Int. J. Dev. Biol. 43: 329-334 (1999)

Original Article

Evidence that Platelet derived growth factor (PDGF) action is required for mesoderm patterning in early amphibian *(Xenopus laevis)* embryogenesis

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ABSTRACT Mesoderm induction is one of the major events of early vertebrate embryonic patterning. It appears to be controlled by sequential and combinatorial actions of several kinds of peptide growth factors. These include activin, fibroblast growth factor (FGF), and transforming growth factor-β (TGF-β), among others. In the present study, the function of platelet-derived growth factor (PDGF) in early *Xenopus laevis* embryogenesis was investigated. In the animal-cap assay, PDGF caused pre-ectodermal tissue to develop a mesoderm specific morphology (elongation) and to express the mesoderm marker genes, *MyoD* family and *α-cardiac actin*. In addition, two other genes were expressed -related serum response factor *SL1* (a dorsal mesodermal marker) and myosin light chain (*MLC2*-heart marker). A role for PDGF in normal *(in vivo)* mesoderm induction is implicated because injection of PDGF receptor α antisense RNA into 2-cell embryos erased the animal cap's mesoderm marker expression. Those injected embryos also exhibited morphological abnormalities including incomplete gastrulation, failure of neural fold closing, and abnormal somitogenesis.

KEY WORDS: *mesoderm induction, mesoderm patterning, PDGF, PDGF receptor α*

Introduction

In amphibian embryos, the induction and patterning of the mesoderm lie at the heart of early development. During the blastula stage, the presumptive mesoderm area is specified in the marginal zone which is located between the vegetal and animal hemisphere (Woodland, 1989; Heasman, 1997). Much recent work on mesoderm induction has taken advantage of the animal cap assay in that animal cap can be induced into mesoderm under the influence of either vegetal pole explants or various soluble factors. The molecules for mesoderm induction include members of the fibroblast growth factor (FGF) family and members of the transforming growth factor-β (TGF-β) family (Kimelman and Kirschner, 1987; Sive, 1993; Cornell and Kimelman, 1994; Kessler and Melton, 1994). It is widely believed that these and other growth factors might act in combination to induce and pattern the embryo. Other intercellular signaling molecules have been identified that pattern the mesoderm by altering the competency of the cells to mesoderm-inducing agents (Christian *et al.*, 1992). These include members of the Wnt family (Christian *et al.*, 1991), noggin (Lamb *et al.*, 1993; Smith *et al.*, 1993), and chordin (Sasai *et al.*, 1994). Although the mesoderm is known to be formed during blastula stages by signals from the vegetal endoderm, the precise mechanism of its polarization and subdivision remains unclear. Animal caps isolated from embryos that have been injected with truncated FGF receptor mRNA and cultured with activin do not extend (Amaya *et al.*, 1991). Also, some mesoderm specific genes, including *α-cardiac actin, Xbra, XMyoD* and *XNot* are not induced, while the induction of other genes, including the head organizer-specific genes, *goosecoid* and *Xlim-1*, are less sensitive (De Robertis *et al.*, 1992; Cornell and Kimelman, 1994). Experiments with a dominant negative form of the activin type II receptor suggest that molecules with activin-like activities function in *Xenopus* mesoderm formation (Hemmati-Brivanlou *et al.*, 1992; Kessler and Melton, 1994). However, in mice, elimination of genes involved in the activin pathway has not resulted in defects in mesoderm induction (Matzuk *et al.*, 1995), lending support to the suggestion that other molecules, either of the TGF-β family or of an unidentified family, might be involved in mesoderm induction. Platelet derived growth factor (PDGF) is known to be produced from mesenchymal cells in many vertebrates.

Abbreviations used in this paper: PDGF, Platelet derived growth factor; TGF-β, transforming growth factor beta; FGF, Fibroblast growth factor; SL1, related serum response factor; MLC2, myosin light chain.

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0214-6282/99/$15.00
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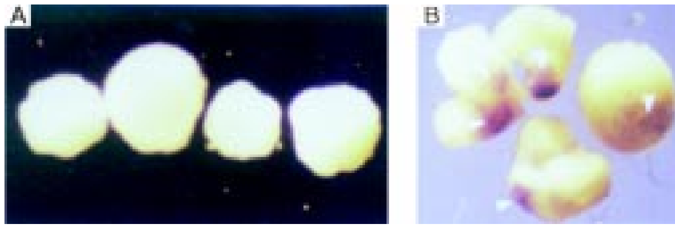

Fig. 1. PDGF causes elongation and induces the expression of α-cardiac actin in animal caps. *Animal caps were removed at the blastula stage, cultured for 25 h, then assayed by whole-mount in situ hybridization to detect the expression of α-cardiac actin. The result of in situ hybridization using sense probe (not shown) is the same as that in (A).* **(A)** *The control untreated animal caps were not elongated and did not express α-cardiac actin.* **(B)** *The PDGF-treated animal caps were elongated and α-cardiac actin expressed (white arrow head).*

