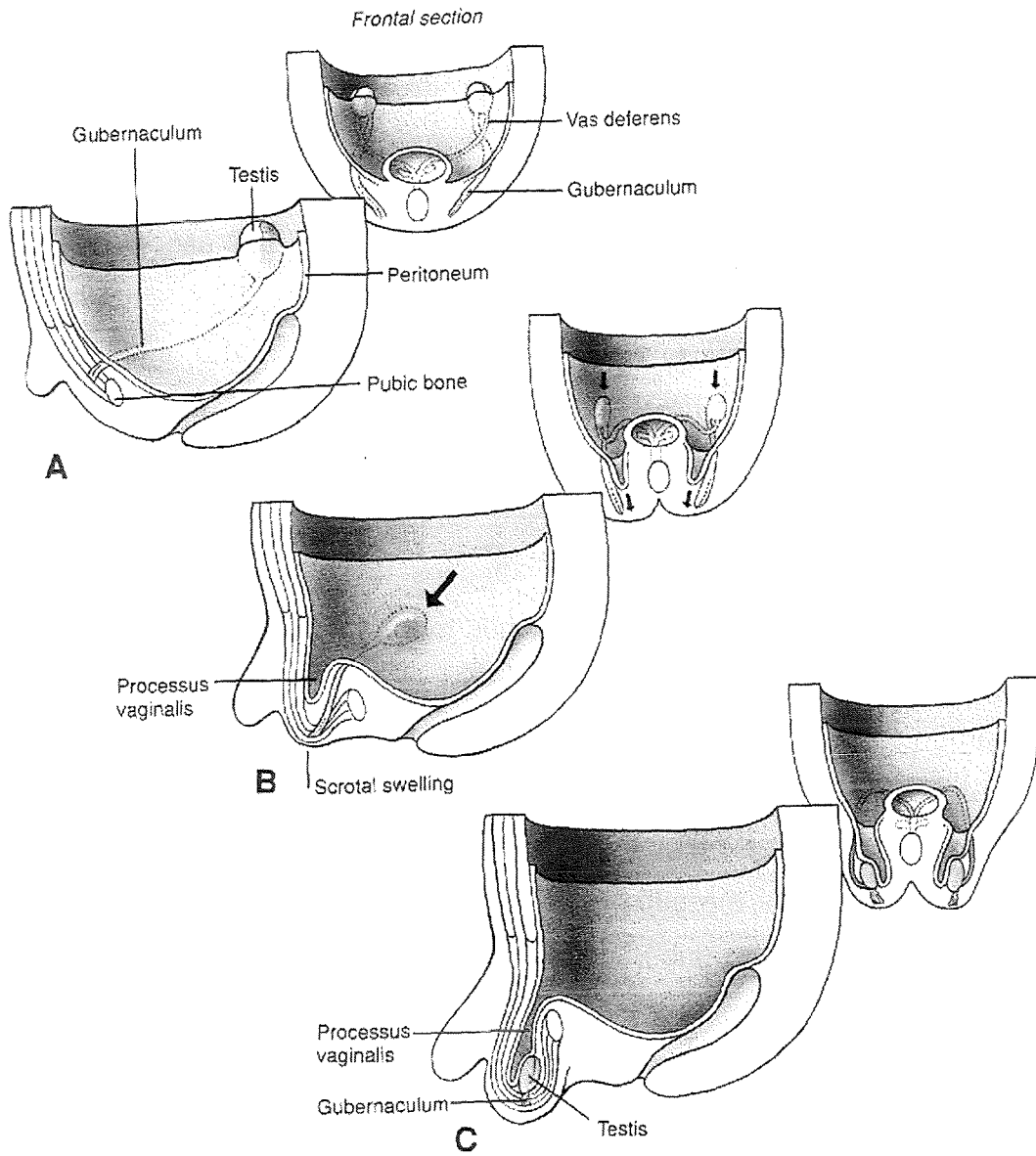
Fig. 5. Histologic transverse section of normal structure of hip joint from wild-type newborn mouse. af: acetabular fossa; f: femur; fo: fovea capitis femoris; h: head of the femur; l: ligament of the head of the femur.

Malformation of the hip joint became apparent in histological sections of newborn mutant mice (Zhu et al., 1998). In wild-type mice the ligament of the head of the femur, a triangular flat band, is attached anterosuperiorly in the fovea capitis femoris, a pit on the femoral head (Fig. 5). Both the fovea and the ligament were not detected in mutant animals (Zhu et al., 1998). This correlates with the expression of *gooseoid* in the space between acetabular fossa and femur at E14.5. Also in agreement with the expression pattern, an adductor muscle initiating at the os pubis was apparently underdeveloped in *gooseoid* null mice, as was an adductor

muscle connecting with the ischial bone (Zhu et al., 1998). Thus, mutant animals displayed aberrant development of structures in the pelvic region and hip joint that express *gooseoid* during organogenesis stages.

2.1.5 Expression of *gooseoid* in the labioscrotal folds

A new site of *gooseoid* gene expression was detected at E14.5 in the labioscrotal swellings, which develop one on each side at the base of the phallus (Fig. 4 E/F). At E15.5 a strong positive signal was detected in the mesenchyme of the labioscrotal folds, which at this stage have extended to and have fused in the midline (Fig. 4 I/J). In males these folds give rise to the scrotal sac, in females they become the labia major. Interestingly, *gooseoid* expression sites in the labioscrotal mesenchyme are destined to give rise to the external and internal muscles underlying the epithelium of labioscrotal swellings. Between the two muscles a fascia is located which serves as the attaching tissue for the gubernaculum, a ligamentous cord, which in both sexes is crucial for the proper descent of the gonads. The superior end of this cord attaches to the gonad and its expanded inferior end (the gubernacular bulb) attaches to the fascia between the developing external and internal oblique muscles in the region of the labioscrotal swellings. In human embryos, the gubernaculum condenses during the seventh week within the subserous fascia. Between the 7th and 12th weeks, the extrainguinal portions of the gubernacula shorten and pull the testes down to the vicinity of the deep inguinal ring within the plane of the subserous fascia (Fig. 6). Like the male embryo, the female embryo develops a gubernaculum extending initially from the inferior pole of the gonad to the subcutaneous fascia of the presumptive labioscrotal folds. In the female, the gubernaculum does not shorten, but deforms, or regresses. Nevertheless, it causes the ovaries to descend during the third month and to be swept out into a peritoneal fold called the broad ligament of the uterus (Larsen, 1997). The expression pattern of *gooseoid* in the labioscrotal mesenchyme suggests a role for the proper development of the external and internal muscles and the intervening fascia. It is tempting to examine if *gooseoid* null mutant mice display any abnormalities with respect to the descent of either testes or ovaries.



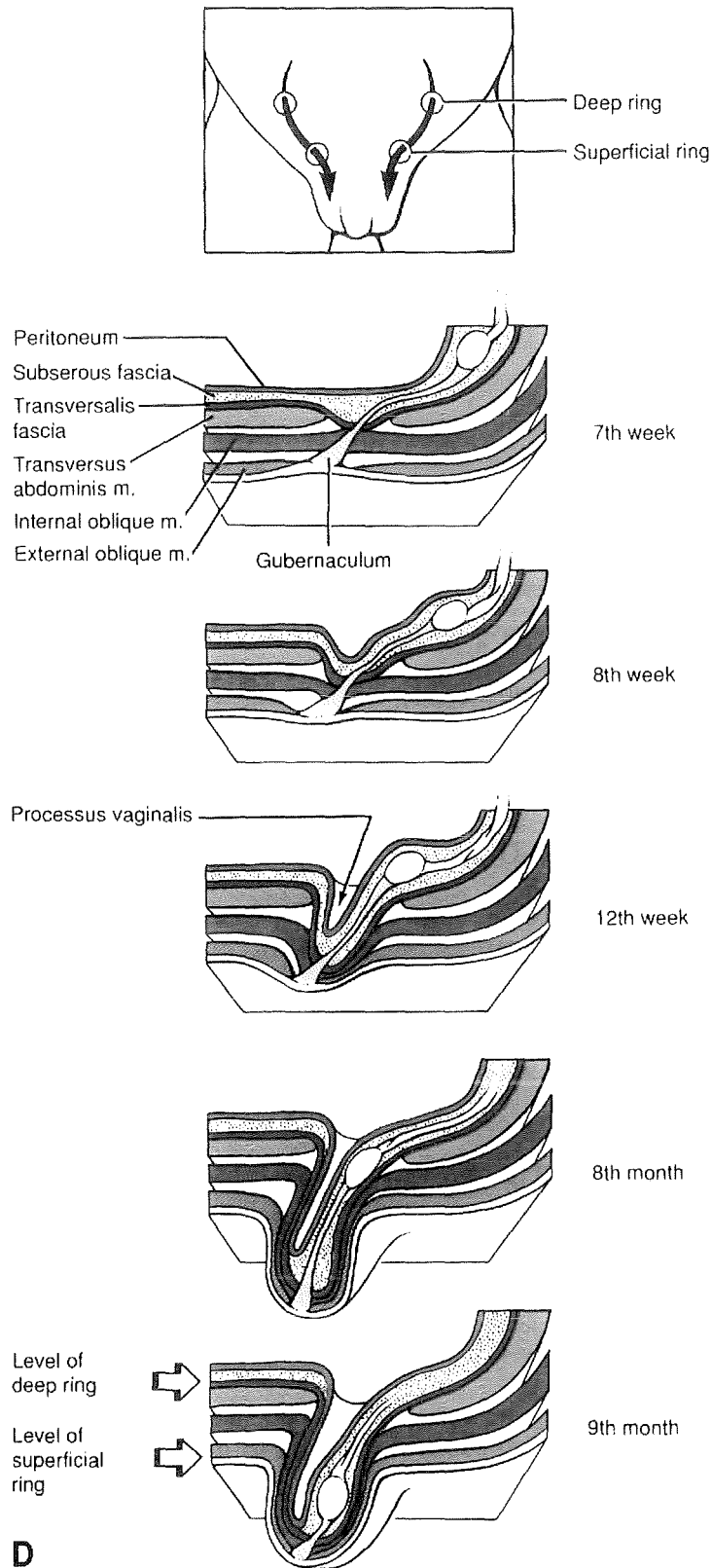


Fig. 6. Descent of the testes in human embryos. (A-C) Between the seventh week and birth, shortening of the gubernaculum testis causes the testes to descend from the 10th thoracic level into the scrotum. The testes pass through the inguinal canal in the anterior abdominal wall. **(D)** After the eighth week, a peritoneal evagination called the processus vaginalis forms just anterior to the gubernaculum and pushes out sock-like extensions of the transversalis fascia, the internal oblique muscle, and the external oblique muscle, thus forming the inguinal canal. The inguinal canal extends

from the base of the everted transversalis fascia (the deep ring) to the base of the everted external oblique muscle (the superficial ring). After the processus vaginalis has evaginated into the scrotum, the gubernaculum shortens and simply pulls the gonads through the canal. The gonads always remain within the plane of the subserous fascia associated with the posterior wall of the processus vaginalis (from Larsen, 1997).

2.1.6 Abnormal epithelial development of the labioscrotal swellings in *gooseoid* mutant mice

A comparative histological analysis was performed for newborn wild-type and mutant animals. Transversal sections of newborn *gooseoid* null mutant mice revealed aberrant epidermal structures in the midline where fusion of the labioscrotal folds had occurred. In normal animals, three layers can be distinguished in the epidermis of scrotum and labia, an outer stratum corneum, a thick middle layer, the stratum spinosum, and the innermost thin stratum germinativum, consisting mostly of a single cell layer. The scrotal epidermis of wild-type male newborn animals appears smooth except for some regularly spaced indentations, the sulci cutis. The three layers show equal thickness along the circumference of the scrotal sac. In mutant mice, however, the middle stratum spinosum displayed a massive hyperproliferation, which was clearly visible in histological sections of male animals, but less pronounced in females. In addition, deep sulci were found which were not observed in normal mice. As *gooseoid* expression in the forming labioscrotal swellings was confined to the mesenchyme, the observed malformations of the epidermal cell layers represent non-cell autonomous effects, as described above for the submucosal layer of the trachea (see discussion). It remains to be elucidated whether mutant scrotal mesenchyme displays abnormalities as well.

Taken together, *gooseoid* gene was found to be expressed in the mesenchyme surrounding the thyroid, cricoid and tracheal cartilages, in the shoulder joint, hip joint, and adductor muscles connecting pubic and ischial bones, in the perichondrium tissue surrounding pubic and ischial bone as well as the perichondrium surrounding tibia and fibia. Another notable expression site was found in the mesenchyme of the labioscrotal swellings. The phenotypes related to the expression profile in *gooseoid* null mutant mice were found in trachea and

pelvic region. The submucosal layer was missing, and the ligament of the head of the femur in the hip joint was not formed.

2.2 Part II. The homeobox gene *gooseoid*: a mediator of retinoic acid teratogenic effects in mouse embryos

gooseoid null mutant mice displayed rib defects and craniofacial abnormalities, including defects of the tongue, nose and mandible, a loss of the tympanic ring and malformation of the malleus (Rivera-Perez et al., 1995; Yamada et al., 1995). These phenotypes resembled some of the teratogenic effects of RA. For example, in mouse embryos that were treated *in utero* with RA at E8 plus 5 to 7 h, the middle ear bones did not develop or became malformed (Mallo, 1997). It was tempting to speculate that *gooseoid* might play a role in mediating some of the RA teratogenicity. Additionally, *gooseoid* was shown to become down-regulated in early *Xenopus* embryos (Cho et al., 1992) and mouse gastrulae (Blum, 1998) following RA-treatment. Three sets of experiments were performed in order to investigate such a link. First, mouse embryos were treated *in utero* with 20 mg/kg all-trans RA (tRA) at E8 + 5 h, a treatment that was shown to result in malformation or loss of the tympanic ring (13-15), and *gooseoid* expression in both control embryo and RA-treated embryos was analysed at E10.5. Second, mouse embryos were treated at E10.5 when *gooseoid* showed strong expression in nasal mesenchyme, branchial arches, ventral body wall and proximal limb bud in order to examine the immediate effect of RA on the expression of *gooseoid* at that stage. Third, the mechanism of down-regulation of *gooseoid* by RA was investigated in P19 teratocarcinoma cells.

2.2.1 *gooseoid* gene expression in branchial arches at E10.5 was altered when mouse embryos were treated with RA at E8 + 5 h

In order to understand if *gooseoid* plays a role in mediating RA teratogenicity in the development of middle ear bones, mouse embryos at E8 + 5 h were treated with 20 mg/kg tRA, and the expression of *gooseoid* in control and tRA-treated embryos was analysed at E10.5. Fig. 7 shows *gooseoid* mRNA expression in E10.5 mouse embryos that were treated with either vehicle solution (10 % DMSO in sesame oil; Fig. 7A, A'), or 20 mg / kg tRA (Fig. 7B, B' ,C, C'). Control treated embryos displayed the normal *gooseoid* expression pattern (Fig. 7A') in the mesenchymal cells of branchial arch I and the anterior third of branchial arch II, as described by Gaunt et al. (1993). In contrast, these hybridization signals were severely altered in RA-treated embryos. Two types of changes were observed. In most cases *gooseoid* mRNA expression became restricted to cells in the branchial cleft between arch I and arch II, as shown for the embryo depicted in Fig. 1B, B'. The second type of change was characterized by a reduction of *gooseoid* transcripts to background levels in the branchial region of the embryo, as shown in Fig. 7C, C'. The change of *gooseoid* expression following RA-treatment was specific for the branchial region. The expression pattern of *gooseoid* in the nasal mesenchyme, in the proximal limb buds and in the adjacent body wall was not affected by RA treatment at E8 + 5 h (Fig. 7 B', C' and data not shown).

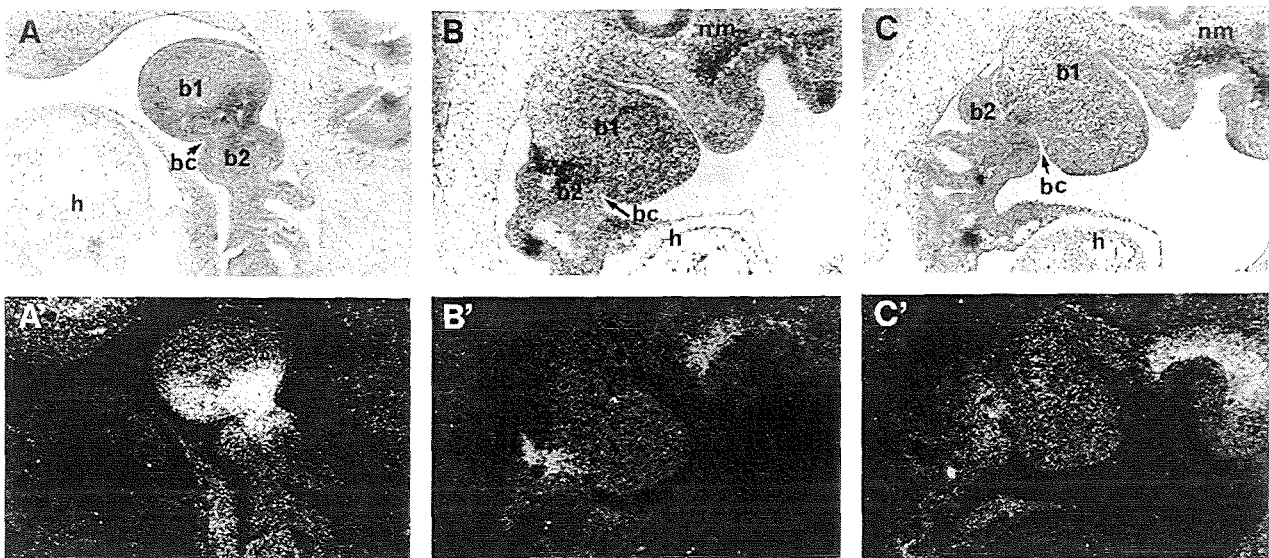


Fig. 7. Alteration of *gooseoid* gene expression following RA treatment of mouse embryos *in vivo* at E8 + 5 h. Mouse embryos were treated with 20 mg/kg tRA and analyzed at E10.5 for *gooseoid* gene expression by *in situ* hybridization of sectioned embryos. **A/A'** : control treated embryo. **B/B'** and **C/C'**: RA-treated embryos. Note that expression of *gooseoid* in branchial arches I (b1) and II (b2) of RA-treated embryos was either restricted to the branchial cleft

region (bc, B'), or reduced to levels close to background (C'), whereas the signal in the nasal mesenchyme (nm) was unaffected. h: heart; A/B/C: bright field; A'/B'/C': dark field.

2.2.2 Malformation and loss of middle ear bones upon RA treatment of mouse embryos at E8 + 5 h

To analyze if and how this alteration of *gooseoid* gene expression was related to teratogenic effects of RA, embryos that were treated *in utero* in parallel under identical conditions were analyzed for skeletal defects at E17.5 by staining with dyes specific for bone (alizarin red) and cartilage (alcian blue). Fig. 8 shows the result of such an experiment. While treatment with vehicle solution did not affect the skeleton (Fig. 8 A), RA-treated mouse embryos revealed severe alterations. In all cases the cartilage primordia of the middle ear ossicles, malleus, incus and stapes, could not be detected. Cartilaginous condensations were sometimes present. According to their anatomical position relative to Meckel's cartilage and other bones in the otic region these cartilages should be hypoplastic forms of the middle ear bones, but they never showed the typical morphology of malleus, incus or stapes (see arrows in Fig. 8 B, C). The tympanic ring was either grossly reduced in size and malformed (Fig. 8 B), or absent (Fig. 8 C), although a remnant of a cartilaginous condensation could also indicate the position of the tympanic ring (arrow in Fig. 8 C). A complete loss of the tympanic ring was seen less frequently (<20 %) than a partial reduction to varying degrees. Other skeletal elements in the craniofacial region that were affected by RA-treatment included the gonial bone, squamosal bone and styloid cartilage (Fig. 8 B, C).

