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Bmp, Fgf and Wnt signalling in programmed cell death and chondrogenesis during vertebrate limb development: the role of *Dickkopf-1*

LARS GROTEWOLD and ULRICH RÜTHER*

Institut für Entwicklungs- und Molekularbiologie der Tiere (EMT), Heinrich-Heine-Universität, Düsseldorf, Germany

ABSTRACT Dickkopf-1 (Dkk-1) is a potent head inducer in *Xenopus*. This effect can be attributed to its capability to specifically inhibit Wnt/β-catenin signalling. Recent data point to a crucial role for Dkk-1 in the control of programmed cell death during vertebrate limb development. In this paper, we present a comparative expression analysis of *Dkk-1*, *Bmp-4* and *Sox-9* as well as data on the regulation of *Dkk-1* by Wnt. Finally, we summarize the current knowledge of its potential function in the developing limb and present a model how the interplay of the Bmp, Fgf and Wnt signalling pathways might differentially regulate programmed cell death versus chondrogenic differentiation in limb mesodermal cells.

KEY WORDS: *Bmp, Dkk-1, limb development, programmed cell death, Wnt*

Introduction

The vertebrate limb provides a paradigm for developmental programmed cell death (PCD). Morphogenesis of this structure critically depends on the endogenous suicide program eliminating cells in very confined regions within the early limb bud (Hurle *et al.,* 1996). These regions include the so-called anterior necrotic zone (ANZ), its posterior counterpart (PNZ) as well as the interdigital mesenchyme (INZ). Not much is known about the molecules that control PCD in the aforementioned areas. It seems that a complex interplay between different members of the Tgfβ and Fgf families largely contributes to this activity (Merino *et al.,* 1998; Macias *et al.,* 1999; Montero *et al.,* 2001). Members of the Bmp subfamily have been identified as components triggering PCD, especially within the INZ (Yokouchi *et al.,* 1996; Zou and Niswander, 1996). Remarkably, Bmps have originally been identified by their ability to induce bone structures (Wozney *et al.,* 1988). Indeed, these signalling molecules are also crucial for the formation of condensations in the vertebrate limb (Pizette and Niswander, 2000). Thus, in very close vicinity to the regions where Bmps promote PCD, the activity of the very same molecules leads to a completely different reaction of the mesodermal cells. Interestingly, both activities of Bmps seem to be mediated by BmpR-Ib (Zou *et al.,* 1997a). The use of different receptors might thus not be the basis for this dual function, the nature of which remains obscure until today.

Dickkopf-1 (Dkk-1) is a member of a new family of secreted proteins which was isolated in *Xenopus* (Glinka *et al.,* 1998). Homologues have now been identified in different vertebrate species like chick, fish, mouse and also humans (Glinka *et al.,* 1998; Krupnik *et al.,* 1999; Hashimoto *et al.,* 2000; Shinya *et al.,* 2000; Mukhopadhyay *et al.,* 2001). Dkk-1 represents a potent antagonist of the Wnt/β-catenin signalling pathway (reviewed in Zorn, 2001). We and others have previously described the dynamic expression pattern of *Dkk-1* as well as its potential function in modulating PCD during vertebrate limb development (Grotewold *et al.,* 1999; Mukhopadhyay *et al.,* 2001; Grotewold and Rüther, 2002). In this paper, we present a comparative expression analysis of *Dkk-1*, *Bmp-4* and the cartilage-specific *Sox-9*. We also analyzed *Dkk-1* expression and the extent of cell death in different mouse limb mutants and its transcriptional regulation by diverse ligands of the Wnt family. Finally, we summarize the recent advances of our understanding of Dkk-1 function during vertebrate limb development and present a model involving Dkk-1 that might explain how limb mesodermal cells become determined to either undergo chondrogenic differentiation or to be commited to apoptotic cell death in response to Bmp signals.

Abbreviations used in this paper: AER, apical ectodermal ridge; ANZ, anterior necrotic zone; Bmp, bone morphogenetic protein; Dkk-1, Dickkopf-1; ES cell, embryonic stem cell; Fgf, fibroblast growth factor; Ft, Fused toes; Hx, Hemimelic extra toe; INZ, interdigital necrotic zone; PCD, programmed cell death; PNZ, posterior necrotic zone; Tgfβ, transforming growth factor β; Xt^J, Extra-toes ^{J}.