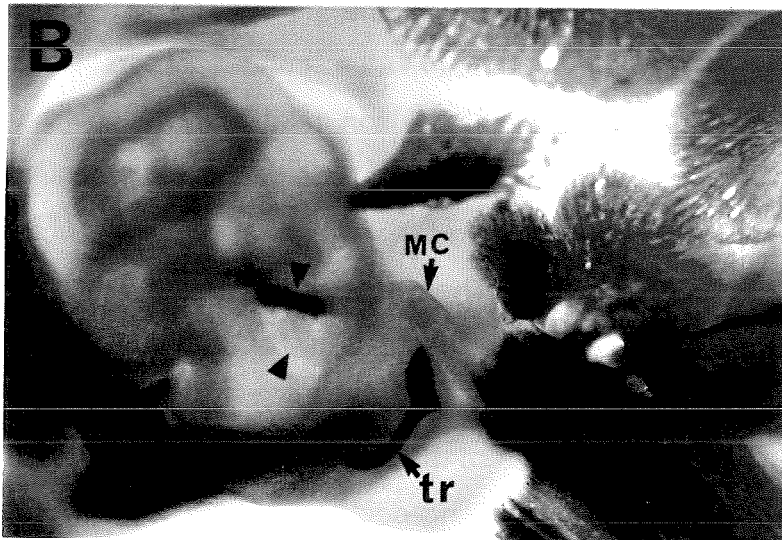
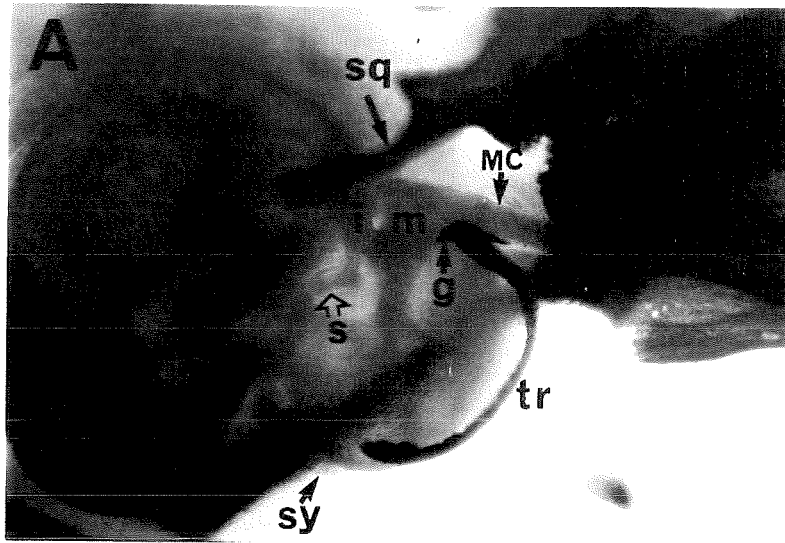


Fig. 8. Loss of tympanic ring and middle ear ossicles following RA treatment of mouse embryos *in vivo*. Mouse embryos were treated at E8 + 5h with 20 mg/kg tRA and analysed at E17.5 for skeleton abnormalities by bone and cartilage staining. **A:** control embryo. **B/C:** RA-treated embryos. Note that RA-treatment resulted in either a partial (B) or complete loss of the tympanic ring (r), whereas the middle ear ossicles, malleus (m), incus (i) and stapes (s) were deleted, and styloid cartilage (sy), gonial bone (g) and squamosal bone (sq) were affected in all embryos analyzed. MC: Meckel's cartilage. Arrows in (B) and (C) indicate cartilaginous condensations reminiscent of middle ear ossicles and tympanic ring.

2.2.3 Down-regulation of *gooseoid* 4 h after treatment of mouse embryos with RA at E10.5

Treatment of rodent embryos from embryonic day 9.5 to 11.5 results in digit truncations and long bone reductions of limbs (Kochhar, 1973, 1985; Tickle et al., 1985) when RA is delivered to embryos through maternal treatment and reaches systemic rather than localized levels throughout the limb buds. This malformation caused by systemic application of RA to the limb buds is different from localized application. In contrast to the malformation of limb buds caused by localized application of RA (see introduction), the cause of malformations resulting from systemic RA application is not well known.

Since *gooseoid* is expressed in the proximal limb bud and in the limb mesenchyme surrounding radius and ulna (Fig. 4 H), and because misexpression of *gooseoid* in the chick limb bud caused malformation of limb long bones (Heanue et al., 1997), I analysed if and how *gooseoid* is involved in the RA teratogenic pathway in limb development.

In order to investigate if RA can regulate *gooseoid* expression at E10.5, mouse embryos at E10.5 were treated with 20 mg/kg all trans retinoic acid (tRA) for 4 h. The dosage of tRA is a standard dosage that has been used by other investigators (Marshall et al., 1992). Control mice were treated with vehicle solution, namely 10% ethanol in sunflower oil. As shown in Fig. 9, E10.5 control embryo had normal *gooseoid* expression pattern in nasal pit, branchial arches and ventral body wall (Fig. 9 A, A'), while *gooseoid* expression was dramatically reduced 4 h after RA treatment (Fig. 9 B, B'). The repression of *gooseoid* by RA appears uniform at all *gooseoid* expression sites in E10.5 mouse embryos, including the limb mesenchyme (Fig. 10) where teratogenic effects occur. In order to know how long the repression of *gooseoid* expression lasts following a single treatment of mouse embryos with 20 mg/kg tRA, mouse embryos were isolated 24 h after a

single dose treatment with 20 mg/kg tRA at E10.5. As shown in Fig. 9 C, C', *gooseoid* was no longer repressed 24 hours after the treatment (Fig. 9 C, C').

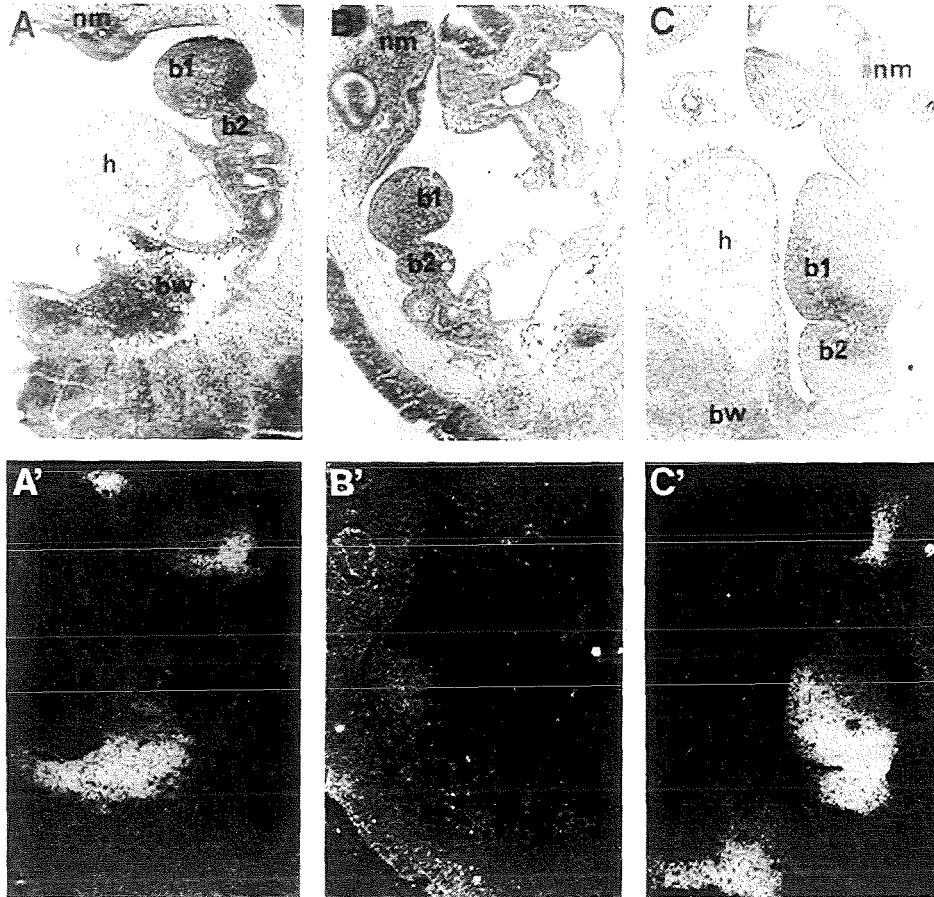


Fig. 9 Transient repression of *gooseoid* in mouse embryos by RA at E10.5. Embryos were treated with vehicle solution (A/A') or with 20 mg / kg tRA at E10.5 *in vivo* and analyzed for *gooseoid* gene expression after 4 h (B/B') or 24 h (C/C'). b1/b2: branchial arch I and II; bw: body wall; h: heart; nm: nasal mesenchyme.

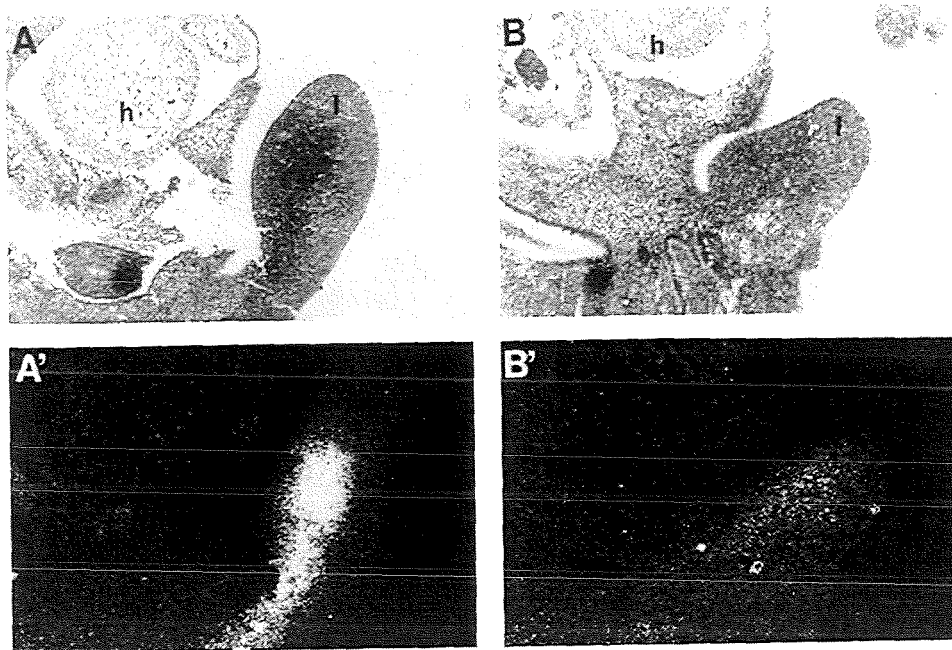


Fig. 10. Down-regulation of *gooseoid* by RA in the developing limb bud 4 h after RA treatment. A: limb bud from a control E10.5 mouse embryo; **B:** limb bud from a E10.5 mouse embryo treated with 20 mg/kg tRA.

2.2.4 RA teratogenic effect following treatment of mouse embryos at E10.5

To show that RA treatment is effectively teratogenic to developing mouse embryos, some litters of mouse embryos treated maternally with a single dosage of 20 mg/kg tRA at E10.5 were kept for further development until birth. All of the newborn mouse embryos after tRA treatment at E10.5 died within 24 hours after birth. Ninety percent of newborn babies showed cleft palate and other developmental defects (data not shown).

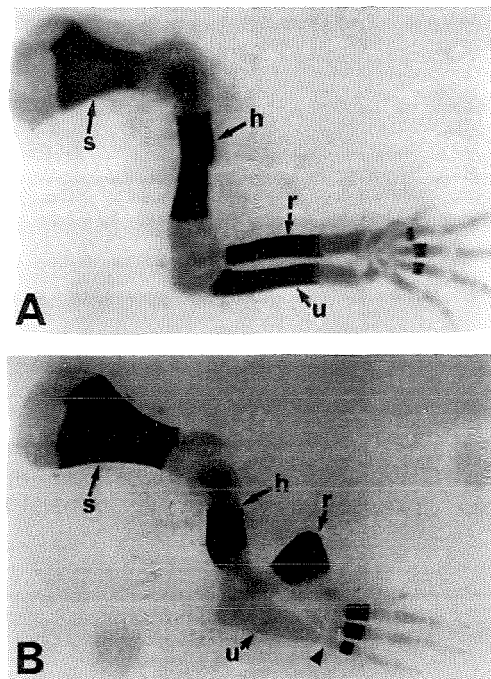


Fig. 11. Teratogenic effect of RA on limb development at E10.5. Wild-type mouse embryos were treated with control solution (A) and 3 times 20 mg/kg tRA (each time for 8 h) (B). The skeleton was prepared from E17.5 control mouse embryo (A) and tRA-treated mouse embryo (B). Note the defects of humerus, radius, ulna and digit in tRA-treated forelimb (B). h: humerus; r: radius; s: scapula; u: ulna

Besides a single dose treatment, mouse embryos at E10.5 were also treated with 20 mg/kg tRA for three times with an interval of 8 hours between each treatment. Mouse embryos were collected at E17.5 for skeleton analysis after tRA treatment. All of the tRA-treated embryos had long bone defects in forelimbs including severe malformation of humerus, radius and ulna as depicted in Fig. 11. Successive low dosage tRA (20 mg/kg) treatment of mouse embryos induced forelimb long bone malformation in the same way as a single high dosage (100 mg/kg). A single high dosage tRA treatment not only resulted in forelimb and hindlimb long bone defects but also caused severe truncations of the caudal vertebrae of developing mouse embryos (data not shown). In contrast, successive low dosage treatment did not cause severe teratogenic effect on the development of mouse hind limbs and caudal vertebrae.

2.2.5 Mechanism of down-regulation of *gooseoid* by RA

The rapid and transient down-regulation of *gooseoid* by RA suggests that the RA effect may be mediated by its receptors, and that down-regulation of *gooseoid* may occur at the transcriptional level.

To test if the down-regulation of *gooseoid* by RA was mediated through the *gooseoid* promoter, a 4.8 kb fragment of the mouse *gooseoid* promoter was cloned into the luciferase reporter vector PXP2 (Fig. 12).

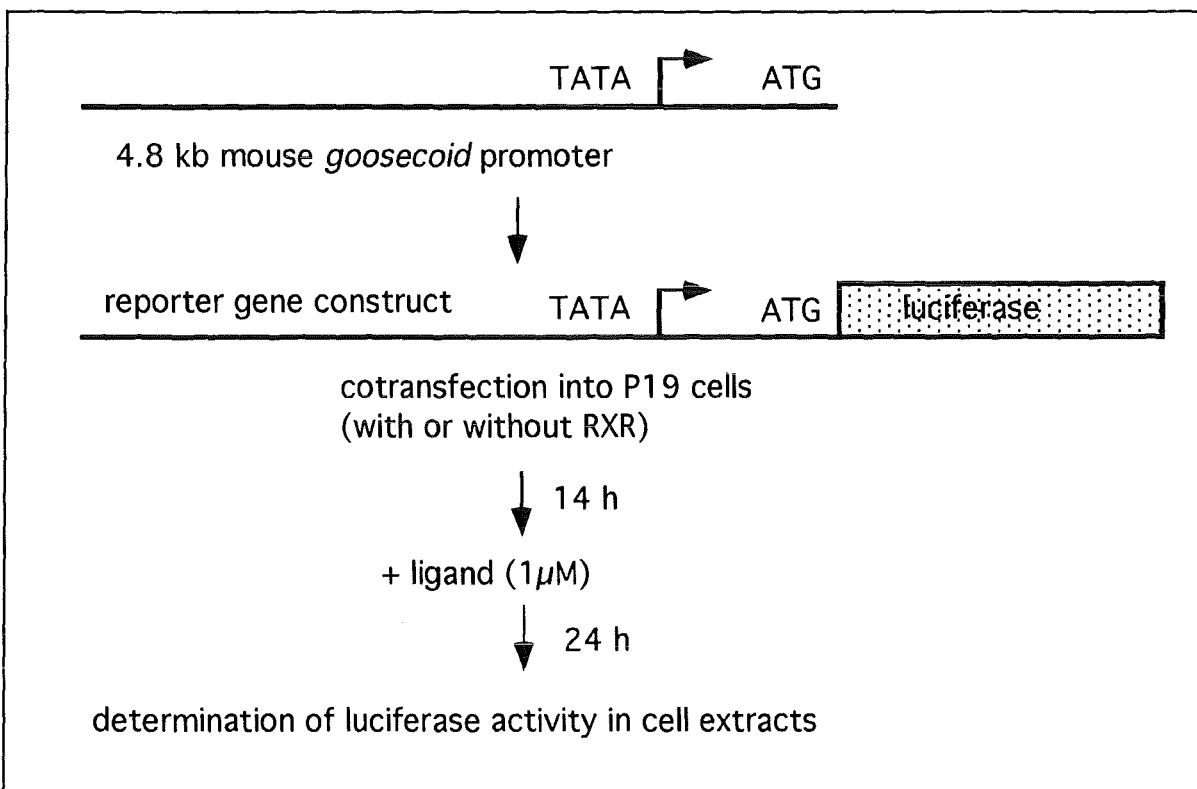


Fig.12. Diagram of *gooseoid* promoter-luciferase reporter construct and DNA transfection procedure.

The 4.8 kb *gooseoid* promoter-luciferase reporter was transfected into P19 mouse embryonic teratocarcinoma cells because this reporter gene was shown to be active in P19 cells (Danilov et al., 1998; Fig. 13). Treatment of the reporter gene-transfected P19 cells with 1 μM tRA or 1 μM 9-cis retinoic acid (9-cis RA) for 24 hours did not result in repression of the luciferase activity of the reporter gene. RA signaling is transduced by RARs and RXRs receptors. In P19 cells, both RAR and RXR are expressed but at a low level (Durand et al., 1992). RAR β can be up-regulated by RA (Bhattacharyya et al., 1997) whereas RXR isoforms can not. I

reasoned that RXR may be a limiting factor for mediating RA signaling in P19 cells. Therefore, in the following experiments, a RXR α expression vector (Chambon, 1994) was cotransfected with the reporter gene. Fig. 13 shows that the reporter gene could be down-regulated 2 fold by 1 μ M tRA or 3 to 4 fold by 1 μ M 9-cis RA when a RXR α expression vector was cotransfected (Fig. 13). 9-cis RA was more potent in the down-regulation of *goosecoid* promoter activity than tRA under the same conditions.

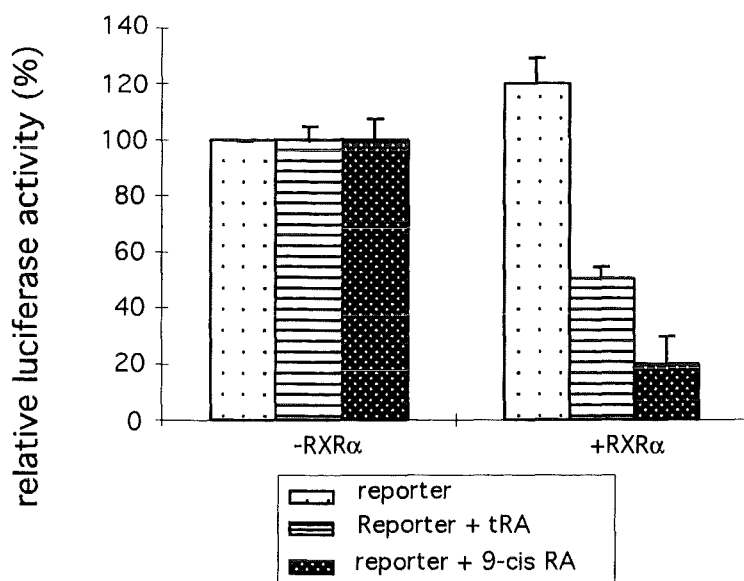


Fig. 13. Down-regulation of *goosecoid* promoter-luciferase reporter in P19 cells by RA. P19 cells were transfected with a 4.8 kb mouse *goosecoid* promoter-luciferase reporter gene either with (+RXR α) or without (-RXR α) cotransfection of RXR α . After washing of the DNA precipitates, cells were treated with control solution, 1 μ M tRA or 1 μ M 9-cis RA for 24 hours.

In order to investigate which RXR isoform was able to mediate RA repression of *goosecoid*, RXR β and RXR γ were included in the study as well. Fig. 14 shows the result from the experiments. RXR α , RXR β and RXR γ were able to mediate the repression of the *goosecoid* reporter gene. However, RXR α was found to be the most potent in mediating the down-regulation of the reporter gene, followed by RXR β and RXR γ (Fig. 14).

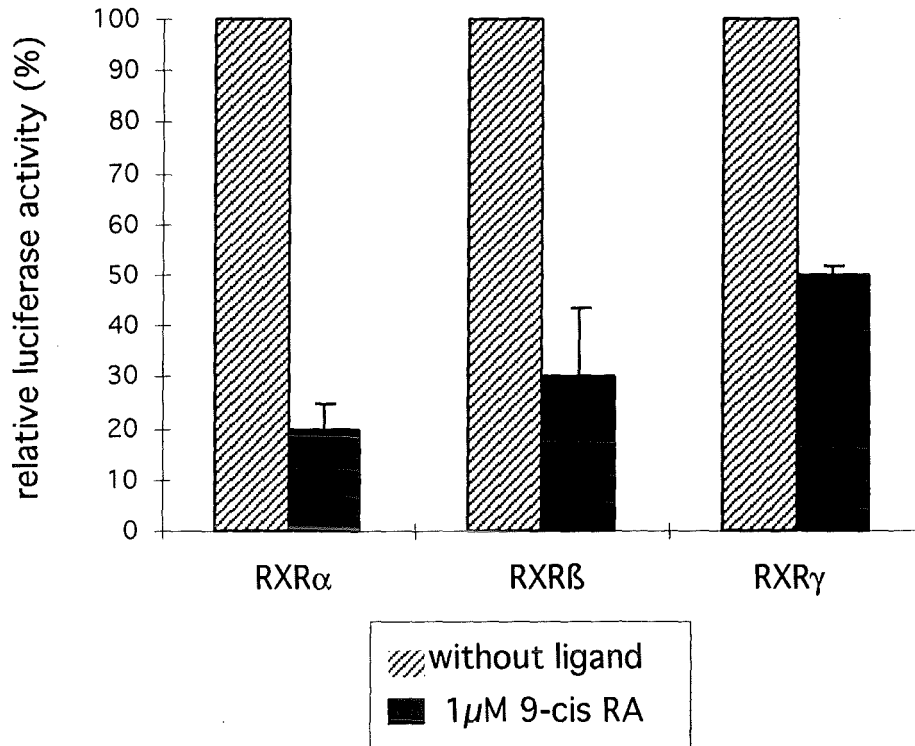


Fig. 14. RXR α is more potent in mediating the down-regulation of the *gooseoid* reporter gene than RXR β and RXR γ . P19 cells were transfected with 4.8 kb mouse *gooseoid* promoter-luciferase reporter and RXR α , RXR β or RXR γ .