***Address correspondence to:** Dr. Ulrich Rüther. Institut für Entwicklungs- und Molekularbiologie der Tiere (EMT), Heinrich-Heine-Universität, 40225 Düsseldorf, Germany. Tel. +49-211-81-11391. Fax +49-211-81-15113. e-mail : ruether@uni-duesseldorf.de

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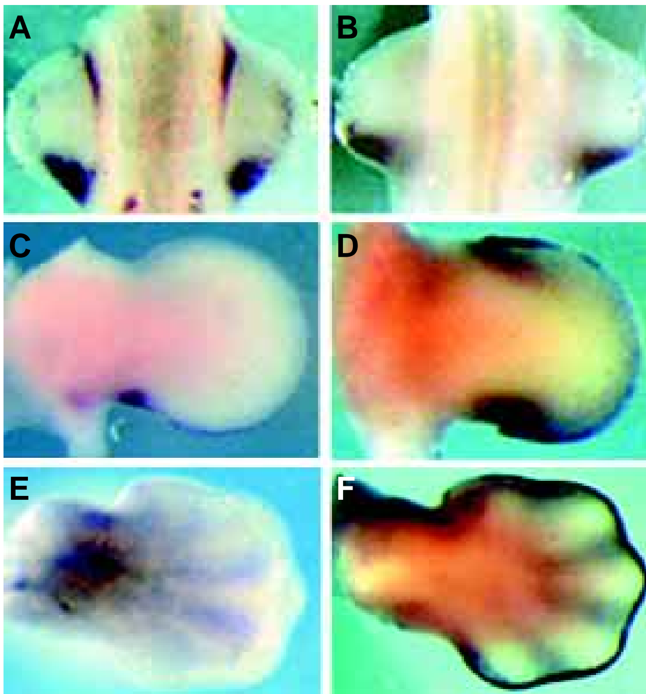

Fig. 1. Co-expression of *Dkk-1* (A,C,E) and *Bmp-4* (B,D,F). *(A,B) Dorsal views of E10.5 forelimb buds.* **(A)** Dkk-1 *is expressed in a stripe in the anterior/proximal mesoderm and in a region corresponding to the PNZ.* **(B)** Bmp-4 *is asymmetrically expressed in the mesoderm with a strong posterior and a faint anterior domain of transcription. (C-F) Dorsal views, anterior is towards the top.* **(C)** Dkk-1 *transcription in the posterior mesoderm and the AER at E11.5.* **(D)** Bmp-4 *expression in overlapping domains at E11.5. In contrast to* Dkk-1*, transcription in the anterior mesoderm is maintained.* **(E)** *Interdigital expression of* Dkk-1 *at E12.5.* **(F)** *Expression of* Bmp-4 *in the interdigital mesenchyme and AER at E12.5.*

Results

Dkk-1 *is co-expressed with* Bmp-4

At E10.5 *Dkk-1* is expressed in an anterior as well as a posterior mesodermal domain in the mouse limb bud (Fig. 1A). Later on, these domains become more confined and transcripts can additionally be detected in the apical ectodermal ridge (AER, Fig. 1C) before expression in the interdigital mesenchyme starts (Fig. 1E). As this pattern of expression seemed to be quite similar to that of some members of the *Bmp* family we undertook a comparative analysis of *Bmp-4* transcription at the corresponding time points. Indeed, at all developmental stages examined (E10.5-E12.5) the expression domains of *Bmp-4* and *Dkk-1* overlapped to a high degree (compare Fig. 1 A,B; C,D; E,F).

Dkk-1 *Expression is associated with the Sites of PCD*

We have previously shown that the areas of *Dkk-1* expression correspond to the sites of PCD in both, mouse and chick limb development (Grotewold and Rüther, 2002). Interestingly, *Dkk-1* is ectopically expressed in limb buds of polydactylous Xt^J/Xt^J mutant embryos while it is normally transcribed in limb buds of *Hx*/+ embryos (Grotewold and Rüther, 2002) which also develop ectopic preaxial digits. We asked whether the enhanced activity of *Dkk-1* might affect PCD in the anterior mesoderm of Xt^J/Xt^J limb buds. The TUNEL stainings in Fig. 2 show that while the extent of cell death is slightly reduced in the anterior mesoderm of *Hx*/+ limb buds (Fig. 2B) compared to the wild-type (Fig. 2A), it is clearly increased in the Xt^J/Xt^J limbs (Fig. 2C). The increased PCD might not affect digit number in the polydactylous Xt^J/Xt^J embryos but rather digit length as the ectopic digits are significantly shorter than the preaxial extra digit of *Hx*/+ embryos (data not shown). Thus, in normal as well as mutant limb development, the sites of *Dkk-1* expression are associated with high PCD.

Dkk-1 *Expression is excluded from Regions of Chondrogenesis*

To analyze the relationship between the regions of *Dkk-1* expression and sites of PCD and on the other hand the areas of chondrogenesis in more detail, we carried out a comparative analysis of *Dkk-1* and *Sox-9* expression which marks the cartilagenous skeleton (Bi *et al.,* 2001). At E12.5 transcription of the two genes is clearly mutually exclusive with *Dkk-1* being expressed in the INZ and *Sox-9* within the digital rays (Fig. 3 A,C). This pattern is maintained one day later in development (Fig. 3 E,G). In order to ask whether this complementary expression is also realized during mutant limb development, we analyzed expression of the two genes in limb buds of *Ft*/+ embryos. These mice develop a syndactyly due to ectopic bone elements connecting digits 1-4 (Heymer and Rüther, 1999). At E12.5 *Dkk-1* is ectopically expressed in the anterior/distal part of *Ft*/+ limb buds (Fig. 3B). We have previously shown that PCD is also enhanced in the corresponding region (Grotewold and Rüther, 2002). In parallel, *Sox-9* starts to be misexpressed in the anterior/distal part of *Ft*/+ limb buds. The domain of ectopic *Sox-9* transcripts, however, does not seem to overlap with that of ectopic *Dkk-1* expression but to border proximally on this domain (compare Fig. 3 B,D). Thus, the region of ectopic bone formation is separated from the area of enhanced *Dkk-1* expression and PCD. At E13.5 *Dkk-1* transcripts are lost

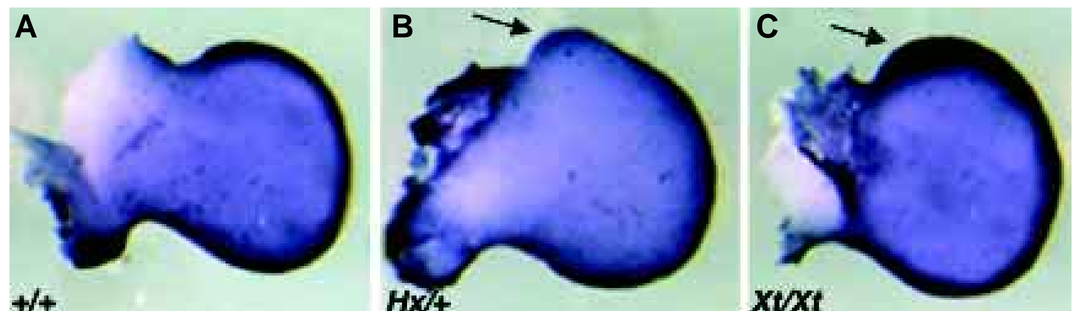

Fig. 2. PCD in polydactylous mouse mutants. *All panels show TUNEL stainings of E12.0 limb buds. Dorsal views, anterior is towards the top.* **(A)** *Wild-type pattern of cell death in the AER and distal-most mesoderm.* **(B)** *PCD is reduced in the anterior outgrowth in* Hx/+ *limb buds (arrow).* **(C)** *Massive increase in the extent of PCD in the anterior mesoderm of* Xt^J/Xt^J *limb buds (arrow).*