Am80 is a selective ligand for RAR α because it can bind RAR α specifically and transactivate a reporter gene through RAR and RXR heterodimer (Delescluse et al., 1991). SR11237 is an RXR specific ligand. It can only activate RXR homodimer but not RAR and RXR heterodimers (Xiao et al., 1995). To distinguish the RAR and RXR retinoid signaling pathway, the RAR α specific ligand Am80 and the RXR specific ligand SR11237 were used in this study. P19 cells were co-transfected with the *gooseoid* reporter gene and the RXR α expression vector (Fig. 12). After transfection cells were treated with 1 μ M Am80 or 1 μ M SR11237, respectively, for 24 h. Fig. 15 shows that both the RAR α specific ligand Am80 and RXR specific ligands were able to repress the luciferase activity of the *gooseoid* reporter gene. However, the RXR-specific ligand SR11237 was more potent than Am80 in down-regulation of the reporter gene. In order to analyse if there is any synergistic effect of Am80 and SR11237 in down-regulation of *gooseoid* reporter gene, both ligands were added to the reporter gene transfected P19 cells simultaneously. As shown in Fig.15, no synergistic effect of RAR and RXR specific ligands was observed.

However, the result implicates both RAR and RXR in the down-regulation of *gooseoid* promoter activity.

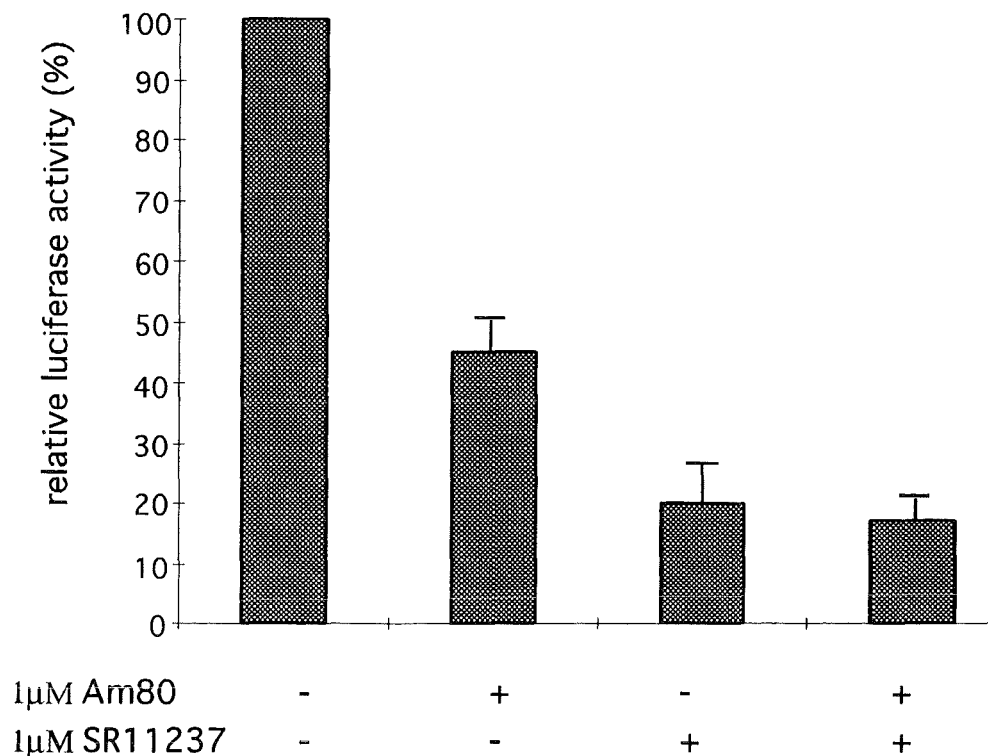


Fig. 15. Repression of *gooseoid* reporter gene by RAR or RXR specific ligands. P19 cells were transfected with 4.8 kb mouse *gooseoid* promoter-luciferase reporter gene together with RXR α . Transfected cells were treated with 1 μ M RAR-specific ligand Am80 or 1 μ M RXR-specific ligand SR11237, or both.

RXRs along with other members of the nuclear hormone receptor superfamily have a highly conserved DNA binding domain (DBD), a ligand-binding domain (LBD), as well as a less conserved amino-terminal domain and a hinge region present between the DBD and LBD (Mangelsdorf et al., 1995; Beato et al., 1995). The schematic structure of RXR α is shown in Fig. 16 A. The LBD not only binds to a specific ligand but is also involved in dimerization with other receptors. The LBD also contains a ligand-dependent transcriptional activation domain called AF-2 to distinguish it from the less well characterized amino-terminal domain AF-1, present in many receptors (Mangelsdorf et al., 1995; Beato et al., 1995). To investigate which domain of RXR is required for the repression of the reporter gene, a dominant negative RXR α that lacks the AF-2 transactivation domain (Feng et al., 1997) was cotransfected with the reporter gene in P19 cells. Fig. 16 B shows that the dominant negative RXR α could not mediate the repression of the reporter gene by either tRA

or 9-cis RA. This result indicates that the AF-2 transactivation domain of RXR α is implicated in the repression of the reporter gene.

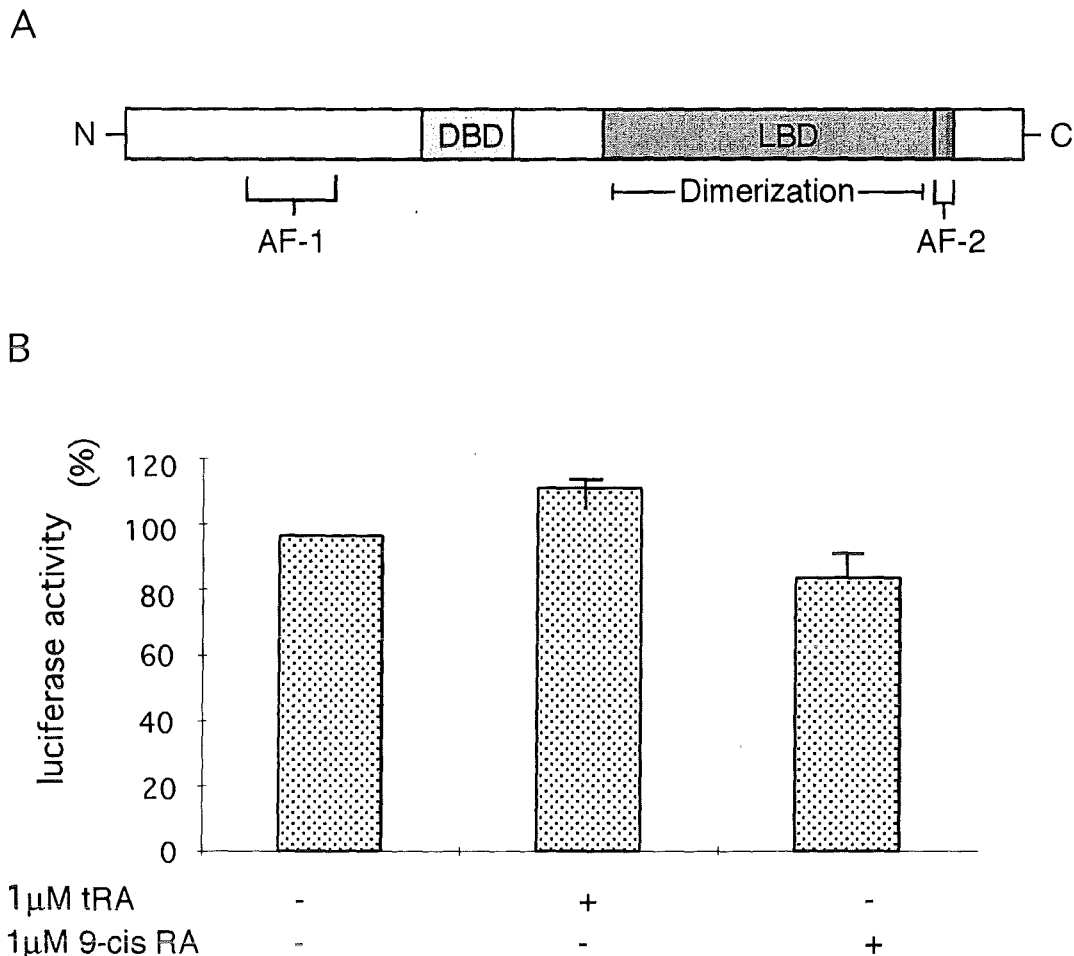


Fig. 16. Dominant negative RXR α abolishes the repression of *gooseoid* reporter by RA. A. Schematic structure of RXR. B. P19 cells were transfected with 4.8 kb mouse *gooseoid* promoter-luciferase reporter gene with a dominant negative RXR α that lacks AF2 transactivation domain. Cells were treated with 1 μ M 9-cis RA for 24 hours.

In summary, in part two I showed that first, *gooseoid* expression in branchial arches was specifically altered following tRA treatment of E8 + 5 h mouse embryos, and the alteration of *gooseoid* expression in branchial arches correlates with the loss or malformation of middle ear bones; second, *gooseoid* was repressed by tRA in E10.5 mouse embryos 4 h after tRA treatment; third, down-regulation of a 4.8 kb mouse *gooseoid* promoter-luciferase reporter by tRA was RXR dependent. AF-2 domain of RXR was indispensable for the down-regulation of the reporter gene. 9-cis RA is more potent in the down-regulation of the reporter gene than tRA. RXR α is

the most potent RXR isoform in mediating the repression of the reporter gene followed by RXR β and then RXR γ .

2.3 Part III. Down-regulation of BMP-4 by RA in mouse embryos and in F9 teratocarcinoma cells

The expression pattern of BMP-4 during *Xenopus* gastrulation revealed a transcript distribution which is complementary to that of genes transcribed in the Spemann organizer, such as *gooseoid*. Ectopic expression of *gooseoid* in *Xenopus* marginal zone led to repression of BMP-4 expression (Fainsod et al., 1994). On the other hand, in mouse embryogenesis, the expression of *gooseoid* and BMP-4 at all developmental stages is also mutually exclusive. This suggests a similar genetic interaction between them as found in early *Xenopus* gastrula embryos. A simple hypothesis would be that as an antagonizing molecule of *gooseoid*, BMP-4 would be up-regulated when *gooseoid* was down-regulated following RA treatment of mouse embryos.

To test if down-regulation of *gooseoid* by RA in mouse embryos would lead to up-regulation of BMP-4, and what is the effect of RA on BMP-4 expression in mouse embryos, the following experiments were performed.

2.3.1 *gooseoid* represses BMP-4 promoter activity in F9 cells

The homeodomain protein *gooseoid* can act as transcriptional repressor (Danilov et al., 1998). It can bind TAAT and ATTA DNA sequence motifs and negatively regulate transcription. As a transcription factor with repressor property, *gooseoid* may regulate BMP-4 directly at the transcriptional level. In order to test this assumption, a 1.5 kb BMP-4 promoter fragment was cloned from mouse genomic DNA by PCR. After examination of the promoter, several TAAT and ATTA

sequences were found in the promoter (Fig.17 A). To analyse the regulation of the BMP-4 promoter by *goosecoid*, the 1.5 kb BMP-4 promoter fragment was cloned into the PGL2 vector which has an SV40 minimal promoter in front of the luciferase reporter gene. The resulting heterologous BMP-4 promoter-luciferase reporter gene (Br) was transfected into F9 mouse embryonic teratocarcinoma cells. As shown in Fig. 17B, the reporter gene is active in F9 cells. A *goosecoid* expression vector was used as described by Danilov et al. (1998) in which the *goosecoid* cDNA is driven by the CMV promoter. Co-transfection of the *goosecoid* expression vector together with the BMP-4 promoter-luciferase reporter construct resulted in 4 to 5 fold repression of the luciferase activity of the reporter gene (Fig. 17B). This result suggests that 1.5 kb of the BMP-4 promoter is able to mediate the repression of BMP-4 by *goosecoid*.

A

ATCCATTAA GGCAAAGCTG CACCCCTAAG GACAACGAAT CGCTGCTGTT
 TGTGAGTTTA AATATTAAGG AACACATTGT GTTAATGATT GGAGCAGCAG
 TGATTGATGT AGTGGCATTG GTGAGCACTG AATCCGTCCT TCAACCTGCT
 ATGGGAGCAC AGAGCCTGAT GCCCCAGGAG TAATGTAATA GAGTAATGTA
 ATGTAATGGA GTTTTAATTT TGTGTTGTTG TTTTAAATAA TTAATGTAA
TTTTGGCTGT GTTAGAAGCT GTGGGTACGT TTCTCAGTCA TCTTTTCGGT
 CTGGTGTAT TGCCATACCT TGATTTAATCG GAGATTAAA GAGAAGGTGT
 ACTTAGAAAC GATTTCAAAT GAAAGAAGGT ATGTTTCCAA TGTGACTTCA
 CTAAAGTGAC AGTGACGCAG GGAATCAATC GTCTTTAAT AGAAAGGGCT

B

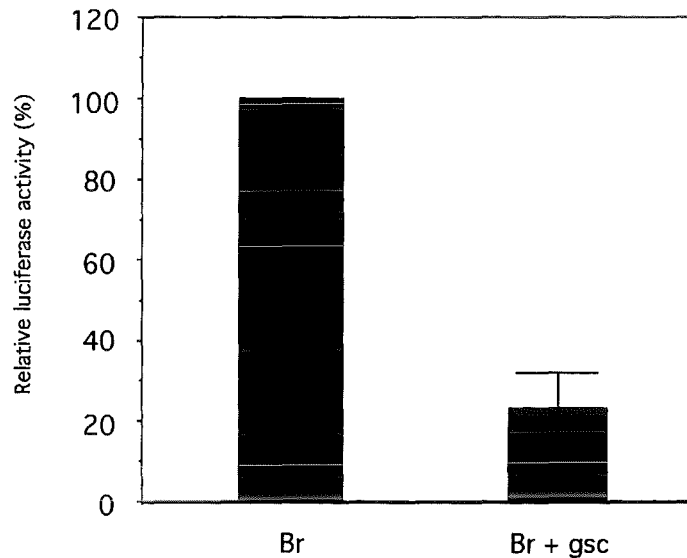


Fig. 17. Down-regulation of BMP-4 promoter activity by *gooseoid* in F9 cells. A: putative homeodomain binding sites in the BMP-4 promoter (TAAT and ATTA,boxed). B: Down-regulation of BMP-4 promoter-luciferase reporter activity (Br) by *gooseoid* (*gsc*).

2.3.2 Down-regulation of BMP-4 by RA in mouse embryos

In order to test if down-regulation of *gooseoid* by RA would lead to up-regulation of BMP-4 because of the antagonism between the two genes found in early frog embryos, E10.5 mouse embryos were treated with either vehicle control solution or 20 mg/kg tRA by oral gavage. Mouse embryos were isolated 4 h and 24 h after tRA treatment respectively. Radioactive *in situ* hybridization was performed

on sagittal sections from both control and tRA-treated E10.5 mouse embryos. Fig. 18 A, A' shows the normal BMP-4 expression in the epithilium of branchial arches, heart and in the apical ectodermal ridge (AER) of the limb bud at E10.5. This normal expression was down-regulated 4 hours after treatment of embryos with tRA (Fig. 18 B, B'). In order to test the duration of the down-regulation of BMP-4 by RA, mouse embryos were isolated 24 hours after RA treatment. The expression of BMP-4 was analysed in these embryos as well. Fig. 18 C, C' shows that the expression pattern of BMP-4 was normal again at all expression sites 24 hours after RA treatment of mouse embryos at E10.5. This result showed that down-regulation of *goosecoid* by RA did not result in up-regulation of BMP-4. On the contrary, BMP-4 was rapidly repressed by RA treatment.

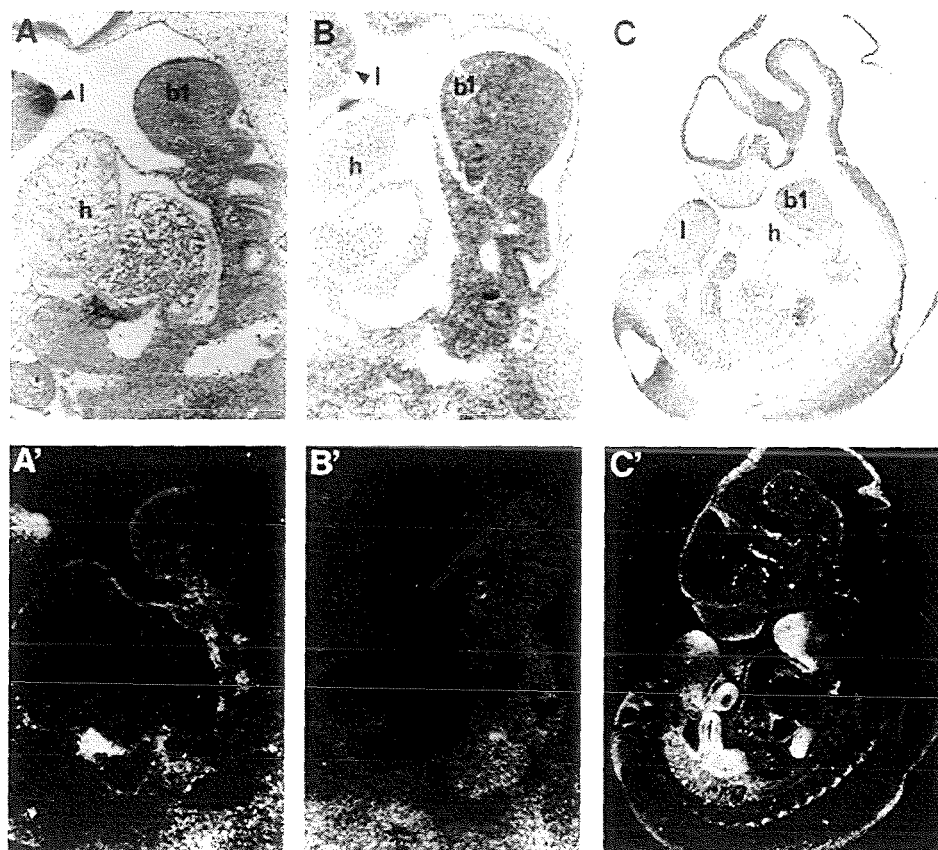


Fig. 18. Transient repression of *BMP-4* by tRA at E10.5 mouse embryos. Embryos were treated with vehicle solution (A/A') or with 20 mg / kg tRA at E10.5 *in vivo* and analyzed for *BMP-4* gene expression after 4 h (B/B') and 24 h (C/C'). A, B, C: bright field; A', B', C': dark field. b1: branchial arch I; bw: body wall; h: heart; l: limb bud.

2.3.3 Mechanisms of the RA-mediated down-regulation of BMP-4

Mouse embryos *in utero* are not suitable for well-defined study of the mechanisms for the regulation of certain endogenous genes by ligands. This is because the amount of the ligand that an embryo receives can not be controlled very well. An alternative way to study the regulation of genes by hormones or by other ligands is to turn to cell lines. F9 mouse teratocarcinoma cells are suitable for the study of the regulation of BMP-4 by RA because BMP-4 is expressed endogenously in F9 cells (Rogers et al., 1992) and RAR and RXR are expressed in this cell line as well (Aneskievich et al., 1992).

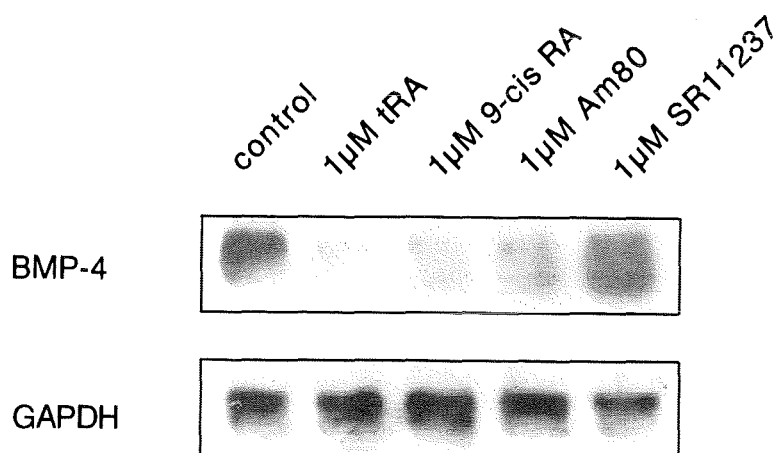


Fig. 19. Down-regulation of BMP-4 24 h after treatment of F9 cells with RA. F9 cells were treated with different ligands for 24 hours. Total RNA was isolated from each treatment. 20 μg of total RNA from each sample was separated on a 1% agarose formaldehyde gel. The RNA was transferred to a nylon membrane. After cross-linking, the membrane was probed with ^{32}P -labeled BMP-4 and GAPDH probe successively.

In order to study if BMP-4 can be down-regulated in F9 cells by RA and which receptor is involved in the repression of BMP-4, F9 cells were treated with tRA (1 μM), 9-cis RA (1 μM), RAR-specific ligand Am80 (1 μM) or RXR-specific ligand SR11237 (1 μM) for 24 hours. Total RNA was isolated and analysed on a Northern blot. The blot was hybridized successively with ^{32}P -labeled BMP-4 and glyceraldehyd 3-phosphate dehydrogenase (GAPDH) probes, respectively.

GAPDH, as a housekeeping gene serves as loading control. As shown in Fig. 19, BMP-4 was expressed in this experiment in F9 cells. The expression was down-regulated by tRA, 9-cis RA, and by the RAR-specific ligand Am80 24 h after treatment of the cells with these ligands. The RXR-specific ligand SR11237, however, could not repress BMP-4 expression in F9 cells as efficiently as the other ligands. Taken together, the data indicates that repression of BMP-4 by RA is mainly mediated by RAR. However, participation of RXR can not be excluded because RXR could be a silent partner for RAR (Xiao et al., 1995). RXR homodimers may not be implicated in the repression of BMP-4 by RA. Induction of one additional transcript might be due to activation of RXR homodimers by the RXR-specific ligand SR11237.

A - 1459 **5'-AGGACTTAGTCAGGGGC-3'**
 - 367 **5'-GGTGATTACTCAGTGTTT-3'**
AP-1 consensus sequence 5'-ATGAGTCAG-3'

B

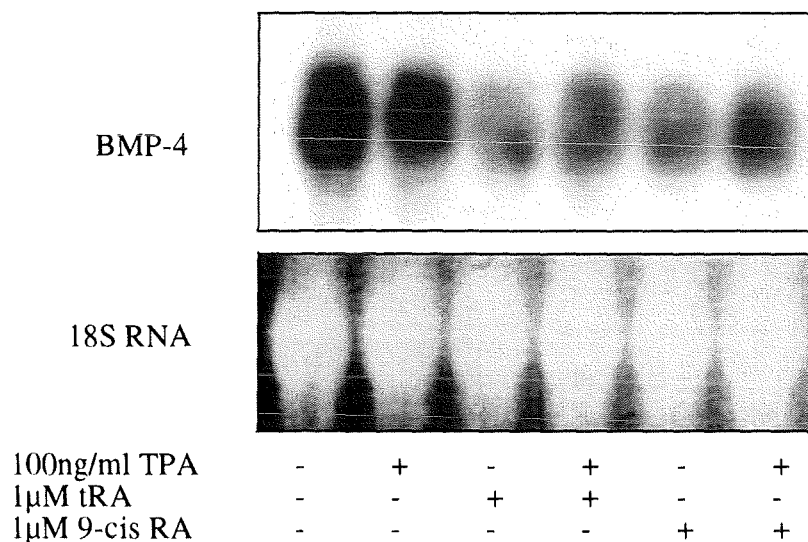


Fig. 20. The expression of BMP-4 is not up-regulated by TPA treatment in F9 cells. A: AP-1 like sites in the BMP-4 promoter. **B:** F9 cells were treated with different combinations of TPA (100 ng/ml), tRA (1 μM) and 9-cis RA (1 μM) for 4 h. Total RNA was isolated, and 20 μg total RNA from each sample was analysed by Northern blot with a BMP-4 probe. 18S RNA serves as loading control.

Down-regulation of genes by RA could be due to cross-talk between RAR or RXR and AP-1 transcription factors (Nicholson et al., 1990; Schüle et al., 1991; Yang-yen et al., 1991; Salbert et al., 1993). There are two AP-1 like sites in BMP-4 promoter (Fig. 20 A). These AP-1 like sites suggest putative positive regulation of BMP-4 expression by AP-1, and indicate the possibility that RA-mediating down-regulation of BMP-4 occurs through this mechanism. Several studies indicate, however, that F9 cells lack AP-1 activity (van Dam et al., 1995; Diccianni et al., 1992). F9 cells do not express c-jun (van Dam et al., 1995), and nuclear extract proteins from F9 cells do not bind to AP1 response elements (Diccianni et al., 1992).

In order to prove that BMP-4 expression in F9 cells is independent of AP-1 transcription factors, F9 cells were treated with or without 100 ng/ml tumor promoter 12-O-tetradecanoyl phobol-13-acetate (TPA) and in a combination with tRA and 9-cis RA for 4 hours, a treatment which stimulates AP1 activity in many cell lines. Total RNA was isolated for Northern blot analysis. The blot was hybridized with a ³²P-labeled BMP-4 probe. 18s ribosomal RNA was used as a loading control. As shown in Fig. 20 B, treatment of F9 cells with 100 ng/ml TPA did not up-regulate BMP-4 gene expression. This suggests that AP-1 is not implicated in the activation of BMP-4 expression in F9 cells. Therefore, down-regulation of BMP-4 by RA can not be mediated by antagonizing AP-1 activity.

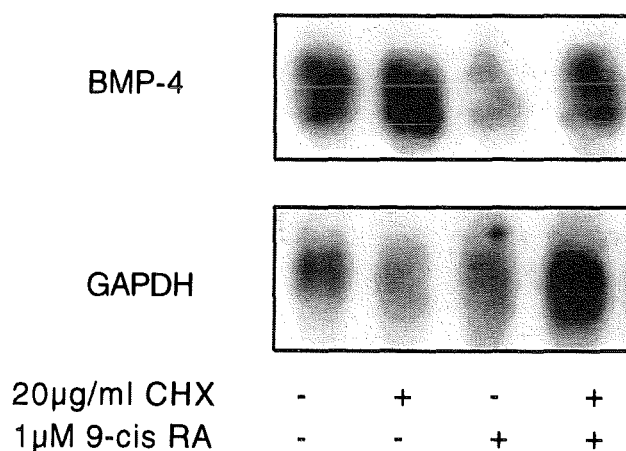


Fig. 21. Down-regulation of BMP-4 by 9-cis RA in F9 cells does not require new protein synthesis. F9 cells were treated without (control) or with 20 µg/ml cycloheximide (CHX) for 30 min before addition of 9-cis RA and cultured for an additional 4 h. 20 µg total RNA from

each sample was used for a Northern blot. The blot was hybridized successively with ^{32}P -labeled BMP-4 and GAPDH probes.

To test if down-regulation of BMP-4 by RA treatment is due to up-regulation of a repressor or down-regulation of an activator for BMP-4 transcription, F9 cells were treated with 9-cis RA in combination with the inhibitor of protein synthesis, cycloheximide (20 $\mu\text{g}/\text{ml}$) in order to inhibit *de novo* protein synthesis. After treatment of F9 cells with different reagents as shown in Fig. 21, total RNA was isolated from control and 9-cis RA treated F9 cells for Northern blot analysis. The blot was hybridized with ^{32}P -labeled BMP-4 and GAPDH probes successively. In Fig. 21 loading of total RNA was not equal for each lane as shown by GAPDH signal, so it was necessary to normalize the signal of BMP-4 by GAPDH. After normalization, BMP-4 was shown to be repressed 4 hours after treatment with 9-cis RA, and cycloheximide did not inhibit the repression of BMP-4 by RA.

2.3.4 Effect of RA treatment on *gooseoid* and BMP-4 expression in RXR α ^{-/-} mice

Mutations of two alleles of the RXR α gene resulted in mouse embryonic lethality around E15.5 due to cardiomyocyte hypoplasia (Sucov et al., 1994; Kastner et al., 1994). Interestingly, RXR α null mutant mice were resistant to the teratogenic effects of RA in limb development compared with wild type and heterozygous mutant embryos (Sucov et al., 1995). RXR α mutant embryos are therefore good tools for analysing the mechanism of RA teratogenesis on limb development.

Both *gooseoid* and BMP-4 are expressed in the developing mouse limb bud. They have different roles for normal limb development. BMP-4 is expressed in the AER of developing limbs and in limb mesenchyme (Fig. 18 A' and C'). Heterozygous BMP-4 mice showed digit defects (Dunn et al., 1997). BMP-4 plays a role in chondrogenesis of developing mouse limbs as well since application of BMP-4 protein to developing chick limb buds induced ectopic bone formation. It was therefore interesting to investigate if down-regulation of *gooseoid* and BMP-4 by RA in limb buds is RXR α dependent, and if *gooseoid* and BMP-4 are involved in RXR α -mediated RA teratogenesis pathway in developing limbs.

Three additional homeobox genes *Msx1*, *Msx2* and *Hoxd-11* were included in this study because of their known role in limb development. *Hoxd-11* is a regulator of limb development (Davis et al., 1994). Double knock-out of *Hoxd-11* and *Hoxa-11* in mice revealed that both genes are required for the normal development of radius and ulna (Davis et al., 1995) in forelimb. This phenotype is reminiscent of RA teratogenic effect on radius and ulna development.

$RXR\alpha^{+/-}$ male and female mice were mated in order to analyse the effect of RA on the expression of *goosecoid*, *BMP-4*, *Msx1*, *Msx2* and *Hoxd-11*. Embryos were treated at E10.5 with either vehicle solution (10% ethanol in sunflower oil) or 20 mg/kg tRA for 4 hours. Embryos were genotyped by PCR of genomic DNA isolated from the yolk sac tissue of each embryo. Fig. 22 shows the DNA fragments of the genotypes of $RXR\alpha^{+/-}$ and $RXR\alpha^{-/-}$ -embryos.

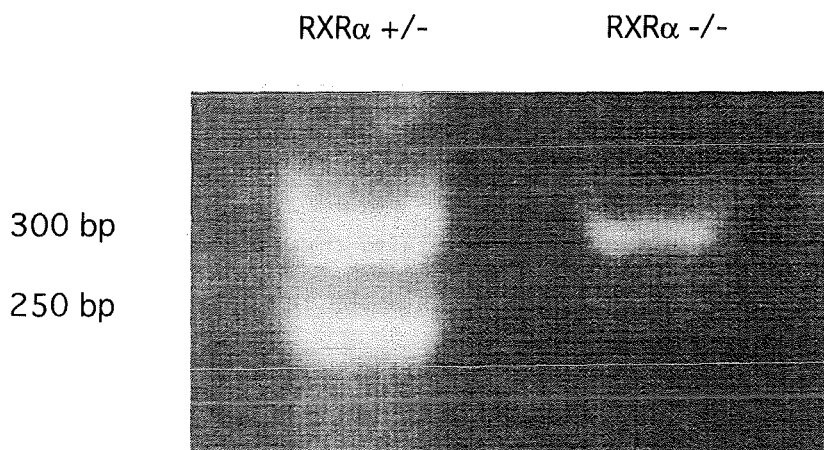


Fig. 22. Genotyping of $RXR\alpha^{+/-}$ and $RXR\alpha^{-/-}$ mouse embryos. Genomic DNA was isolated from embryonic yolk sac tissue. PCR was performed to distinguish the genotypes of $RXR\alpha$ null mutant embryos and $RXR\alpha$ heterozygous embryos. The 300bp fragment represents the mutant allele, and a 250 bp amplification fragment is indicative of the wildtype gene.

Radioactive *in situ* hybridization was performed with the $\alpha^{35}\text{S}$ -labeled probes for *goosecoid*, *BMP-4*, *Msx1*, *Msx2* and *Hoxd-11* on sagittal sections from both control E10.5 mouse embryos and tRA-treated E10.5 mouse embryos with $RXR\alpha^{+/-}$ and $RXR\alpha^{-/-}$ genetic background, respectively (Fig. 23 and Fig. 24).

As shown in Fig. 23, *goosecoid* was normally expressed in the nasal mesenchyme, first and second branchial arches, and the intervening branchial arch cleft, and in the proximal limb bud in E10.5 $RXR\alpha^{+/-}$ control embryos (Fig. 23 A, A'). This expression profile was repressed by tRA within 4 h following tRA treatment of mouse embryos (Fig. 23 B, B'). *goosecoid* expression in $RXR\alpha^{-/-}$ null mutant mice

appeared normal (Fig. 24 A, A'), but it was also repressed in $\text{RXR}\alpha^{-/-}$ null mutant mice (Fig. 24 B, B').

BMP-4 was expressed in the epithelium of the maxillary, the epithelium of the first branchial arch and in the atrium of heart of control E10.5 $\text{RXR}\alpha^{+/-}$ mouse embryos (Fig. 23 C, C'). At all sites the expression was down-regulated by tRA in $\text{RXR}\alpha^{+/-}$ embryos (Fig. D, D'). Although $\text{RXR}\alpha^{-/-}$ mutant embryos had heart defects, BMP-4 transcripts were still present in the atrium of the E10.5 heart (Fig. 24 C, C').

Msx1 was expressed in the upper facial process, the first, second and third branchial arches, and in limb buds in control E10.5 $\text{RXR}\alpha^{+/-}$ embryos (Fig. 23 E, E'). The expression of *Msx1* was repressed in RA-treated E10.5 $\text{RXR}\alpha^{+/-}$ mouse embryos (Fig. 23 F, F'). In $\text{RXR}\alpha^{-/-}$ mutant mouse embryos, *Msx1* was normally expressed (Fig. 24 C, C'), as in $\text{RXR}\alpha^{+/-}$ embryos. The normal expression pattern of *Msx1* was down-regulated by tRA in E10.5 $\text{RXR}\alpha^{-/-}$ mutant embryos (Fig. 24 F, F').

Msx2 was normally expressed in the upper facial process, in the anterior of the first branchial arch and in the otic vesicle of $\text{RXR}\alpha^{+/-}$ E10.5 control embryos (Fig. 23 G, G'). Only very faint signal of *Msx2* is visible in tRA-treated E10.5 $\text{RXR}\alpha^{+/-}$ embryos (Fig. 23 H, H'). The normal expression of *Msx2* in tRA-treated E10.5 $\text{RXR}\alpha^{+/-}$ embryos was down-regulated 4 hours following tRA treatment of mouse embryos (Fig. 23 H, H'). In E10.5 $\text{RXR}\alpha^{-/-}$ mutant embryos *Msx2* was normally expressed (Fig. 24 G, G'). Following RA treatment of E10.5 $\text{RXR}\alpha^{-/-}$ mutant embryos for 4 h, there was still strong *Msx2* expression signal in maxillary (Fig. 24 H, H'). In contrast to the effect of RA on *Msx2* expression in $\text{RXR}\alpha^{+/-}$ embryos, *Msx2* was not repressed by RA treatment in E10.5 $\text{RXR}\alpha^{-/-}$ mutant embryos.

Hoxd-11 was expressed in the distal limb bud of control E10.5 $\text{RXR}\alpha^{+/-}$ mouse embryo (Fig. 23 I, I'). It was repressed by tRA in treated E10.5 $\text{RXR}\alpha^{+/-}$ mouse embryos (Fig. 23 J, J'). The normal expression of *Hoxd-11* in $\text{RXR}\alpha^{-/-}$ mouse embryos was down-regulated as well (Fig. 24 I, I', J and J').

Part III showed that BMP-4 was down-regulated following RA treatment in both mouse embryos and in F9 cells. In mouse embryos, BMP-4 was repressed 4 h after RA treatment. In F9 cells tRA, 9-cis RA and RAR-specific ligand efficiently repressed BMP-4 expression. Remarkably RXR-specific ligand could not repress BMP-4 expression. Down-regulation of BMP-4 by RA did not require new protein synthesis.

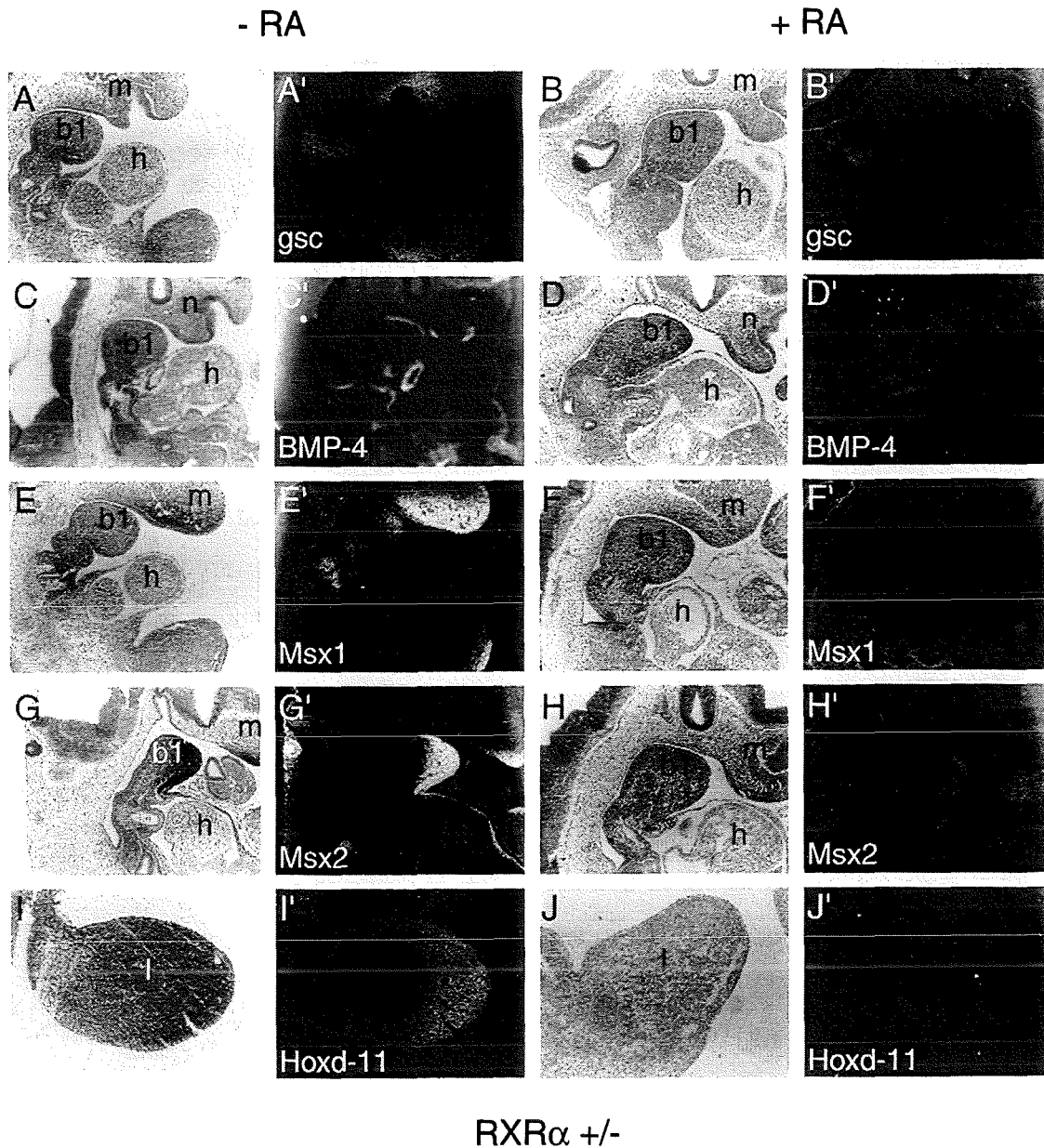


Fig. 23. Down-regulation of genes by RA in RXR α +/- mouse embryos. Radioactive *in situ* hybridization was carried out on sagittal sections of RXR α +/- E10.5 mouse embryos with probes for *goosecoid* (A/A', B/B'), BMP-4 (C/C', D/D'), *Msx1* (E/E', F/F'), *Msx2* (G/G', H/H'), and *Hoxd-11* (I/I', J/J'). Sections of A/A', C/C', E/E', G/G' and I/I' were from control embryos treated with vehicle solution. Sections of B/B', D/D', F/F', H/H' and J/J' were from 20 mg/kg tRA-treated embryos. m: maxillary; n: nasal mesenchyme; b1: branchial arch 1; b2: branchial arch 2; h: heart; l: limb bud.

In RXR α +/- mouse embryos *goosecoid* and BMP-4 together with *Msx1*, *Msx2* and *Hoxd-11* were repressed by RA. However, in RXR α -/- mouse embryos *Msx2* expression was not repressed by RA, and the repression of BMP-4 by RA was far less pronounced in RXR α -/- mouse embryos than in RXR α +/- mouse embryos. In

$RXR\alpha^{-/-}$ mouse embryos *gooseoid*, *Msx1* and *Hoxd-11* were still down-regulated following RA treatment.