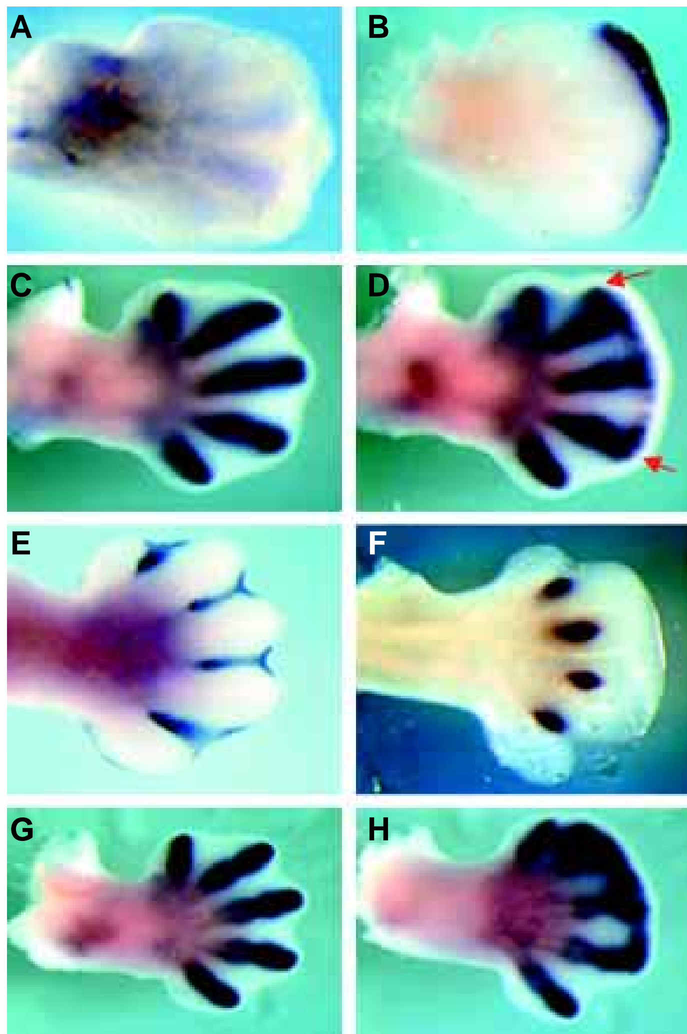

Fig. 3. Mutually exclusive expression of *Dkk-1* and *Sox-9* in wild-type (A,C,E,G) and mutant (B,D,F,H) mouse limb buds. *All panels show dorsal views, anterior is towards the top.* **(A)** *Wild-type expression of Dkk-1 at E12.5 in the INZ.* **(B)** *Ectopic* Dkk-1 *activation at E12.5 in the anterior/distal part of* Ft/+ *limbs.* **(C)** Sox-9 *is expressed in the digit anlagen at E12.5 in the wild-type.* ***(D)*** *Ectopic expression of* Sox-9 *at E12.5 connecting the tips of the presumptive digits 2-4 (arrows). Note that the region of ectopic* Dkk-1 *transcription is devoid of* Sox-9 *transcripts (compare to B).* **(E)** *Interdigital expression of* Dkk-1 *in an E13.5 wild-type limb bud.* **(F)** Dkk-1 *expression is lost from the distal part of corresponding* Ft/+ *limbs and restricted to the interdigital regions.* **(G)** Sox-9 *expression at E13.5 in the digital rays of a wild-type limb.* **(H)** *Massive ectopic expression of* Sox-9 *in the anterior/distal part of an E13.5* Ft/+ *limb prefiguring the fusion of digits.*

from the distal-most part of *Ft*/+ limb buds but are restricted to the interdigital regions (Fig. 3F). At this time point, the ectopic *Sox-9* expression domain has expanded to cover the complete region of the presumptive fusion of digits 1-4 (Fig. 3H). Thus, also in *Ft*/+ mutant limbs *Dkk-1* expression is excluded from the regions of chondrogenesis and the dynamics of its transcription correlates with the temporary alterations of PCD.

Regulation of* Dkk-1 *by Wnt

It seems to be a recurrent theme in animal development that secreted signalling molecules induce the expression of inhibitors of their own activity, probably to limit the range of their action. This is e.g. true for the Bmp-inhibitor Bambi (Onichtchouk *et al.,* 1999, Grotewold *et al.,* 2001) and the Fgf-antagonizing Sproutys (Minowada *et al.,* 1999). We wanted to analyze whether *Dkk-1* which inhibits Wnt/β-catenin signals might be transcriptionally induced by members of the Wnt family. Arnold *et al.* (2000) and Lickert *et al.* (2000) recently reported a co-culture system of embryonic stem (ES) cells with NIH/3T3 fibroblasts that express different *Wnt* genes. The induction of Wnt target genes like e.g. *Cdx-1* could then be observed in the ES cells (Lickert *et al.,* 2000). Using this system we could reproduce the induction of *Cdx-1* by Wnt-1, -3a and -4 and slightly also by Wnt-7a and -7b (Fig. 4), verifying that this system worked in our hands. When we monitored *Dkk-1* transcription by RT-PCR we observed a transcriptional induction of the gene by Wnt-1, -3a and -4 (Fig. 4).
Thus, *Dkk-1* expression responds to the activity of a subset of Wnt ligands.