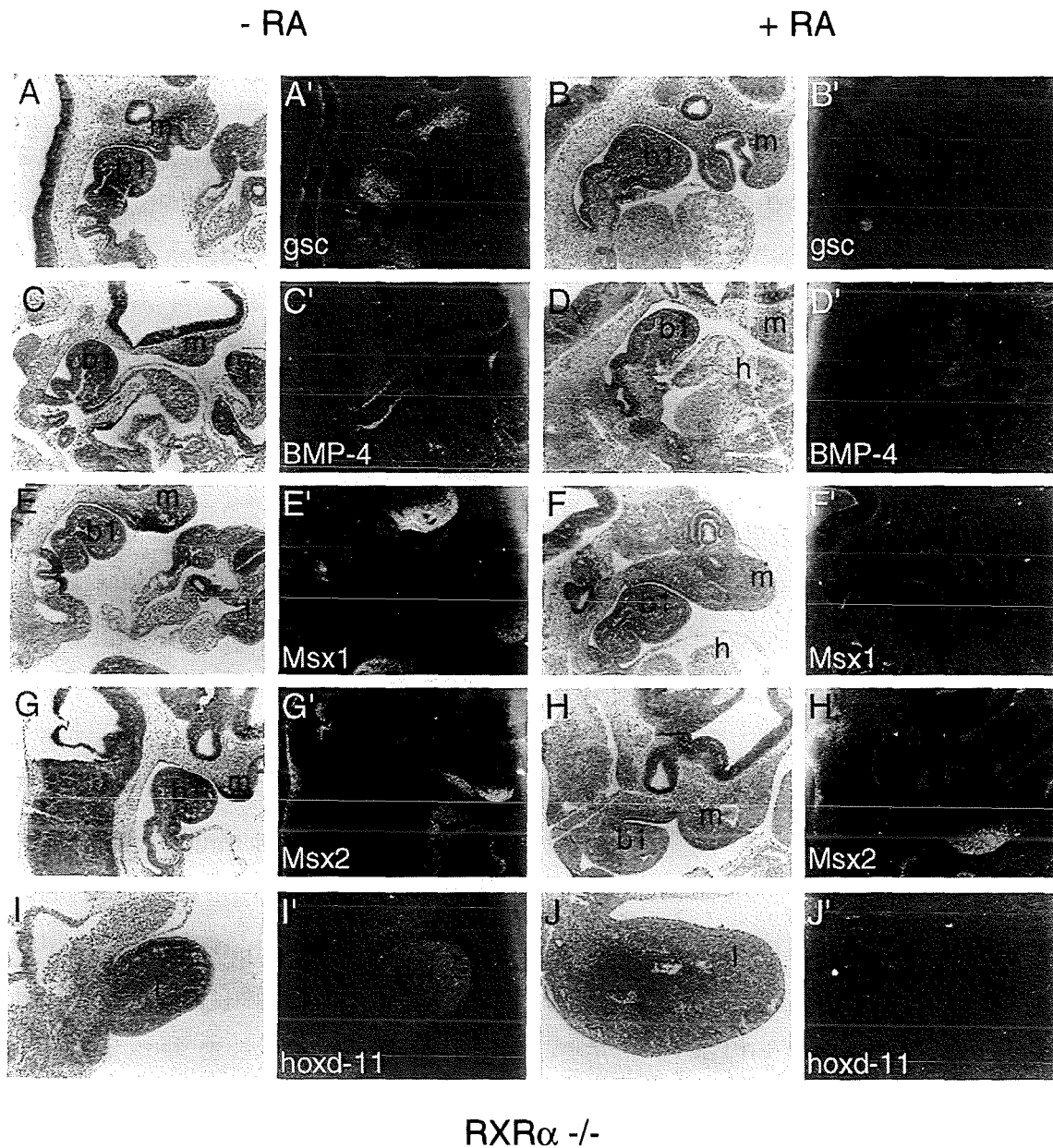


Fig. 24. Down-regulation of genes by RA in $RXR\alpha^{-/-}$ genetic background. Radioactive *in situ* hybridization was carried out on sagittal sections of $RXR\alpha^{-/-}$ E10.5 mouse embryos with probes of *gooseoid* (A/A', B/B'), *BMP-4* (C/C', D/D'), *Msx1* (E/E', F/F'), *Msx2* (G/G', H/H'), and *Hoxd-11* (I/I', J/J'). Sections of A/A', C/C', E/E', G/G' and I/I' were from control embryos treated with vehicle solution. Sections of B/B', D/D', F/F', H/H' and J/J' were from 20 mg/kg tRA-treated embryos. m: maxillary; n: nasal mesenchyme; b1: branchial arch 1; b2: branchial arch 2; h: heart; l: limb bud

3. Discussion

This study provides an in-depth analysis of *gooseoid* expression at mid-embryogenesis in the mouse. Phenotypes corresponding to the newly described expression in trachea and pelvic region are described. In addition, a role for *gooseoid* in mediating RA teratogenic effect was studied at two different time windows E8 + 5 h and E10.5. BMP-4 together with other three homeobox genes *Msx1*, *Msx2* and *Hoxd-11* was analysed in the study for their possible role in mediating RA teratogenic effects. The mechanisms for the down-regulation of *gooseoid* and BMP-4 were studied in P19 and F9 mouse teratocarcinoma cells.

3.1 ***gooseoid* is required for the correct development of the submucous layer of the trachea.**

The homeobox gene *gooseoid* plays an important role during organogenesis stages of mouse embryogenesis. This has been previously demonstrated for craniofacial and rib cage development (Yamada et al., 1995, 1997; Rivera-Perez et al., 1995).

gooseoid null mutant mice died within 24 h after birth with air in their stomach. The absence of the submucous layer of the tracheal tunica mucosa most likely contributes to the neonatal death of homozygous null animals because of breathing problems. The submucous layer of the trachea contains secretory glands. These provide the mucus of the respiratory tract with highly glycosylated molecules, which protect airways and alveoli from injury and infections. In fact, respiratory defects have been described previously in the *gooseoid* mutant mouse (Yamada et al., 1997). It is well established that the physiological functions of the tracheal mucosa and glands are essential for proper respiratory function. In the case of cystic fibrosis, for example, which is the most common fatal hereditary disease of Caucasian populations, the primary pathological defect is a hypertrophy of the submucosal glands of the trachea (Scriver et al., 1995), which thus could be considered the reverse of the *gooseoid* phenotype. Therefore, further analysis of the *gooseoid* tracheal phenotype may provide insights in the understanding of the trachea-mucosal system. During development of the trachea the endodermal lining

of the laryngotracheal tube differentiates into the epithelium and glands of the trachea and pulmonary epithelium, whereas cartilage, connective tissue and muscles of the trachea are derived from the splanchnic mesoderm surrounding the laryngotracheal tube (Moore and Persaud, 1993). It is conceivable that cell-cell interactions between these two tissues play a crucial role for the proper development of the trachea. The absence of the submucous layer of the tracheal tunica mucosa, therefore, likely represents a secondary phenotype.

3.2 The role of *gooseoid* in the development of the shoulder and hip joint and associated adductor muscles of mouse embryos.

No defects were reported in the appendicular skeleton of *gooseoid* mutant mice in the initial description of the mutation (Yamada et al., 1995; Rivera-Perez et al., 1995). Upon re-evaluation of the expression pattern I found that the earlier expression in the proximal limb bud at E10.5, and in the shoulder region at E12.5 continues in the shoulder and hip joint regions, and in ligaments and muscles attached to the humerus and femur (Fig. 3). Strictly related to this expression profile, I found a deletion of the fovea capitis femoris and the ligament which is attached to this depression in the head of the femur, as well as an underdevelopment of the adductor muscles extending to the femur. The hip joint is an anatomical and functional unit. Primitive condensed sclerotomic mesenchyme transforms into cartilage that shapes in a genetically determined pattern to form the femur and os innominatum in continuity. In human, the joint space develops by autolytic degeneration in the 7-8 week of gestation. By the 11th week, labrum and ligamentum teres are differentiated from the joint cavity. (Ferrer-Torrelles et al., 1990). The homeobox gene *gooseoid* is the first transcription factor implicated in the normal development of the ligament attached to the head of femur.

Although a detailed analysis was not performed as yet, I would like to speculate that the expression in the perichondrium and mesenchyme around the cartilage primordia of the tibia and fibula (Fig. 3 G/H) may also result in defective ligaments and muscle alignments. The bones, however, do form normally (not shown). The malformation of the innominate bone, particularly the os ischium, may

represent secondary effects, since - as is the case for most skeletal elements apart from neural crest derived craniofacial bones (Gaunt et al., 1993) - *gooseoid* expression is restricted to the perichondrium of the cartilage primordia, and the ligaments and muscles attached to these bones.

The function of *gooseoid* in limb development has also been investigated by a gain-of-function study in the chick limb bud (Heanue et al., 1997). Over-expression of *gooseoid* by retroviral infection led to an alteration in the angle of femur outgrowth from the main body axis and the overall decrease in the size of *gooseoid*-infected limbs, with cartilage elements being small, misshapen and bent. Hox gene expression was down-regulated in *gooseoid* infected limbs. The combined evidence of these gain- and loss-of-function studies establishes *gooseoid* as an important determinant of vertebrate limb patterning.

3.3 A role of *gooseoid* in the development of the genital ridge?

The significance of the expression of *gooseoid* in the genital is not clear at this time. Because homozygous mutant animals die 24 to 48 hours after birth, we were not able, for example, to assess possible abnormalities of sexual functions. However, we would like to speculate on a role of *gooseoid* in epithelial cell fusions and the descent of the testis.

gooseoid-expressing cells in the mesenchyme of the genital tubercle are fated to give rise to the external and internal muscles between which the fascia is located which is supposed to be the attaching tissue for the gubernaculum. In both sexes, the descent of the gonads depends on the gubernaculum, a ligamentous cord (Larsen, 1997). It remains to be examined if the descent of testis and ovary is impaired in *gooseoid* null mutant newborn mice.

Epithelial cell adhesion plays an important role within the three layers of the scrotal epidermis, stratum corneum, stratum spinosum and stratum germinativum, as well as for the process of the fusion of the labioscrotal folds during development. Highly glycosylated cell surface molecules play a decisive role in this process. It has previously shown that *gooseoid* mutant mice have altered cellular carbohydrate profiles at the site of the palatal-nasal fusion, and it was argued that these defects may constitute one of the elements for the lack of fusion of the nasal septum with the

secondary palate in the *gooseoid* mutant (Yamada et al., 1997). It is, therefore, tempting to speculate that abnormal cell-surface characteristics are involved in aberrant labioscrotal fusions in homozygous mutant mice. As *gooseoid* is expressed in the mesenchyme, and the defects arise in the overlying epithelium, these defects represent non-cell autonomous effects.

A non-cell autonomous function of *gooseoid* was previously described in the context of axial development of the frog *Xenopus* during gastrulation. Ectopic expression of *gooseoid* in ventral blastomers of 32-cell frog embryos by microinjection of synthetic mRNA resulted in fate changes of neighbouring cells which were consequently recruited into secondary embryonic axes (Niehrs et al., 1993). Thus, both during early and late phases of vertebrate embryogenesis *gooseoid* exerts some aspects of its function through the modulation of cell-to-cell signaling, probably by transcriptional activation or repression of genes encoding elements of signaling cascades.

The *gooseoid* expression site in the labioscrotal mesenchyme colocalized in part with the expression site of the androgen receptor (Crocoll et al., 1998). Androgen receptor is a steroid hormone receptor that is required for proper male sex development. Hox genes are expressed in the developing genital tubercle (Dolle et al., 1991; Lyons et al., 1992; Zelster et al., 1996; Hostikka and Capecchi, 1998) and the requirement for *Hoxa-10* for the reproductivity of mice has been demonstrated (Satokata et al., 1995). However, *Hox* genes are expressed in the urogenital system in a broader domain than *gooseoid*. It is not clear at this moment if there is a genetic interaction among these genes in the development of the genital tubercle. It will be interesting to investigate the genetic hierarchy of these genes in the development of the genital tubercle in the future.

In summary, there is a fairly good correlation between the sites of *gooseoid* expression at mid-embryogenesis and phenotypic alterations in mutant mice. However, as for the early phase of gene activity during gastrulation, where no axial defects were detected in homozygous null embryos, there are structures that express *gooseoid* during organogenesis and that are definitely normal in the mutant, as for instance the incus (compare Fig. 3 A/B) and the secondary palate (Gaunt et al., 1993; Yamada et al., 1995; Rivera-Perez et al., 1995). This indicates that complementing gene activities should exist, as was postulated for the gastrula.

3.4 A family of *gooseoid* related genes

In a systematic screen for *gooseoid*-related sequences a new gene has been cloned from genomic DNA by PCR using degenerate primers (Schweickert and Blum, unpublished). Recently, the same gene was also cloned by Galili et al. (1997), who have cloned and sequenced 38 kb of the syntenic region to the DiGeorge syndrome on mouse chromosome 16. The *gooseoid*-related gene, which was one of seven genes in this region, was named *gooseoid*-like (*Gscl*). Although expression of the human homolog of this gene was reported for the ninth and tenth week of development (Gottlieb et al., 1997), a detailed expression analysis of this gene was not performed as yet. Therefore it is not clear at the moment if this gene overlaps with *gooseoid* during gastrulation and/or organogenesis. However, the DiGeorge syndrome is characterized by defects related to branchial arches III and IV (Conley et al., 1979; Lammer and Opitz, 1986). Thus, if *Gscl* plays a role in the DiGeorge syndrome, it is likely to be expressed in the same region as *gooseoid* during organogenesis.

Another *gooseoid*-related gene, *GSX*, was described in the chick (Lemaire et al., 1997). Within the homeo domain, *gooseoid*, *Gscl* and *GSX* are quite similar, with 74 to 87 percent identical amino acid residues. Outside the homeodomain, the sole region with significant homology is a seven amino acid stretch in the amino-terminal part of the protein, which shows homology to engrailed and has been described as a repression domain (Smith and Jaynes, 1996).

GSX and *gooseoid* during gastrulation in the chick are initially co-expressed in Koller's sickle and in the primitive streak. The expression domains segregate in the process of gastrulation, such that *gooseoid* is expressed in the prechordal plate, and *GSX* in a non-overlapping manner in the developing neural plate (Lemaire et al., 1997). The individual roles of the different *gooseoid* genes in the process of gastrulation and organogenesis remain to be uncovered.

Recently, early phenotypes in axial patterning have been observed in *gooseoid* and *HNF-3 β* compound knock-out mice. In *gooseoid* *-/-* embryos, removal of one copy of *HNF-3 β* resulted in early phenotypes in day 8.75 embryos, including reduction of the forebrain and severe ventralization of the brain, accompanied by loss or reduction of *shh* and FGF-8 gene expression (Filosa et al.,

1997). These phenotypes indicate that *gooseoid* together with other genes is required for axial patterning.

3.5 The antagonism between the dorsal transcription factor *gooseoid* and the ventral signaling molecule BMP-4

As a transcription factor, mouse *gooseoid* has been shown to be a strong repressor (Danilov et al., 1998; Simth et al., 1996). This property of *gooseoid* is conserved from *Drosophila* to mouse (Mailhos et al., 1998). *gooseoid* protein can bind TAAT and ATTA DNA sequences. This has been shown for the binding of *gooseoid* to its own promoter (Danilov et al., 1998). A 1.5 kb BMP-4 promoter contains several TAAT sequences. In *Xenopus* gastrula embryos, misexpression of *gooseoid* led to repression of BMP-4 (Fainsod et al., 1994). In this study, I showed that BMP-4 promoter was able to mediate the repression by *gooseoid*. Although a detailed analysis has not been performed as yet, the data suggests that the repression of BMP-4 by *gooseoid* may be a direct regulation effect. It remains to be seen if mutation of TAAT and ATTA sequences abolishes the repression of the BMP-4 reporter gene by *gooseoid*.

In early *Xenopus* embryos *gooseoid* and BMP-4 are expressed on opposing dorsal and ventral sides of the embryo (Fainsod et al., 1994; Steinbeisser et al., 1995). Mis-expression of *gooseoid* in the ventral marginal zone of *Xenopus* embryo repressed BMP-4, and resulted in dorsalized embryos (Fainsod et al., 1994). In addition, it was shown that the secreted molecule *chordin*, which binds to BMP-4 and prevents its interaction with its receptors, is a (direct or indirect) target of *gooseoid*. Thus, *gooseoid* antagonizes BMP-4 by two mechanism, transcriptional repression and activation of the secreted molecule *chordin*. In contrast, the nature of the repression of *gooseoid* by BMP-4 remains elusive.

3.6 Neural crest cells are the primary targets of retinoic acid teratogenicity in early mouse embryogenesis

gooseoid gene expression in the branchial region was altered at E10.5 when embryos were treated with 20 mg / kg tRA at E8 + 5 h. This change in gene

expression correlated with specific teratogenic effects in the middle ear region, particularly affecting the tympanic ring and the three middle ear ossicles. For RA-treatment a regime was chosen that was shown previously to result in alterations of the tympanic ring (Kessel, 1992; Mallo, 1997), because this bone was specifically lost in *gooseoid* mutant mice (Yamada et al., 1995; Rivera-perez et al., 1995). However, as free and non-metabolized RA does not persist in the embryo after gavage application for more than approximately 8 h (Satre and Kochhar, 1989), and because *gooseoid* is not present at E8.5, or expression is below the detection level of radioactive *in situ* hybridization (Gaunt et al., 1993), the observation of RA-mediated changes in the expression of this gene seem to be an indirect event.

A number of reports have demonstrated specific effects of RA on neural crest cell formation and migration in the mouse (Mallo, 1997; Thorogood et al., 1982; Webster et al., 1986; Pratt et al., 1987; Lee et al., 1995; Gale et al., 1996). Mallo (1997) has shown in a recent paper that the middle ear ossicles were sequentially lost by RA treatment in a small time window between E8 plus 4.5 and E8 plus 7.5 h, and that these phenotypic changes were related to the RA-dependent transient inhibition of neural crest cell migration. In the light of this study I interpret the changes of *gooseoid* gene expression following RA treatment as the result of an altered population of neural crest derived mesenchymal cells in the branchial arches at E10.5. The two different patterns, either restriction of *gooseoid* gene expression to the branchial cleft region or complete absence of transcripts from arch I and II, most likely reflect differences of developmental stages of embryos at the time of RA administration.

This study was initiated because of the striking correlation of *gooseoid* expression at organogenesis, the knock-out phenotype, and specific RA teratogenic effects. A number of other genes have been described that - like *gooseoid* - are expressed in the forming middle ear bones and show specific malformations or deletions in knock-out mutant mice. The malleus, derived from arch I, was affected in mouse mutants of the genes *endothelin-1* (Kurihara et al., 1994), *msx-1* (Satokata and Mass, 1994), *Hoxa1* (Chisaka et al., 1992) and *Mhox* (Martin et al., 1995), the arch II derived ossicles incus and stapes were malformed or absent in the case of *endothelin-1* (Kurihara et al., 1994), *Mhox* (Martin et al., 1995), *Hoxa1* (Chisaka et al., 1992) and *Dlx-2* (Qiu et al., 1995), and the tympanic ring was changed or deleted in *Mhox* (Martin et al., 1995) and *endothelin-1* mutants (Kurihara et al.,

1994). It will be of relevance to analyze the expression pattern of these genes following RA administration in the sensitive time window on the ninth day of pregnancy (Mallo, 1997) in order to identify other candidate genes that could mediate the RA teratogenicity, particularly in the ossicles where *gooseoid* is not expressed. As the majority of these other factors - like *gooseoid* - represent homeobox genes it seems possible that they might govern the formation of middle ear ossicles and tympanic ring by utilizing similar molecular pathways. It is intriguing to analyze if and how these factors interact genetically in the development of the middle ear.

Teratogenic effects of RA have been described for several susceptible phases of mouse embryogenesis. Administration shortly after implantation between E4.5 and E5.5 resulted in the duplication of the genital region (Rutledge et al., 1994). Whereas *gooseoid* is not expressed at E4.5, we see transient expression in the developing genital eminence at E14.5 and E15.5 (Zhu et al., 1998). It will be particularly interesting to analyze if duplications of the genitals by RA treatment is associated with ectopic expression of *gooseoid* mRNA. RA treatment during and after gastrulation led to homeotic transformations of the axial skeleton (Kessel and Gruss, 1991; Kessel, 1992). As an effect of RA on *gooseoid* transcription has been described in *Xenopus* (Cho et al., 1991), RA effects during gastrulation and organogenesis (starting at E10.5) might in part be directly mediated by a change of *gooseoid* mRNA expression.

3.7 Perturbation of normal development at E10.5 by RA via alteration of expression of developmental genes

Treatment of mouse embryos at E10.5 with a single dosage of 20 mg/kg tRA resulted in craniofacial defects, like cleft palate. Three successive times treatments of mouse embryos at the same stage with the same dosage led to forelimb long bone malformations without perturbation of caudal vertebrae development compared with a single dosage of 100 mg/kg tRA treatment (data not shown). This is consistent with the notion that RA teratogenic effect is dosage and stage dependent.

Normal development of embryos is governed by coordinated expression of developmental genes. In the facial development, a number of signaling molecules and transcription factors are determining components. *gooseoid* is expressed in

the craniofacial region (Gaunt et al., 1993) and required for normal development of craniofacial structures such as middle ear bones, mandible, tongue, and nose (Yamada et al., 1995; Rivera-Perez et al., 1995) which are potential targets of teratogens. As a signaling molecule, BMP-4 is an antagonist of *gooseoid*, as was shown in early frog development. A role for BMP-4 in craniofacial development is supported by the fact that BMP-4 heterozygotes mutant mice exhibits craniofacial malformations (Dunn et al., 1997). Ectopic application of recombinant BMP-2 and BMP-4 proteins changed the patterning of the developing chick facial primordia (Barlow et al., 1997). Both loss- and gain-of-function studies implied that the dosage of BMP-4 is important for craniofacial development. The long arm of chromosome 14q22.1-q23.2 was deleted in a patient with craniofacial defects (Lemyre et al., 1998), and BMP-4 was mapped to this region of the chromosome.

Mouse embryonic day 10.5 is one of the sensitive time windows for RA-induced craniofacial defects. In the present study both *gooseoid* and BMP-4 were found to be repressed by RA treatment at this stage. The discovery itself implicates *gooseoid* and BMP-4 as two of the target genes of RA in mediating teratogenic effect in craniofacial development.

Other genes that have been found to be required for the development of craniofacial structures include the homeobox genes *Msx1* and *Msx2*. The expression of these two transcription factors are colocalized with BMP-4, and they have been shown to be target genes of BMP-4 (Vainio et al., 1993). In this genetic network, one part of the function of BMP-4 is mediated by the transcription factors *Msx1* and *Msx2*. Targeted mutagenesis of *Msx1* in mouse embryos resulted in cleft palate and malformation of middle ear ossicles (Satokata and Maas, 1994) which is reminiscent of RA teratogenic effect. Recently it was found that *Msx-1* and *Msx2* were down-regulated in developing chick facial primordium following RA treatment (Brown et al., 1997). In my study, *Msx1* and *Msx2* were found to be repressed rapidly by RA in mouse embryos as well. RA teratogenesis likely shares similar molecular mechanism among different vertebrate species.

Interestingly, all these genes are not only important for craniofacial development but also for limb development. All of the examined genes are expressed in the limb field. The function of these genes in limb development has been addressed by different approaches (Heanue et al., 1997; Belo et al., 1998; Zhu et al., 1998; Dunn et al., 1997; Ferrari et al., 1998). Developing forelimbs are

particularly sensitive to RA if mouse embryos are exposed between E10.5 to E11.5. The rapid down-regulation of *gooseoid*, BMP-4, *Msx1*, *Msx2* correlates with RA-induced malformation of limbs.