Discussion

Until recently, not much was known about potential functions of *Dkk-1* outside head induction (Glinka *et al.,* 1998). We and others could show that the spatiotemporal expression of the gene during vertebrate limb development coincides significantly with the sites of PCD (Grotewold *et al.,* 1999; Mukhopadhyay *et al.,* 2001; Grotewold and Rüther, 2002). This is not only true for normal but also mutant mouse limb development as shown for syndactylous *Ft*/+ mutant embryos and the polydactylous Xt^J/Xt^J embryos (Grotewold and Rüther, 2002 and this study). Moreover, the co-expression of *Dkk-1* with some members of the *Bmp* family and their target genes suggested a role for Dkk-1 in the Bmp-triggered PCD cascade. Indeed, *Dkk-1* is transcriptionally regulated by Bmp (Mukhopadhyay *et al.,* 2001; Grotewold and Rüther, 2002). Remarkably, *Dkk-1* is only induced by Bmp under PCD-inducing conditions but not when the Bmp signal promotes the formation of bone. Moreover, Bmp activity is also necessary for the endogenous expression of *Dkk-1* (Grotewold and Rüther, 2002).

Dkk-1 is also positively regulated by and most likely dependent on the activity of Fgf signals from the AER (Grotewold and Rüther, 2002). We now show that also Wnt-1, Wnt-3a and Wnt-4 induce the expression of *Dkk-1*. Wnt-1 and -3a activate an intracellular signaltransduction pathway which leads to the stabilization of β-catenin (Shimizu *et al.,* 1997). Evidence has also been reported for Wnt-4 to signal via β-catenin in chick limb development (Hartmann

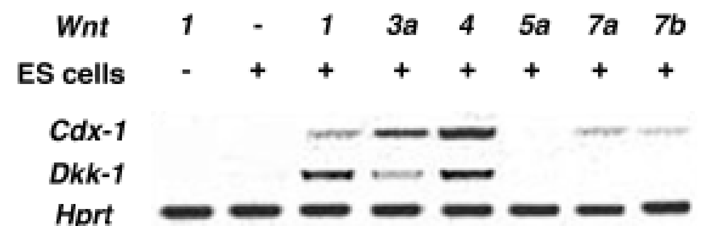

Fig. 4. Regulation of *Dkk-1* by different Wnt ligands. *RT-PCR analysis.* Cdx-1 *is induced in ES cells when co-cultured with NIH/3T3 stably transfected with expression constructs for* Wnt-1, Wnt-3a, Wnt-4, Wnt-7a, Wnt-7b. Dkk-1 *is induced by Wnt-1, -3a and -4. As controls, NIH/3T3 transfected with a lacZ-expression plasmid* (Wnt -) *and NIH/3T3 expressing* Wnt-1 *but without co-cultured ES cells were used.* Hprt *was used for standardization.*

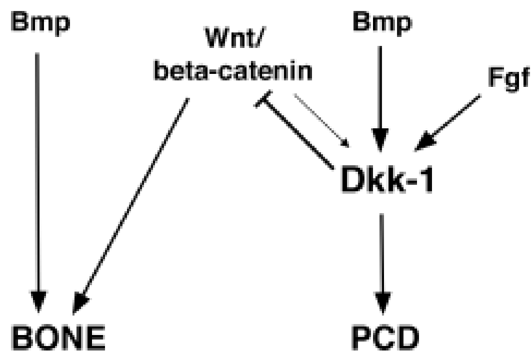

Fig. 5. Model for the dual role of Bmp signalling in limb mesodermal cells. *We propose that the reaction of a mesodermal cell in the vertebrate limb on a Bmp signal largely depends on the balance of Wnt/β-catenin and Bmp signalling. The induction of* Dkk-1*, which obviously depends on both, Bmp and Fgf signalling, leads to an inhibition of the Wnt/β-catenin pathway and subsequent PCD. Thus, the co-ordinated interplay of Bmp, Fgf and Wnt signalling seems to be crucial for the fate of limb mesodermal cells.*

and Tabin, 2000). As Dkk-1 specifically interferes with the Wnt/β-catenin pathway (Zorn, 2001), it thus seems, that these ligands limit their range of action by the induction of *Dkk-1*.