The vertebrate forelimb is divided into three zones: the stylopod (humerus), zeugopod (radius and ulna), and autopod (carpals, metacarpals and phalanges). RA treatment mainly results in malformation of humerus, radius and ulna in forelimb. It almost phenocopies the defects caused by single or double *Hoxa-11* and *Hoxd-11* mutations in mouse forelimbs (Davis et al., 1995). Activation of *Hox* genes in the developing limb is in a spatial and temporal sequence with the 3' genes expressed first and the 5' genes last, creating a transcriptional cascade of *Hox-9*, *10*, *11*, *12* and then *13* (Izpisua-Belmonte and Duboule, 1992). The development of limbs occurs in a sequence such that it proceeds through the humerus, the ulna and then anteriorly through the wrist, and that the anterior distal carpals are the last to be made (Duboule, 1994). *Hox 10-13* genes specify the development of each part of limb in a sequential manner from humerus to autopod. RA specific teratogenic effect on humerus, radius and ulna are presumably mediated either directly or indirectly by alteration of the expression of certain *Hox* genes in the limb.

Because the phenotypes of *Hoxa-11* and *Hoxd-11* closely resemble RA teratogenic effect at E10.5 on forelimb, both genes became interesting candidate molecules for the study. *Hoxd-11* is expressed at the right time and the right place when and where RA teratogenesis occurs in the developing limb. In the study I showed that the expression of *Hoxd-11* in the limb bud was repressed by RA treatment. This result suggests a role for the gene in mediating RA teratogenicity in developing limbs, especially in radius and ulna. RA treatment led to shortening and expansion of ulna and radius bones. This malformation is similar to the knockout phenotypes of *Hoxa-11* and *Hoxd-11*, but not identical. As observed for *gooseoid* and BMP-4, RA repression of *Hoxd-11* gene may be also in a ligand-dependent way. Ligand-dependent repression of genes is a temporary process. It is primarily different from gene targeting in embryos. RA teratogenesis involves a large spectrum of genes in embryos, like *gooseoid*, BMP-4, *Msx1*, *Msx2* and *Hoxd-11*. RA teratogenesis is a result of the alteration of all the genes caused by RA treatment.

Other signaling molecules and transcription factors also play important roles in craniofacial and limb development. The signaling molecule *shh* is implicated in

epithelial and mesenchymal interactions in craniofacial, body axis and limb development. Targeted disruption of *shh* revealed a critical role in patterning vertebrate embryonic tissues (Chiang et al., 1996). In *shh* null mutant mice the normal facial structures like nose, eyes and oral structures were not identifiable, and the sole remaining external feature of the mutant head is a proboscis-like extension that protrudes from the rostral midline. The development of limbs was severely affected as well. Application of RA in chick facial primordia down-regulated the expression of *shh* and delimits the out-growth of the facial primordium (Helms et al., 1997). Contrary, local application of RA to developing limb buds up-regulates *shh* expression and creates a new ZPA, and therefore leads to duplication of digits (Riddle et al., 1993). The knock-out phenotype of the transcription factor *AP-2* demonstrated important roles for craniofacial and limb development (Zhang et al., 1996; Schorle et al., 1996). One of the distinct phenotypes is the lack of the radius (Zhang et al., 1996) although not with 100 percent penetrance. This phenotype resembles to some extent the phenotype of *Hox11* knock-out mice and RA teratogenic effect. In chick embryos it was shown that *AP-2* was repressed by local application of RA (Shen et al., 1997). It seems that RA execute its teratogenic effect by alteration of expression of numerous key developmental genes.

Functional analyses of RARs and RXRs by gene targeting not only uncovered the important functions of these genes in normal development but also shed light on the functions of some of the receptors in mediating RA teratogenic effects. Mice null for retinoic acid gamma receptor (*RAR γ*) exhibit axial defects, including homeotic transformation of several vertebrae (Lonhes et al., 1993). Interestingly, *RAR γ* null mutants are completely resistant to RA-induced spina bifida (Lonhes et al., 1993), neural tube defects and craniofacial malformations (Iulianella and Johnes, 1997), suggesting that this receptor specifically transduces at least a subset of the teratogenic effects of retinoids in spina bifida, neural tube and craniofacial development. Interestingly, another retinoic acid X receptor (*RXR α*) is a component in the teratogenic process in the limbs (Sucov et al., 1995). Mouse embryos homozygous for a mutation in the *RXR α* gene appear normal in limb development although they have defects in heart development (Sucov et al., 1994; Kastner et al., 1994). RA treatments that cause limb defects in 100% of wild-type embryos fail to elicit malformations in *RXR α* homozygotes, and heterozygous embryos are

intermediate in sensitivity to RA, suggesting the importance of RXR α gene dosage in limb teratogenesis.

These two discoveries indicate clearly that RA teratogenesis is under genetical control. It is not a simple mass killing of cells by excess RA. Therefore, it is interesting to uncover the genes in mediating RA teratogenic effect and reveal the mechanisms of how RA teratogenesis takes place. In this study, the expression of *goosecoid*, BMP-4, *Msx1*, *Msx2* and *Hoxd-11* were analysed in both RXR α ^{+/-} and RXR α ^{-/-} genetical background with and without RA treatment of mouse embryos. All of the genes examined were normally expressed in both RXR α ^{+/-} and RXR α ^{-/-} mouse embryos. Although RXR α ^{-/-} mouse embryos had heart defects, expression of BMP-4 in developing heart was not changed in these embryos. All of the five genes were repressed following RA treatment of RXR α ^{+/-} mouse embryos, suggesting that these genes are involved in the RA teratogenesis process. However, in RXR α ^{-/-} genetic background, *Msx2* was not repressed by RA and BMP-4 was less repressed by RA than in RXR α ^{+/-} mouse embryos, suggesting that *Msx2* and BMP-4 are downstream of RXR α in mediating RA teratogenic effect. They might be two of the genes downstream of RXR α in transducing RA teratogenesis in limb. RA signaling is transmitted by RAR and RXR receptors. It is conceivable that inappropriate activation of these receptors in developing embryos by RA causes either ectopic expression or down-regulation of a large number of developmental genes that would lead to malformation of developing embryos, like the alteration of expression of the genes discovered in this study. Investigation of the mechanism how these genes are repressed by RA could help select those ligands that are effective in treatment of disease without inducing RA teratogenesis at the same time.

3.8 Mechanisms of down-regulation of *goosecoid* and BMP-4 by RA

Down-regulation of both *goosecoid* and BMP-4 by RA in mouse embryos at E10.5 was achieved 4 hours following RA treatment. A 4.5 kb mouse *goosecoid* promoter-luciferase reporter gene was found to be able to mediate RA repression in P19 cells. The repression appears to be in part mediated by RXR since cotransfection of RXR is necessary for the repression, and because a dominant

negative RXR α that lacks the AF-2 transactivation domain abolishes the repression. The latter result indicates that the AF-2 domain is required for the repression of *goosecoid* by RA.

Several studies have demonstrated that RA can repress the expression of genes by antagonizing *AP-1* activity (Schüle et al., 1991; Nicholson et al., 1990). However, no consensus *AP-1* binding site was found in 4.8 kb of the mouse *goosecoid* promoter. This suggests that other mechanisms may be involved in the repression of *goosecoid* by RA. Since *goosecoid* is not expressed in P19 cells, it is not possible to study the regulation by analysing endogenous *goosecoid* expression following RA treatment in P19 cells.

In mouse embryos BMP-4 was repressed by RA treatment. This effect was also seen on the endogenous BMP-4 gene in F9 cells. The BMP-4 promoter contains *AP-1* like sites, but one component of the *AP-1*, c-JUN is not expressed in F9 cells (van Dam et al., 1995). Nuclear protein extract from F9 cells can not bind to consensus *AP-1* response elements (Diccianni et al., 1992), indicating that F9 cells do not have *AP-1* activity. Therefore, down-regulation of BMP-4 by RA in F9 cells is not due to antagonizing *AP-1* activity by RA receptors. The fact that TPA did not increase the transcription of BMP-4 in F9 cells further supports this notion. Down-regulation of BMP-4 by RA does not require *de novo* protein synthesis, implying that the down-regulation may not be due to up-regulation of a repressor or repression of an activator by RA.

In F9 cells, the RAR α specific ligand Am80 can repress BMP-4 expression efficiently, while no repression was found with a RXR specific ligand. This suggests that RXR may not mediate the repression of BMP-4 expression alone. It does not exclude the possibility that RAR and RXR form heterodimer to transduce the repression of BMP-4 by RA.

Recently, it was reported that TR4, an orphan receptor, was up-regulated by RA in F9 cells (Lee et al., 1998). It recognizes the AGGTCA direct repeat (DR) of the hormone response element spaced by 1 and 5 base pairs (DR1 and DR5), which are also the response elements for RAR and RXR. TR4 represses RA-induced transactivation by competing the DNA binding site with RAR and RXR (Lee et al., 1998). In the BMP-4 promoter there is DR1 site that has been shown to be bound by another type of orphan receptor, COUP-TF1 (Feng et al., 1995). However, COUP-TF1 was not up-regulated in F9 cells by RA, so it is unlikely that down-regulation of

BMP-4 is mediated by COUP-TF1. The DR1 in the BMP-4 promoter could be a potential binding site for TR4. It remains to be seen if TR4 is the mediator for the repression of BMP-4 in F9 cells by RA.

A. Embryonic day 7.5 to 8.5



B. Embryonic day 8.0 + 4 to 7 h



C. Embryonic day 10.5 to 11.5

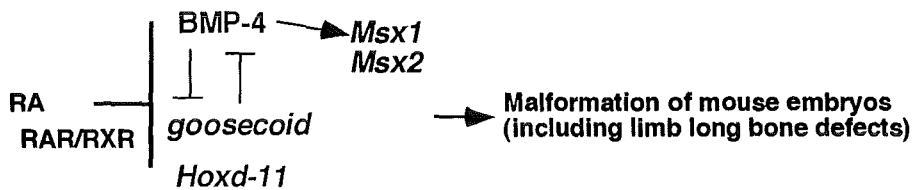


Fig. 25. A model for RA teratogenic effect on developing mouse embryos at different stages.

RA teratogenicity is dosage and stage-dependent for developing embryos. If mouse embryos are exposed to RA from E7.5 to E8.5, RA induces ectopic expression of Hox genes. Ectopic expression of Hox genes results in homeotic transformation of vertebrae (Fig. 25 A). Up-regulation of many Hox genes is due to ectopic activation of RA receptors that will bind to RAREs in the regulatory sequences of Hox genes. Treatment from E8 + 4 to 7 hours inhibits neural crest cell migration and causes different types of malformations of middle ear bones (Fig. 25 B). If mouse embryos are treated at E10.5 to E11.5 with RA, RA down-regulates many genes, such as, *goosecoid*, *BMP-4*, *Msx1*, *Msx2*, *Hoxd-11* and others. This treatment results in cleft palate, limb defects (Fig. 25 C). Down-regulation of genes by RA could be mediated via antagonizing AP-1 activity by RARs or RXRs.

Alternatively, RA may activate transcription factors that act as potent repressors. Another possible mechanism may involve in the degradation of certain general transcription factors following RA treatment, like SP1 that would result in down-regulation of many genes.

Understanding of the mechanisms of RA teratogenicity is particularly clinically relevant. It will help to develop and screen for RA analogs that exhibit the beneficial feature for the treatment of disease while lacking teratogenic potential.

4. Materials

All of the chemicals used in the study was of high quality from different companies. Below is only a list of special reagents and materials that were utilized in this study.

Reagents for radioactive *in situ* hybridization:

| Reagent | Company |
|---------------------------------------|------------------|
| Tespa (3-Aminopropyltriethoxy-silene) | Sigma |
| ATP, GTP, CTP | Boeringer Manham |
| Silicone solution | Serva |
| Triethanolamine | Sigma |
| Hypercoat nuclear emulsions | Amersham |
| Sodium Thiosulfate | Sigma |
| Entellan Neu | Merk |
| Mayer's Hematoxylin solution | Sigma |
| RNase inhibitor | Amersham |
| α - ³⁵ S-UTP | Amersham |

DNA Plasmids

- 1.2 kb *gooseoid* cDNA in pBluescript vector (from Dr. Martin Blum): for antisense RNA probe, digest the plasmid with SacI and transcribed with T3 RNA polymerase; for sense control probe, digest the plasmid with Hind III and transcribe with T7 RNA polymerase.
- 1.55 kb BMP-4 full length cDNA in pSP72 vector (ampicillin resistant, from Dr. Brigit Hogan): for antisense RNA probe, digest the plasmid with Acc I and transcribe with T7 RNA polymerase; for sense RNA probe, linearize with EcoRV and use Sp6 RNA polymerase for transcription.
- Msx1 cDNA with Sph fragment deleted in pTZ19 vector (from Dr. Richard L. Maas). Linearize the plasmid with EcoNI and transcribe with T7 RNA polymerase to generate antisense probe (about 500 bp).
- Msx2 full length cDNA in PCR II vector (from Dr. Richard L. Mass). Linearize the plasmid with BamHI and transcribe with T7 RNA polymerase for antisense probe; and digest the plasmid with EcoRI and transcribe with Sp6 RNA polymerase for sense probe.
- Hoxd-11 plasmid was got from Dr. Pascal Dollé. Antisense probe for Hoxd-11 was transcribed from HindIII-digested plasmid with T7 RNA polymerase, and sense probe was transcribed from BamHI-digested plasmid with Sp6 RNA polymerase.
- GAPDH plamid was linearized with PstI to isolate 1.3 kb GAPDH insert for Northern blot probe.
- mRXR α , mRXR β , mRXR γ and dnRXR α were offered by Prof. Dr. Pierre Chambon.

Retinoic acid

| | |
|-------------------------|------------------------|
| All-trans retinoic acid | Sigma |
| 9-cis retinoic acid | Sigma |
| Am80 and SR11237 | Dr. Hinrich Gronemeyer |

PCR primers

PCR primers for amplifying a 1.5 kb BMP-4 promoter: 5' primer: 5'-GCT CGA GAA TTC GCT AGG TAG AC-3'; and 3' primer: 5'-TAA GCT TTT AGG CCA TGT AGA-3'.

PCR primers for genotyping RXR mutant embryos: primer 1: 5'-ACT GCC TGA TCG ACA AGA GAC AGC G-3'; primer 2: 5'-GGG CTC TGC TGA GCA GGA GGA CA-3'; primer3: 5'-GCC CAT CCC TCA GGA AAT ATG-3'; primer4: 5'-GAT CAG CAG CCT CTG TTC CAC ATA C-3'. Wild-type embryos gave rise to a 250 bp DNA fragment, heterozygous RXR embryos showed a 300 bp and a 250 bp DNA fragment, and homozygous RXR embryos had a 300 bp DNA fragment.

Mouse embryos

1. Wild-type mouse embryos were derived from matings between C57BL/6J strain mice.
2. *gooseoid* null mutant embryos were provided by Dr. Gen Yamada.
3. RXR α +/- and RXR α -/- mouse embryos came from matings between RXR α +/- mice with FP104 genetic background (generously provided by Prof. Dr. Pierre Chambon's lab).

5. Methods

Preparation of competent cells

Inoculate a single colony of XL1 blue cells from an agar plate into 2.5 ml of LB or SOB medium, grow overnight at 37 °C. Subculture the cells from overnight growth in 1:100 ratio (i.e. add the 2.5 ml overnight growth cells into 250 ml SOB + 20 mM MgSO₄) in SOB +20 mM MgSO₄ and grow the culture until OD₅₉₀=0.4 - 0.6 (about 3.5 to 4.5 h). Centrifuge the culture at 5000 rpm for 5 min at 4°C. Gently resuspend pellet in 100 ml (40 ml for 100 ml culture in step 2) ice cold TFB1 and work on ice from this step onward. Incubate on ice for 5 min. Centrifuge at 5000 rpm for 5 min at 4 °C. Gently resuspend the pellet in 10 ml (4 ml for 100 ml culture from step 2) ice cold TFB2. Incubate on ice for 40 - 50 min. Aliquote the cells with chilled pipette tip in 100 µl volume in ice-colded eppendorf tubes and immediately freeze in liquid N₂. Store at -80°C.

Solutions:

LB medium: Per liter, add

| | |
|---------------------|------|
| bacto-tryptone | 10 g |
| bacto-yeast extract | 5 g |
| NaCl | 10 g |

add distilled H₂O to 950 ml, adjust the pH to 7.0 with 5 N NaOH (about 0.2 ml). Adjust the volume of the solution to 1 liter with distilled H₂O, and autoclave.

SOB medium: For one liter, in 950 ml distilled H₂O add

| | |
|---------------------|-------|
| bacto-tryptone | 20 g |
| bacto-yeast extract | 5 g |
| NaCl | 0.5 g |
| 250 mM KCl | 10 ml |

Adjust the pH to 7.0 with 5 N NaOH (about 0.2 ml). Add dH₂O to 1 liter.

Dissolve all components and autoclave.

Just before use, add 5 ml of a sterile solution of 2 M MgCl₂. Both KCl and MgCl are made sterile by autoclaving.

| | | |
|------------------|----------------------|-------|
| TFB1: for 100 ml | RbCl ₂ 1M | 10 ml |
| | MnCl ₂ 1M | 5 ml |
| | KAc 1M | 3 ml |
| | CaCl ₂ 1M | 1 ml |
| | Glycerol | 15 ml |
| | H ₂ O | 66 ml |

add one drop of HAc to pH 5.8, sterilize by filtering through 0.2µm filter.

| | | | |
|------------------|----|---------------------------|---------|
| TFB2: for 100 ml | 1M | MOPS (pH 6.5 with 1N KOH) | 1 ml |
| | 1M | RbCl ₂ | 1 ml |
| | 1M | CaCl ₂ | 7.5 ml |
| | | Glycerol | 15 ml |
| | | H ₂ O | 75.5 ml |

sterilise by filtering through 0.2µm filter.

Transformation

Add plasmid DNA (1 - 5 µl, up to 0.1 µg DNA is enough) to 100 µl competent cells and mix with pipette tip. Store the tubes on ice for 30 min. Controls include competent bacteria that receive no plasmid DNA and competent bacteria that receive a known amount of the standard preparation of supercoiled plasmid DNA. For cloning, two enzyme-digested plasmid after ligation should be included as a negative control. Transfer the tubes to a rack placed in a circulating water bath that has been preheated to 42 °C. Leave the tubes in the rack for exactly 90 seconds (37 °C for 40 seconds also works). Don't shake the tubes. Rapidly transfer the tubes to an ice bath. Allow the cells to chill for 1-2 min. Add 800 µl LB medium to each tube. Transfer the tubes to a shaking incubator set at 37 °C. Culture the bacteria at 37 °C with less than 225 cycles/min for 45 min. Add all of each in 200 ml LB ampicillin or other antibiotics depending on the plasmid (100 µg/ml) medium for maxiprep growth. Or centrifuge each tube at 2500 rpm for 3 min, remove about 700 µl medium and resuspend the bacteria pellet in the rest 200 µl medium and plate all of each onto an agar LB medium plate containing 100 µg/ml ampicillin for selection of positive clones. For maxiPrep, grow bacteria at 37 °C with 250 rpm/min overnight. For cloning, invert plates and grow at 37°C overnight.

MiniPrep of plasmid DNA

Pick colony from plate, grow overnight at 37 °C in 3 ml LB -ampicillin medium. Transfer 1.5 ml into an Eppendorf tube, and spin 1 min at room temperature. Suck off the supernatant. Add 100 µl Solution 1 to bacterial pellet, vortex until suspended. Open lid and add 200 µl Solution 2. Close lid, and invert tube 3-4 times. Add 150 µl Solution 3, close lid, turn in inverted position for 10 sec. Spin 5 min at RT. Transfer the supernatant to fresh tube. Add equal volume (450µl) phenol-chloroform (1:1), vortex, spin 2 min at RT. Transfer aqueous phase to new tube, add 1 ml ethanol, vortex, incubate 2 min at RT. Spin 5 min at RT, wash pellet with 70% ethanol (don't vortex). DNA pellet is dried in air. Dissolve DNA in 50 µl TE-RNAase A (20µg/ml), vortex and spin down. Digest about 5 µl for verifying positive clones. Run on agarose gel.

| | | |
|--------------------|-------|-------------------|
| 100 ml Solution 1: | 5 ml | 1M Glucose |
| | 2.5ml | 1M Tris.Cl pH 8.0 |
| | 2 ml | 0.5M EDTA pH8.0 |

dd H₂O to 100 ml

Always prepare fresh Solution 2, for 200 µl, add 40 µl 1N NaOH
 10 µl 20% SDS
 150 µl ddH₂O

100 ml Solution 3: 60 ml 5M Potassium acetate (KAc)
 11.5 ml Glacial acetic acid
 28.5 ml ddH₂O

MaxiPrep of DNA

Maxi preparations of plasmid DNA were done by either QIAGEN column and reagents or Nucleobond column and reagents. Inoculate 200 ml LB ampicillin (100 µg/ml) medium with bacteria carrying the plasmid. Grow overnight at 37°C. Next day add bacteria culture to a plastic tube, centrifuge for 10 min/5000 rpm/4 °C. Drain the pellet by inverting the tubes.

QIAGEN protocol:

Resuspend the bacteria pellet in 10 ml of buffer P1 + 1000 µg RNase A. Add 10 ml of buffer P2, mix gently and incubate at room temperature for 5 min. Add 10 ml of buffer P3, mix immediately but gently, and centrifuge in a 30 ml-volume tube at 4 °C for 45 min with a speed of 12000 rpm. Equilibrate a QIAGEN-tip 500 with 10 ml of buffer QBT and allow it to empty by gravity flow. Pour the supernatant from the centrifuge tube through a cotton cloth to the equilibrated QIAGEN-tip and allow it to enter the resin by gravity flow. Wash the QIAGEN-tip with 30 ml of buffer QC. Elute the DNA from the tip with 15 ml buffer QF. Precipitate the DNA with 0.7 volumes of isopropanol (10.5 ml), previously equilibrated to room temperature, and centrifuge in a 30 ml-volume glass tube at 4 °C for 30 min at a speed of 9000 rpm.

Composition of buffers

Buffer P1 50 mM Tris/HCl, 10 mM EDTA, pH8.0, add RNase A to 100 µg/ml, store at 4°C

Buffer P2 200 mM NaOH, 1%SDS

Buffer P3 2.55 M KAc, pH4.8

Buffer QBT 750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100

Buffer QC 1.0 M NaCl, 50 mM MOPS (pH7.0, 15% ethanol, pH 7.0

Buffer QF 1.25 M NaCl, 50 mM Tris/HCl (pH8.5), 15% ethanol, pH 8.2

Store buffers P2, P3, QBT, QC, and QF at room temperature.

Nucleobond protocol as described by the manufacturer.

Digestion of DNA with restriction enzymes

Digest 1 to 10 µg DNA with restriction enzymes in a volume of 20 to 100 µl. Restriction enzyme could be 1 to 3 µl (10 to 30 U). One U of restriction enzyme is designated as the

amount of the enzyme that can digest 1 μg λ DNA at 37 °C within 1 hour. The volume of the total restriction enzymes in the reaction tube should not exceed 10% of the total volume. Otherwise, the glycerol from the stock enzyme will inhibit the reaction. Add corresponding 10 x restriction buffer in 1/10 of the total reaction volume. Add ddH₂O to the desired reaction volume. Vortex the reaction mixture and spin down shortly. Incubate the tubes in 37 °C warm room for 1 hour to overnight (For certain enzymes, other special reaction temperatures are required). Here is an example of digestion of 5 μg DNA (1 $\mu\text{g}/\mu\text{l}$ plasmid DNA in TE buffer) with HindIII restriction enzyme:

5 μl plasmid DNA

10 μl 10 x restriction buffer E

2 μl Hind III (10u/ μl)

83 μl dd H₂O

vortex and spin down, incubate at 37 °C for 1 hour or longer.

Repairing 3' or 5' overhanging ends to generate blunt ends

In a 20 μl reaction, digest 0.1 to 4 μg DNA with a restriction endonuclease. Add 1 μl of 0.5 mM of each dNTP. Add 1 to 5 U of the Klenow enzyme and incubate at 30 °C for 45 min. Stop the reaction by heating to 75 °C for 10 min or by adding 1 μl of 0.5 M EDTA.

Dephosphorylation of DNA

Calf intestine phosphatase (CIP) catalyzes the hydrolysis of 5'-phosphate residues from DNA, RNA, and rib and deoxyribonucleoside triphosphates. The dephosphorylated products possess 5'-hydroxyl termini which can subsequently be radioactively labeled using [γ -³²P]ATP AND T4 polynucleotide kinase. On the other hand, dephosphorylation of vector alone can prevent it from self-ligation. CIP requires Zn²⁺ for activity. CIP is readily inactivated by heating to 70°C for 10 min. CIP reaction conditions for dephosphorylation of DNA:

For 50 μl reaction:

20 mM Tris/HCl, pH 8.0

1 mM Mg Cl₂

1 mM ZnCl₂

1 to 20 pmol DNA termini (1 μg of a 3kb linear DNA contains 1pmol of 5' termini.)

0.1 U CIP

Incubate at 37 °C for 30 min, and stop reaction by heating to 75 °C.

Extraction of protein from nucleic acid

Add an equal volume of phenol/chloroform (1:1) to DNA solution, vortex and centrifuge at 13000rpm for 5 min in Eppendorf centrifuge. DNA is in upper phase (aqueous phase), and protein is in inter phase. Carefully transfer the upper aqueous phase to a new tube and precipitate the DNA from the solution.

Electrophoresis of DNA in agarose gel

Prepare 1% or 1.5% agarose gel in 1 X TBE buffer in a volume of 50 or 300 ml in microwave until boiling. Cool down the agarose gel solution to about 60°C, and then add 2.5 µl of 10 mg/ml ethidium bromide to 50 ml gel solution. 1% gel is suitable for separating 700 bp to 6 kb DNA fragments, and 1.5% for shorter fragments like 200 bp to 2 kb. Add 10 x loading buffer in a volume of 1/10 of the DNA solution to DNA sample. Load DNA solution in 1 x loading buffer to the gel slots. Run the gel in 1 x TBE buffer at constant voltage around 120 V. Visualize DNA fragments under UV light.

10 X TBE buffer (1 liter): Tris base 108 g
 Boric acid 55 g
 Na₂EDTA.2H₂O 9.3 g
 H₂O to 1000 ml, filter.

Precipitation and purification of DNA fragments

Precipitation of DNA: Add 10 X TNE buffer in a volume of 1/10 of the DNA solution. Then add cold ethanol in a volume of 2.5 times of the total DNA solution (including 10 X TNE buffer) volume. Vortex and centrifuge at 13000 rpm for 10 min. Discard solutions carefully and keep the DNA pellet on the bottom of the Eppendorf tube. Wash the DNA pellet carefully with 70% cold ethanol (don't vortex). Dry the pellet in air.

Purification of DNA fragments: Cut the DNA fragment from agarose gel and put it in a Spin-X column (Costar, Corning Costar Corporation, USA) and spin the DNA down at 13000 rpm for 10 min in Eppendorf centrifuge. The eluted DNA solution could be used directly for cloning.

Another very efficient DNA purification way is to use DNA purification kit (Biozym, Germany): Cut DNA band and measure the volume (100 mg = 100 µl). For TBE gel, add 1/2 volume of **MELT**, then 4.5 volume **SALT**. 55 °C, 5 min incubation and mix (agarose must be completely dissolved). Vortex **BIND**. add 5 µl +1 µl/µg DNA **BIND** (i.e. 6.5 µl **BIND** for 1.5 µg DNA). 5 min incubation at room temperature, mix. Spin 5 seconds, discard the supernatant. Resuspend the pellet in 1 ml **WASH**. Spin 5 seconds, discard the supernatant. Short spin, discard the supernatant. Dry the pellet. Add 10 - 20 µl ddH₂O or TE (pH7.8) buffer, resuspend. 5 min incubation at room temperature. 1 min spin, remove the DNA solution to a new tube and store DNA solution at 4 °C or - 20 °C.

Ligation reaction

Ligation reactions are usually done in a 10 µl volume. Add 1 µl 10 X T4 DNA ligase buffer, 1 to 4 µl of DNA solutions, 1 µl 5 mM ATP, 1 µl T4 DNA ligase, and add ddH₂O to a final

volume of 10 μ l. Mix well and leave the reaction tubes in a 16°C water bath overnight. Perform transformation next day with 5 μ l of the ligation solution.

Sequencing

Use the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Life Science). Components of the kit: **Thermo sequenase DNA polymerase**, 4 U/ μ l, 0.0006 U/ μ l *Thermoplasma acidophilum* inorganic pyrophosphatae, 50 mM Tris.HCl, pH8.0, 1 mM dithiothreitol (DTT), 0.1 mM ethylenediamine tetraacetic acid (EDTA), 0.5% Tween-20, 0.5% NonideTMP-40, 50% glycerol. **Reaction buffer**: 260 mM Tris.HCl, pH9.5, 65 mM MgCl₂. **dGTP nucleotide master mix**: 7.5 μ M dATP, dCTP, dGTP, dTTP. **dITP nucleotide master mix**: 7.5 μ M dATP, dCTP, dTTP, 37.5 μ M dITP. **Stop solution**: 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF.

Brief description of the protocol of sequencing with above mentioned kit. Choose the termination master mix, dGTP or dITP. dGTP is fine for normal GC content sequence, while dITP is designed for GC rich sequence. Prepare the 4 termination mixes using 2.0 μ l of termination master mix and 0.5 μ l of labeled ddNTP for each sequence (see below):

| | 1 sequence | | | | 5 sequence | | | |
|----------------------------------|------------|-----|-----|-----|------------|------|------|------|
| | G | A | T | C | G | A | T | C |
| Termination master mix(μ l) | 2.0 | 2.0 | 2.0 | 2.0 | 10 | 10 | 10 | 10 |
| α - ³³ P ddGTP | 0.5 | | | | 2.5 | | | |
| α - ³³ P ddATP | | 0.5 | | | | 2.5 | | |
| α - ³³ P ddTTP | | | 0.5 | | | | 2.5 | |
| α - ³³ P ddCTP | | | | 0.5 | | | | 2.5 |
| Total | 2.5 | 2.5 | 2.5 | 2.5 | 12.5 | 12.5 | 12.5 | 12.5 |

Dispense termination mixes: label four tubes (`G`, `A`, `T`, `C`) for each sequence. Fill tubes with 2.5 μ l of the appropriate termination mix prepared above and cap. Prepare the reaction mixture: combine the following,

| | |
|---|------------|
| Reaction buffer | 2 μ l |
| DNA (0.5 -1 μ g) | _ μ l |
| Prime (2.5 pmol) | _ μ l |
| ddH ₂ O | _ μ l |
| Thermo Sequenase TM DNA polymerase | 2 μ l |
| Total | 20 μ l |
| Mix well | |

Cycling termination reaction: Transfer 4.5 μ l of the template and polymerase mixture from last step to each termination tube (`G`, `A`, `T`, and `C`). Mix well . Cap and place the tube in the thermal cycling instrument. Cycle 30-60 times as follows:

| dGTP | dITP |
|-------------|----------------|
| 95°C, 30s | 95°C, 30s |
| 55°C, 30s | 50°C, 30s |
| 72°C, 1 min | 60°C, 5-10 min |

Stop the reaction by adding 4 μ l of stop solution.

Heat samples to 70 °C for 2-10 min immediately before loading onto sequencing gel. 3 -5 μ l/lane.

Preparation and running of a sequencing gel: Clean the plates of sequencing apparatus, wash both plates with detergent. Get rid of detergent and H₂O with Kimwipes. Use ethanol to clean the glass (which doesn't have any attachments), then clean it with H₂O, then dry carefully. Siliconize the ear plate with SIGMACOTE (Sigma), and air dry. Assemble the plates with spacers so that the thicker part (0.4mm) is down and the thinner part (0.25mm) is up. Put the rubber slice inside the casting tray, one on the bottom, the other standing on the opposite to the screw, and put a strip of Whatman 3 mm paper on the rubber layer on the bottom. Prepare the polymerizing solution:

| | Small gel | big gel |
|----------|-------------|-------------|
| Urea mix | 90 ml | 150ml |
| 10% APS | 375 μ l | 750 μ l |
| TEMED | 150 μ l | 300 μ l |

Pour it quickly on the paper in the tray, and then put the assembled glass apparatus in the casting tray. Let the solution enter into the interspace between the plates, then fix with the screw. Let it polymerize for 10 min. Prepare gel solution:

| | Small gel | Big gel |
|----------|-------------|-------------|
| Urea mix | 90 ml | 150 ml |
| 10% APS | 750 μ l | 900 μ l |
| TEMED | 75 μ l | 90 μ l |

Mix well, and then pour it slowly into the space between the two plates. Then put the comb upside down (about 0.4 cm) between the two plates, and clip both plates well. Let the gel polymerize for at least 1 h. Disassemble the lower tray and put the glasses into gel running tray. Add 1xTBE buffer. Take away the comb, and add 1xTBE. Use syringe with a needle to displace urea, and add 2 μ l stop buffer in order to see the upper line of the gel clearly. Insert the right side of the comb slightly into the gel and the sequencing reaction solution will be added into each of the comb spaces, and leave the comb there, and add 2 μ l stop buffer to each of several slots to check if leaky or not. Load 2 μ l stop buffer to each lane, and pre-run the gel (small gel: 100 w, 50 °C, big gel: 120 W, 50 °C) about 15 min until the gel reaches 50 °C. After

pre-run, displace Urea again with a needle just before loading the samples. Samples should be heated with open lid at 70 °C for 10 - 20 min and then load them immediately onto sequencing gel. Run the big gel at 120 W, 50 °C, the small gel at 100 W, 50 °C about 2.5 h until the first blue band to the bottom, and the second blue band to 2/3 of the gel. Dispose the lower 1xTBE as radioactive waste, upper 1xTBE as normal waste. Disassemble the plates carefully, leave the gel on one side of the plate(without ears). Put 1 layer of Whatman 3 mm paper on the gel, and remove the glass, put one layer of plastic membrane on the gel. Dry the gel at 80 °C for about 2 h. Put film directly on the gel. Expose the film overnight at -80°C with intensifying screen.

Urea mix (6% acrylamide/N,N'-methylenebisacrylamide mix): for 500 ml

| | |
|-------------------|-----------|
| Urea 7M | 210 g |
| 10XTBE | 50 ml |
| 30% acr/bis mix | 100 ml |
| dH ₂ O | to 500 ml |

Filter through 0.2 µm filter and keep at 4 °C in dark.

Isolation of genomic DNA from mouse tails

Cut 2 cm tail. Add 750 µl of 50 mM Tris-HCl (pH8), 100 mM EDTA, 100 mM NaCl, 1% SDS, 0.5 mg/ml proteinase K. Incubate at 55 °C overnight. Mix 5 min on Eppendorf mixer. Add 300 µl 5M NaCl, Mix 5 min on an Eppendorf mixer. Spin 5 - 10 min 13000 rpm. Take 750 µl without top phase and pellet to a new tube. Add 500 µl isopropanol, vortex, spin 13000 rpm for 10 min. Wash the DNA pellet with 1.5 ml 70% ethanol. Dry the pellet in air. Dissolve the pellet in 100 - 500 µl TE buffer.

PCR reaction to amplify DNA fragment from plasmid

PCR reaction was performed in 100 µl volume. Add 100 ng plasmid DNA, 10 µl of 10xDNA polymerase buffer, 0.2 mM dNTP mix, 1 µg each of 5' and 3' primer, 150 µmol MgCl₂, 5 U of Taq polymerase and ddH₂O to final volume of 100 µl. First denature at 94 °C for 4 min, in each cycle denature at 94 °C for 1 min, annealing for 2 min at the temperature 5 °C lower than the melting temperature of the primer with the lower melting temperature. Primer extension at 72 °C for 1 min. Number of cycles about 25 to 30. The last primer extension time was 10 min.

PCR reaction to amplify DNA fragment from genomic DNA

Embryonic visceral endoderm tissue was used for isolation of genomic DNA. 4µl of the genomic DNA was digested with BamHI in a 20µl volume. Then take 2µl of the digested DNA for amplification of a genomic DNA fragment that has no Bam HI site by PCR. PCR reaction was done in a 20 µl volume in which the following reagents were added: 2 µl of BamHI-digested genomic DNA, 10 pmol of 5' primer, 10 pmol of 3' primer from the promoter, 2 µl of 10xPCR buffer, 0.25 mM dNTPs, 1.5 mM MgCl₂, and ddH₂O to 20 µl. Denature at 94 °C for 10 min, in each cycle denature at 94 °C for 40 seconds, annealing at the temperature 5°C lower

that the primer with lower temperature for 50 seconds, extension at 72 °C for 1 min/kb, at last step extension of primers at 72 °C for 10 min. PCR was done with 40 cycles.

TA cloning

DNA fragment was amplified from genomic DNA by PCR and cloned into pCRII-TOPO vector (Invitrogen) with TA cloning protocol provided by the manufacturer. If concentration of the PCR product is high, 10-20 times dilution is necessary.

Cell culture

P19 cell culture: P19 teratocarcinoma cell line was cultured in gelatinized cell culture dishes (incubate autoclaved 0.1% gelatin in PBS in cell culture dishes for 30 min at RT) supplemented with alpha medium that contains 10% heat inactivated fetal calf serum, 1% glutamax, 1% non essential amino acids and 1% penicillin. Cells were cultured in a cell culture incubator at 37°C with 95% humidity and 5% CO₂. Cells were split when they reach confluency.

Culture of F9 cells: F9 cells were cultured in gelatinized cell culture dishes supplemented with Hans' F-12/DMEM (i.e., F-12/Dobeco's 1:1) medium containing 2 mM glutamine, 150 µM mercaptoethanol and 10% fetal bovine serum. Cell culture condition is the same as that for P19 cells.

DNA transfection with coprecipitates of calcium phosphate and DNA:

Split cells at the density of 5 x 10⁵ cells/9cm dish the day before transfection. Prepare DNA precipitates: for each dish, add 10µg DNA (reporter+internal control+carrier) and ddH₂O in a sterile Falcon tube in a volume of 225 µl, and then add 25 µl 2.5 M CaCl₂, and then use an electric pipette to create air bubbles in the solution and meanwhile add 250 µl of 2xHBS into the tube drop by drop, vortex for 5 seconds, and incubate for 20 min at RT. Before adding precipitates to cells, vortex the tubes and pipette the precipitates up and down several times in order to mix well. Leave DNA precipitates on cells for 12-16 hours (overnight), and wash the precipitates with PBS (Mg²⁺/Ca²⁺ free) and add new medium. If any treatment of cells should be done, such as treatment of cells with 1 µM all-trans retinoic acid, do it at this step. Harvest cells 24 hours or more after washing of precipitates for luciferase assay and β-galactosidase assay.

Luciferase and β-gal assay

Assay of luciferase: Wash cells twice in PBS without Ca²⁺ and Mg²⁺ carefully since Ca²⁺ will inhibit luciferase activity. Remove all PBS from cultures and keep cultures on ice. Add **lysis buffer** (500 µl/10cm dish) to dishes and rock occasionally to distribute buffer evenly over plates. Collect lysate with pipette into precooled Eppendorf tubes. Clear lysate by 5 min

centrifugation at 13000 rpm at 4 °C. Transfer 100 µl of the supernatant for determining luciferase activity. Measurement is done in a luminometer with automatic injection of **luciferin assay solution** of 100 µl and 350 µl of **assay buffer**.

Solutions:

Lysis buffer, 0.1 M Tris acetate pH7.5(dissolve Tris base in ddH₂O, and adjust pH to 7.5 with acetate acid).
2 mM EDTA
1% Triton X100

For 100 ml lysis buffer: add 10 ml 1M TrisAcetate, 400 µl 0.5M EDTA, 1 ml 100% Triton X100 and dH₂O to 100ml.

Glycylglycine buffer: 25 mM Glycylglycine
15 mM MgSO₄
4 mM EGTA
pH7.8

For 1000 ml, dissolve 3.3g Glycylglycine , 3.69g MgSo₄ and 1.52g EGTA in distilled H₂O, adjust pH to 7.8.

Luciferin Stock: 1 mM (0.28 mg/ml) luciferin in glycylglycine buffer, store at -20°C in dark.

Assay buffer(350µl/test): 1 mM DTT
2 mM ATP
in glycylglycine buffer

For 10 ml, add 100 µl of 0.1M DTT and 200µl of 0.1M ATP to 9.7 ml glycylglycine buffer.

Luciferin assay solution (100 µl/test): Dilute luciferin stock 1:5 in glycylglycine buffer, final concentration : 20nmol/test.

Assay for beta-galactosidase in solution: Place 50 µl of cell extract (from the cell extract used for luciferase assay) into u-wells, Use extraction buffer for determination of reagent blank. Add 200 µl of **reagent mix**. Incubate at 37°C in bacteria or cell incubator. Read A₄₂₀ (alternatively A₄₀₅) when all of the reactions have developed a clearly visible yellow colour which might take about more than 30 min.

Solutions:

Sodiumphosphate buffer: 0.1M Sodiumphosphate pH7.5 (0.082M Na₂HPO₄ + 0.018M NaH₂PO₄)

ONPG buffer: 8 mg/ml ONPG (o-nitrophenyl-β-D-galactopyranoside) in 0.1M sodiumphosphat buffer

100xMg: 0.1M MgCl₂, 4.5M 2-Mercaptoethanol

Reagent mix(per sample): 175 μ l of 0.1M Sodiumphosphate
 23 μ l of ONPG buffer
 2 μ l of 100xMg

β -galactosidase staining of cells in culture

In order to know how efficient DNA transfection is, it would be necessary to stain cells for β -galactosidase activity and count the number of blue cells after transfection of cells with CMV-lacZ expression vector. Rinse cells with 1 X PBS. Fix for 5 min at 4°C in 2% paraformaldehyde (in PBS without Ca²⁺ and Mg²⁺). Rinse cells with 1 X PBS 3 - 4 times. Overlay cells with **staining solution**, incubate from 30 min to overnight at 37°C. Check blue cells under microscope.

Preparation of reagents: Dissolve X-gal in DMSO at 40mg/ml, store at -20°C. Prepare 500mM potassium ferricyanide (K3) in and 500mM potassium ferrocyanide (K4) in PBS (withou Ca²⁺ and Mg²⁺) and aliquot and store in dark at -20°C. Thaw K4 or K3 only once. Make staining solution fresh each time. 6 ml **staining solution** is enough for 9cm dish. Add reagents in the following order (important) to make **staining solution**:

| | 10ml | 25ml | 50ml | final concentration |
|--------------------|-------------|-------------|--------------|---------------------|
| X-gal | 250 μ l | 625 μ l | 1250 μ l | 1mg/ml |
| K3 | 100 μ l | 25 μ l | 500 μ l | 5mM |
| K4 | 100 μ l | 250 μ l | 500 μ l | 5mM |
| MgCl ²⁺ | 200 μ l | 500 μ l | 1000 μ l | 2mM |

Add 1 X PBS (Ca²⁺/Mg²⁺ free) to the final volumes.