During chick limb development, Fgf-10 induces the expression of *Wnt-3a* in the AER (Kawakami *et al.,* 2001). The induction of *Dkk-1* by Fgf might thus be mediated by Wnt ligands, in particular Wnt-3a, which is a crucial component for the correct establishment of the AER (Kengaku *et al.,* 1998; Kawakami *et al.,* 2001). Besides, it has been shown in *Xenopus* that Dkk-1 can inhibit the activity of Wnt-3a (Kazanskaya *et al.,* 2000). These interactions might explain the consequences of *Dkk-1* overexpression during chick limb development. Ectopic expression of *Dkk-1* with an adenoviral and a retroviral system led to dramatic distal truncations of the resulting limb buds (Mukhopadhyay *et al.,* 2001; Grotewold and Rüther, 2002). The truncated limbs exhibited massive PCD (Grotewold and Rüther, 2002). This phenomenon, however, seemed to appear secondary to the truncations as we could not detect a significant increase in the number of apoptotic cells until truncations were rather advanced. Thus, we conclude that Dkk-1 blocks the regulatory loop between Fgfs and Wnt-3a which ensures AER maintenance and thus interferes with distal outgrowth. This interpretation is supported by the expansion of the *Fgf-8* expression domain and most likely the AER itself in *Dkk-1*$^{-/-}$ limb buds (Mukhopadhyay *et al.,* 2001).

As mentioned above, overexpression of *Dkk-1* in the limb bud does not seem to directly commit cells towards a death program which is consistent with findings in cultured cells (Wang *et al.,* 2000). Nevertheless, two lines of evidence support a crucial role of Dkk-1 in PCD. First, the ablation of *Dkk-1* function in the mouse led to syndactyly and the variable appearance of ectopic anterior and posterior digits, a phenotype that strongly suggests reduced PCD in the ANZ, PNZ and INZ, respectively, to be the basis for these alterations (Mukhopadhyay *et al.,* 2001). Second, we could recently show that overexpression of *Dkk-1* does have a dramatic influence on Bmp-triggered PCD. When a bead soaked in Bmp protein is implanted into the undifferentiated mesenchyme of early limb buds, PCD is induced in a restricted area around the bead after several hours (Macias *et al.,* 1997; Zou *et al.,* 1997b; Pizette and Niswander, 1999). When limb buds were infected with a retrovirus expressing *Dkk-1* before the bead implantation this led to a dramatic increase in the amount of cells undergoing PCD (Grotewold and Rüther, 2002). Thus, it seems that the prior exposure of a cell to Dkk-1 significantly enhances the ability of Bmp to induce PCD. One could imagine that the Dkk-1-mediated inhibition of Wnt/β-catenin signalling might provide a permissive signal that confers mesodermal limb bud cells the competence to react on an instructive Bmp signal to initiate the endogenous death program. We thus believe that the status of Wnt signalling of a mesodermal cell determines whether the cell undergoes PCD or chondrogenic differentiation in response to a Bmp signal. Thus, under conditions where both, Bmp and Wnt/β-catenin signalling exceed a certain level of activity this would lead to chondrogenesis to occur (Fig. 5). Indeed, enhanced Wnt/β-catenin signalling is associated with accelerated chondrogenesis in chick limb development (Hartmann and Tabin, 2000).

Further experimental proof will be required to evaluate whether our model might hold true, but up to now it certainly provides an attractive possibility to explain the dual role of Bmp signalling in vertebrate limb development.

Materials and Methods

In Situ *Hybridization and TUNEL Staining*

Whole mount *in situ* hybridization has been carried out according to standard procedures (Xu and Wilkinson, 1998) using the following probes: *Bmp-4* (kindly provided by Dr. B. Hogan), *Dkk-1* (Glinka *et al.,* 1998), *Sox-9* (kindly provided by Dr. R. Lovell-Badge). Whole mount TUNEL was performed as described (Grotewold and Rüther, 2002).

Co-Culture of NIH/3T3 and ES Cells

For co-culture experiments we used NIH/3T3 fibroblasts which were stably transfected with expresssion constructs of the following genes: *lacZ*, *Wnt-1*, *Wnt-3a*, *Wnt-4*, *Wnt-5a*, *Wnt-7a* and *Wnt-7b* (kind gifts from Dr. R. Kemler) which were plated at 10^6 cells /10 cm dish one day before addition of the ES cells. 2-3 days before co-culture, ES cells (ES 14-1, Kühn *et al.,* 1991) were transferred to gelatine-coated dishes to remove the feeder cells. $2x10^6$ ES cells were added to the NIH/3T3 cells and incubated for 24h. Cells were washed with PBS and RNA was isolated using TRIZOL (GibcoBRL). First strand synthesis was performed using the Expand RT system (Roche) according to the manufacturer's instructions.

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