Total RNA isolation

Isolation of total RNA was done with peqGOLD TriFast™ solution (peQLab Biotechnologie GmbH). Wash cells with PBS without Ca²⁺ and Mg²⁺. Leave plates on ice. Add 6.3 ml peqGold TriFast solution to the cells in 9cm dish, and pipet cells up and down several times. Transfer all lysate to a 12ml steril Greiner polypropyleve tube. Leave the samples at room temprature for 5 min. Add 1.26ml chloroform to each tube, vortex 15 seconds. Incubate at RT for 3 -10 min. Centrifuge at 12000 rpm at 4 °C for 5min. Transfer 60% the upper aquous phase into a new tube. Add 3.15 ml isopropyl alcohol (2-propanol) to each tube. Incubate at RT for 5 -15 min. Centrifuge 12000 rpm at 4 °C for 10 min. Wash RNA pellet with 75% ethanol. Dry RNA pellet for 5 -10 min (not longer than this. Otherwise, RNA can not be dissolved. Dissolve RNA pellet in a very small amount (30 to 50 μ l) of DEPC H₂O.

Determination of Nucleic acid concentration

DNA or RNA solution is usually diluted in ddH₂O in a 1:200 ratio (2.5µl DNA solution in 497.5µl ddH₂O). The concentration of the diluted DNA solution is measured by its optical density (OD) at 260 and 280. The concentration of the original DNA solution = $OD_{260} \times 10 \mu\text{g}/\mu\text{l}$ and RNA concentration = $OD_{260} \times 8 \mu\text{g}/\mu\text{l}$. An $OD_{260} = 1$ is equivalent to 50 µg/ml of double stranded DNA or 40 µg/ml RNA or 20 µg/ml single stranded oligonucleotide. The OD_{280} is used as an indication of the purity of the nucleic acid.

Northern Blot

Electrophoresis of RNA in horizontal agarose gel: Prepare 1% formaldehyde agarose gel as following: melt 0.5 g agarose in 37.7 ml H₂O in microwave, cool down to about 60°C, add 5.0 ml 10xMOPS buffer and 8.2 ml of 37% formaldehyde. Pour this gel solution into a gel chamber with appropriate comb. Sample preparation, add up to 15 µl of RNA (3 - 5 µg polyA or 20 µg total RNA), add 16 µl of **the Sample Buffer Stock Solution** to each RNA sample and heat at 65 °C for 10 min, cool on ice, add 4 µl **RNA Dye Solution**, spin, load on to the gel which is in 1xMOPS buffer. Run the gel under a fume hood with 100 volts at RT.

Post electrophoresis processing: The gel is photographed while still on the gel tray. Put a plastic ruler along the side of the gel. Minimize the exposure of the gel to UV light. Transfer the gel with the tray to a large baking dish containing distilled H₂O. Change the H₂O after two min. Pour off the dH₂O after 5 min and add enough 50 mM NaOH to cover the gel well and incubate with gentle shaking for 30 min. Pour off the NaOH, add an excess of 100 mM Tris-HCl pH7.0. Make three changes of this buffer, 10 min each. Finally soak the gel in 10xSSC for 5min. Set up capillary blotting apparatus with layers of 3 layers of Whatman 3MM paper in contact with the transfer buffer (10xSSC), the gel, the blotting membrane (nylon membrane: Hybond-N+), put parafilm around the gel to avoid paper towels touching whatman paper and 10xSSC directly, 3 layers of Whatman 3 MM paper, lots of paper towels, heavy books (about 500 g), make sure that there are no air bubbles under the gel, under the blotting membrane or on the top of the blotting membrane. Transfer RNA from gel to membrane overnight with 10xSSC.

UV crosslink and prehybridization: Transfer the blotting membrane to a tray of 2xSSC and remove any adhesive agarose on the gel. Dry the membrane in air at least for 1 h. Put the RNA side to UV light in a Stratalinker device and autocrosslink RNA with the membrane. Transfer the membrane back to 2xSSC and then to H₂O. Use QuickHyb solution (Stratagene) for prehybridization and hybridization, prehybridize the membrane in 5 or 10 ml QuickHyb solution at 68 °C for 20 to 30 min.

Preparation of [α -³²P]dCTP labeled probe: Denature 20 - 50 ng DNA template, 95 °C, 5 min, then on ice. Add denatured DNA in a final volume of 45 µl to a Rediprime tube, flick the tube to

mix and spin briefly, add 5 μ l of [α - 32 P]dCTP, pipet up and down to mix, spin briefly, incubate at 37 °C for 30 min. Purify the probe with QIAGEN Quick Nucleotide Removal Kit. Take 1 μ l of probe for measuring the counts.

Hybridization: Add the labeled probe to 100 μ l of 10 mg/ml sonicated salmon sperm DNA, boil the double-stranded probe and salmon sperm DNA mixture for 2 min and denature the probe on ice. Place the probe into the bag containing prehybridized membrane. Mix the probe in the bag well. Hybridize the membrane with the probe at 68 °C for 1 h.

Wash: Carefully cut one corner of the bag and pour the radioactive solution into radioactive waste tank. Wash the membrane in a metal box containing 2xSSC and 0.1% SDS (2.5 ml/cm² membrane) twice (15 min/each) at RT with gentle agitation. Wash once for 30 min at 60 °C with 0.1xSSC and 0.1% SDS with gentle agitation.

Autoradiography: Wrap the membrane in a plastic wrap and place the wrapped membrane on Kodak X-OMAT AR film with an intensifying screen at -80°C. Expose the film for overnight to several days.

Stripping the membrane for reuse: Heat the 0.1 x SSC and 0.1% SDS wash solution to boiling. Pour it over the membrane in a metal box. Wash the membrane twice for 15 min. Wrap the wet membrane immediately in a plastic wrap for next hybridization. **Never let the membrane get dry if it has to be reprobbed.**

Reagents from Stratagene company:

QuickHyb hybridization solution, Salmon Sperm DNA,

Solutions:

| | | |
|------------------------------|--|------------|
| 10 x MOPS | 0.2M MOPS | 41.85 g/l |
| | 0.05M Na Acetate | 4.1 g/l |
| | 0.01M Na ₂ EDTA | 20 ml 0.5M |
| | adjust pH to 7.0 | |
| | store in a dark bottle or foil wrapped bottle, autoclave | |
| Sample Buffer Stock Solution | 5 μ l of 10mg/ml Ethidium Bromide | |
| | 250 μ l of Formamide | |
| | 50 μ l of 10 x MOPS buffer | |
| | 80 μ l of 37% Formaldehyde | |
| RNA Dye Solution | 40% sucrose | |
| | 0.025% Bromophenol blue (6mg/25ml) | |
| | in DEPC H ₂ O | |

don't autoclave

1M Tris-HCl pH7.0 dissolve Tris base in dH₂O and use HCl to adjust pH to 7.0, autoclave.

Retinoic acid treatment of mouse embryos in utero

C57BL/6J mice were mated in the evening and plugs were checked 2 hours later. Fertilization was assumed one hour after setting up matings. This time point was designated as embryonic day 0. When the development of embryos reached E8+5hours or E10.5, 200 µl of 2.5 mg / ml all-trans retinoic acid (Sigma) were administered to pregnant mice by oral gavage. tRA was dissolved in DMSO at 25 mg / ml and diluted in sesame oil to 2.5 mg/ml. Control embryos were treated with 200 µl 10% DMSO in sesame oil. The dosage administered was 20 mg tRA per kilogram of body weight.

Isolation of mouse embryos, fixation, dehydration, embedding and sectioning

Mouse embryos at different stages were isolated in ice cold PBS. Fixation of embryos was done in 4% paraformaldehyde (dissolved in PBS without Ca²⁺ and Mg²⁺) at 4°C overnight or at RT for 4 h. Dehydration of embryos was done by changing through PBS, 25% methanol-75% PBS (Ca²⁺/Mg²⁺ free), 50% methanol-50% PBS (Ca²⁺/Mg²⁺ free), 75% methanol-25% PBS(Ca²⁺/Mg²⁺), 100% methanol. Embryos were incubated at each step for 10 min at RT. Store dehydrated embryos in methanol at -20°C.

Infiltrate E10.5 embryos with ethanol for 1 h, xylene 1 h, xylene 1 h, wax:xylene(1:1) 1 h and wax 1 h. Then embed embryos in wax. Histo wax or Parafin wax was melted at 60°C. Sectioning of embryos at thickness of 7 µm/section was done with a microtome.

Radioactive in situ hybridization of mouse embryos in sections

tRA-treated and control embryos were isolated at E10.5. Embryos were fixed in 4% paraformaldehyde overnight at 4°C, and dehydrated and embedded in parafin. Sagittal sections (7µm thick) were subjected to in situ hybridization, following standard procedures (Bright Hogen). A 1.2 kb full-length mouse *goosecoid* cDNA clone (Blum, cell) was used as template for synthesis of radioactive α³⁵S-UTP labeled probes by *in vitro* transcription. Hybridization reactions were performed at 60°C. After development slides were counter stained with hematoxylin and eosin. For details, see the radioactive in situ hybridization protocol in the supplement of the thesis (see supplement for a detailed protocol).

Bone and cartilage staining of E17.5 mouse embryos

tRA-treated and control embryos were harvested at E17.5 for skeletal analysis. Embryos were skinned and eviscerated, and fixed in 100 % ethanol overnight. Cartilage staining was

performed by overnight incubation in 0.015% alcian blue 8 GX (Sigma), dissolved in 80% ethanol / 20% acetic acid. After washing of embryos in 100% ethanol for 12 h, they were treated with 2% KOH for 6 h, followed by staining with 0.005% alizarin red S (Sigma) in 2% KOH overnight. Finally, the embryos were incubated in 2% KOH until they are transparent and stored in 25% glycerol-75% ethanol.

1. Preparation of slides for carrying tissue sections

Imerse normal slides in the following solutions step by step:

10% HCl/70% Ethanol
ddH₂O
96% Ethanol

Put slides in an oven at 150 °C for 5 to 10 min (not longer than 10 min)

Cool down the slides to RT

Imerse slides in 2% Tespa (Sigma) in Aceton for 10 sec.

Slides in 100% aceton for twice.

Slides in ddH₂O

Dry slides overnight at 42°C

These slides can be used within two weeks.

Tespa: 3-Aminopropyltriethoxy-silane, C₉H₂₃NC₃Si, store at 0 to 4°C

2. Pre-hybridization

Bake all the glass containers and racks for 2 hours at 250°C or let them autoclaved to make them RNase-free before the experiment. Prepare 22 baked or autoclaved glass containers (9.5x7.5x6cm) for the following procedure, each containing 200ml following solution respectively.

Put slides with sections in glass racks. Each rack can hold 19 slides at most. Incubate the racks with sections in each container according to the following schedule.

- | | |
|--|--|
| 1. Xylol | 10 minutes |
| 2. Xylol | 10 min |
| 3. 100% Ethanol | 2 min |
| 4. 95% Ethanol | 2 min |
| 5. 90% Ethanol | 2 min |
| 6. 80% Ethanol | 2 min |
| 7. 70% Ethanol | 2 min |
| 8. 50% Ethanol | 2 min |
| 9. 30% Ethanol | 2 min |
| 10. 0.86% NaCl | 5 min (6ml 5M NaCl in 194 ml ddH ₂ O |
| 11. PBS | 5 min |
| 12. 4%PFA in PBS(without Mg ²⁺ & Ca ²⁺) | 20 min |
| 13. PBS | 5 min |
| 14. PBS | 5 min |
| 15. Proteinase K(400µl of 10mg/ml Prot K in 200 ml PBS) | 7 min |
| 16. PBS | 5 min |
| 17. 4% PFA in PBS | 5 min |
| 18. PBS | 5 min |
| 19. Acetylation mix | 10 min (2.5 ml triethanol amine + 200 ml ddH ₂ O + 0.5 ml acetic anhydride, make fresh) |
| 20. PBS | 5 min |
| 21. 0.86% NaCl | 5 min |
| 22. 30% Ethanol | 2 min Use ethanol from step 9 |
| 23. 50% Ethanol | 2 min to step 8 |
| 24. 70% Ethanol | 2 min to step 7 |
| 25. 80% Ethanol | 2 min to step 6 |
| 26. 90% Ethanol | 2 min to step 5 |
| 27. 95% Ethanol | 2 min to step 4 |
| 28. 100% Ethanol | 2 min Use fresh ethanol |

Air dry , slides are ready for the probe.

3. Making Radioactive Probe

Set up a transcription reaction in 20 μ l volume:

4 μ l 5xTranscription buffer
 2 μ l 200mMDTT (
 1 μ l placental RNase inhibitor(20-40U/ μ l)(also called RNA guard)
 4 μ l 2.5mMNTP(ATP,GTP&CTP mixture,each at 2.5mM)
 1 μ l 1 μ g/ μ l Template(linear cDNA plasmid)
 2 μ l Autoclaved distilled water(dd water)
 1 μ l T3, T7 orSp6 RNA polymerase(20-40U/ μ l) for antisense or sense probe(according to the template.
 5 μ l alpha[35S]-UTP(800 Ci/mmol)

-> vortex by dipping
 -> spin down quickly
 -> 37°C/1hour

3 μ l tRNA(10mg/ml)
 3 μ l RNase-free DNase (1-5U/ μ l)

-> vortex by dipping
 -> spin down quickly
 -> 37°C/15min

80 μ l dd water
 100 μ l phenol/Chloroform(1:1)

-> vortex well
 -> spin 13000rpm/5min
 -> transfer upper phase (the aqueous layer containing probe) into a new tube(~100 μ l), the following steppes will be done in the new tube

=====

Ethanol precipitation:

33 μ l 7.5M Ammoniumacetate(NH₄OAc)
 300 μ l 100% Ethanol(2-3xVol.)

-> vortex well
 -> dry ice/10min.
 -> spin 13000rpm/10min => pellet
 -> remove ethanol carefully with pipette!!!

Wash the pellet with 1 ml of 70% cold ethanol (to remove salts) (don't vortex)
 Dry the pellet biefly in air

Dissolve the pellet in 50 μ l of 100mM DTT(100 μ l 1MDTT into 900 μ l ddwater)
 Vortex well, spin down quickly

take out 1 μ l for measurement of radioactivity(dissolved in 3ml scintillation cocktail)
 take out 2 μ l for running a 6% polyacrylamide gel
 store the rest of the probe (47 μ l) at -80°C.

4. Treatment of coverslips

Imerse coverslips in **Silicone solution** (Serva)
 Dry the coverslips at RT
 Imerse the coverslips in 99% ethanol
 Back at 150 °C for 2 h or let them autoclaved.

5. Hybridization

Set up 60°C water bath and prepare a good plastic box. Wash the box with 1% SDS and lay 3 layers of Whatman 3 mm paper on the botterm inside the box. Add premix of 20 ml formamide, 4 ml 20 x SSC (autoclaved) and 16 ml of ddH₂O evenly on the paper.

Hybridization: Add **hybridization buffer** in a volume of 9 times of the volume of the probe to the probe. Mix well and heat at 80 °C for 3 min and chill on ice immediately. Add 40 µl to each slide. Use a pipet to spread the probe on a slide evenly and cover the slides with a pretreated coverslip carefully to try to avoid air bubbles between slides and coverslips. Note, forceps used in this step should be flamed before start. It is suggested that 1-2 x 10⁵ cpm/µl count be used. Total count per slide is about 4-8 x 10⁶.

The number of the slides that can be hybridized = Total volume of the probe/ 40 µl, or Total counts of the probe divided by 4-8 x 10⁶.

Hybridize the slides in the plastic box in water bath at 60 °C overnight.

Hybridization buffer: 1ml

| | |
|-------------------------|--------|
| 10 x hybridization salt | 100 µl |
| formamide | 500 µl |
| 50% Dextran sulfate | 200 µl |
| 1M DTT | 100 µl |
| tRNA | 1µl |

10 x Hybridization Salt:

| | |
|---|----------|
| PVP (Polyvinylpyrrolidone) | 0.2 g |
| Ficoll | 0.2 g |
| NaH ₂ PO ₄ pH6.8 | 1.38 g |
| 0.5 M EDTA pH8.0 | 10 ml |
| 5 M NaCl | 58.82 ml |
| 1 M Tris/HCl pH8.0 | 10 µl |
| add ddH ₂ O to 100 ml, autoclave | |

6. Wash

Remove slides from humid chamber , put them directly into racks. Lable containers especially for RNase step. The containers used for washing should not be mixed with other containers. Process racks through the following schedule:

| | |
|---|---|
| 1. 2xSSC/50% Formamide/MeE | 15 min , 37°C in water bath with gentle shake |
| 2. 2xSSC/50% Formamide/MeE | 30 min, 65 °C |
| 3. Transfer container from step 2 to 37°C water bath, incubate for 1 to 2 h | |
| 4. NTE | 15 min, 37°C |
| 5. NTE + 20 µg/ml RNase A(40µl of 100mg/ml RNase Ato 200 ml NTE) | 15 min, 37°C |
| 6. NTE | 15 min, 37°C |
| 7. 2xSSC/50% Formamide/MeE | 15 min , 37°C in water bath |
| 8. 2xSSC | 15 min |
| 9. 0.1xSSC | 15 min |
| 10. 30% ethanol/0.25 M NH ₄ OAc | 2 min |
| 11. 50% ethanol/0.25 M NH ₄ OAc | 2 min |
| 12. 70% ethanol/0.25 M NH ₄ OAc | 2 min |
| 13. 80% ethanol | 2 min |
| 14. 90% ethanol | 2 min |
| 15. 95% ethanol | 2 min |

16. 100% ethanol 2 min
17. Air dry, The slides can now be placed into an X-ray cassette and exposed to a normal XAR-5 film. After an overnight exposure, an extremely faint outline of the section and the signal should be visible. This step helps for an overview and an estimation of the background.

2XSSC/50% Formamide/MeE, for 750 ml, add
 375 ml formamide(100%)
 75 ml 20xSSC
 1 ml Mercaptoethanol
 distilled H₂O to 750 ml

NTE buffer: for 1000 ml, add
 100 ml of 5M NaCl
 10 ml of 1M Tris/HCl pH8.0
 10 ml of 0.5M EDTA pH8.0
 distilled H₂O to 1 liter

7. Dip slides in photoemulsion and autoradiography

Dilute photoemulsion in distilled warm H₂O (43 °C) in 1:1 ratio in dark room. Dip slides twice in diluted warm photoemulsion (43°C). Dry the slides in air in a dark room. Afterwards wrap the slides in a rack and leave the slides at 4 °C for 5-10 days exposure.

8. Develop the slides

Dissolve 32 g Kodak D19 Developer in 200 ml tap water at 36°C, 60 g of Sodium Thiosulfate (Na₂S₂O₃·5H₂O, Sigma) in 200 distilled water and 2 ml acetic acid in 200 ml distilled water.

- | | |
|-------------------------------|--------|
| 1. Kodak D19 solution | 3 min |
| 2. 1% acetic acid | 1 min |
| 3. Sodium thiosulfate (fixer) | 5 min |
| 4. Tap water | 10 min |
| 5. Tap water | 10 min |
| 6. Tap water | 30 min |

Dry the slides in air

9. Hematoxylin and eosin staining

- | | |
|-------------------------------|--------|
| 1. Mayer's Hematoxylin(Sigma) | 2 min |
| 2. dH ₂ O | 5 min |
| 3. 0.02% Eosin(Sigma) | 30 sec |
| 4. 70% ethanol | 30 sec |
| 5. 100% ethanol | 30 sec |

Dry slides by air

Lay Entellan on the slides and cover the slides with coverslips.

Check insitu signal under a microscope with both bright and dark field.

6. Abbreviations

| | |
|---------|--|
| AP-1 | Activator -protein-1 |
| BMP-4 | Bone Morphogenetic Protein 4 |
| cDNA | double-strand copy of mRNA |
| DMSO | dimethylsulfate |
| DNA | deoxyribonucleic acid |
| dNTP | desoxynucleoside triphosphate |
| DTT | dithiothreitol |
| EDTA | ethylenediamine-N, N-tetracetate |
| E8 +5 h | embryonic day 8 plus 5 hours |
| E10.5 | embryonic 10.5 |
| et al. | and others (lat. <i>et alii</i>) |
| FCS | fetal calf serum |
| Fig. | figure |
| g | gram |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GTP | guanosine triphosphate |
| h | hour |
| kb | kilobase (1 kb = 1000 bp) |
| kg | kilogram |
| l | liter |
| M | molar |
| mg | milligram |
| μ l | microliter (10^{-6} l) |
| μ M | micromolar (10^{-6} molar) |
| mRNA | messenger RNA |
| nM | nanomolar (10^{-9} molar) |
| OD | optical density |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| RAR | retinoic acid receptor |
| RARE | retinoic acid response element |
| RXR | retinoic X receptor |
| RA | retinoic acid |
| shh | sonic hedgehog |

| | |
|--------------|---|
| tRA | all-trans retinoic acid |
| 9-cis RA | 9-cis retinoic acid |
| RNA | ribonucleic acid |
| RT | room temperature |
| SDS | sodium dodecylsulfate |
| sec | second |
| TEMED | N,N,N',N'-tetramethylethylenediamine |
| ZPA | zone of polarizing activity in posterior limb bud |
| TGF- β | transforming growth factor β |
| TPA | 12-O-tetradecanoyl-phorbol-13-acetate |
| Tris | tris-(hydroxymethyl)-aminomethane |
| U | unite |

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