Especially PDGF A-chain mRNA has been detected as a maternally encoded transcript in *Xenopus* oocytes and is known to be expressed in the animal cap and marginal zone at the blastula stage. In contrast, Platelet derived growth factor receptor α (PDGFRα) has been found to be expressed in the primitive streak of the mouse which is analogous to the involuting marginal zone of *Xenopus* (Orr-Urtreger and Lonai, 1992; Schatteman *et al.*, 1992). In *Xenopus*, PDGFRα has been found to be expressed in marginal zone cells at gastrula stage, presomitic region at neurula stage and many mesenchymal tissues at various stages of development (Ho *et al.*, 1994). In the mouse embryo, PDGFRα transcript has been detected throughout mesenchymal tissues including the splanchnic mesoderm, surrounding the neural tube, somites, and the developing vasculature (Graham *et al.*, 1992; Schatteman *et al.*, 1992). Recent studies on the PDGFR imply that the downstream components of the PDGFR signaling pathway are p^{21}ras, raf-1, etc. (Wagner and Cochran, 1993; Bowen-Pope and Seifert, 1994). In *Xenopus*, it has been previously demonstrated that dominant negative mutants of c-ras or c-raf inhibit the ability of FGF and activin to induce mesoderm. Curiously, overexpression of activated p^{21}ras or raf-1 is sufficient to induce mesoderm in animal cap assay (Whitman and Melton, 1992). The downstream molecule of both ras and raf is MAP kinase (Blenis, 1993). MAP kinase phosphatase (MKP-1) was used to inactivate endogenous MAP kinase and to prevent the induction of early and late mesodermal markers by both FGF and activin (Gotoh *et al.*, 1995; Labonne *et al.*, 1995, 1997; Rose and Busa, 1998). PDGFRα, as well as FGF receptor, is a tyrosine kinase receptor to activate c-ras, c-raf, and MAP kinase in several cell lines (Ullrich and Schlessinger, 1990; Blenis, 1993; Claesson-Welsh, 1995). These data suggest that PDGF may function in mesoderm induction. In order to investigate this possibility, we carried out animal cap assay to check if PDGF treatment leads to mesodermal specific morphological change and gene expression.

Results

PDGF induces mesoderm from animal cap explants – morphological study

When mesoderm is induced, a critical morphological change-namely elongation occurs. The animal cap explants treated with PDGF showed clear-cut elongation (Fig. 1B), while the control did not show any sign of elongation (Fig. 1A). In addition to that morphological change, whole-mount *in situ* hybridization detected the expression of α-cardiac actin in the animal cap explants treated with PDGF. This data confirm that the elongated explants represent authentic induced mesoderm tissue. Histological analysis showed that untreated animal caps formed atypical epidermis (Fig. 2A,B) but PDGF treated animal caps formed a ventral mesodermal vesicle (Fig. 2C,D) and a mass of tissue containing clumps of muscle-like cells (Fig. 2E,F), similar to those formed in *XBra*-injected animal caps (O'Reilly *et al.*, 1995).

PDGF activates the expression of mesodermal marker genes

To determine whether PDGF is mesoderm inducer, we examined the expression of mesoderm specific markers, *MyoD* family of genes including *XMyoD, XMyf5,* and *XMRF4* by RT-PCR. In normal *Xenopus* embryos, the expression of XMyoD and XMyf5 begins weakly at the early gastrula stage (Hopwood *et al.*, 1992), whereas *XMRF4* expression is first detected only in somites at stage 18 (neural fold stage) and becomes maximum at stage 22-23 (Jennings, 1992). The *XMyoD* family of genes was expressed in the animal caps treated with PDGF. Similar results were obtained in the cases of activin and FGF, which are well known as mesoderm inducers (Fig. 3). The expression of *α-cardiac actin* was also assayed by RT-PCR. This is supporting evidence for the *in situ* study (Fig. 1). Somite mesodermal markers, such as related serum responsive factor (RSRF) family, *SL1* (MEF2D), and a cardiac mesodermal marker, *Xenopus* myosin light chain (*XMLC2*) were studied by RT-PCR. *SL1* and *XMLC2* were both expressed in the animal cap explants treated with PDGF, activin and FGF (Fig. 3). With the mesoderm induction from the PDGF treated animal caps, neural induction also occurred. Microinjection of the XPDGF receptor antisense RNA inhibits the expression of mesoderm-specific genes in animal cap explants. To confirm the above results, the signal of XPDGF receptor was blocked by microinjection of XPDGF receptor antisense RNA. After microinjection at 1 cell stage into *Xenopus* embryos, the animal caps were dissected at the blastula stage (st. 8-9) and RT-PCR assay was carried out. When the control animal caps microinjected with neo pa RNA (microinjection control) were treated with PDGF, *XMyoD, XMyf5, XMRF4* and *α-cardiac actin* were expressed. On the contrary, the animal caps microinjected with antisense RNA of XPDGF receptor and cultured with PDGF did not express any of these genes (Fig. 4).

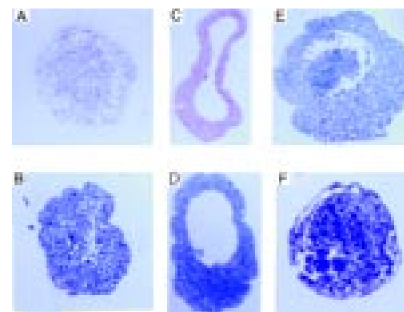

Fig. 2. Histological analysis of animal caps treated with PDGF. *Animal caps were dissected from embryos at the mid-blastula stage and cultured to the equivalent of neural fold stage, when they were fixed, sectioned at 1 μm. An untreated animal cap forms a typical epidermis* **(A,B).** *The animal caps treated with PDGF show ventral tissue* **(C,D)** *and muscle-like tissue* **(E,F**, *white arrow head***)**.

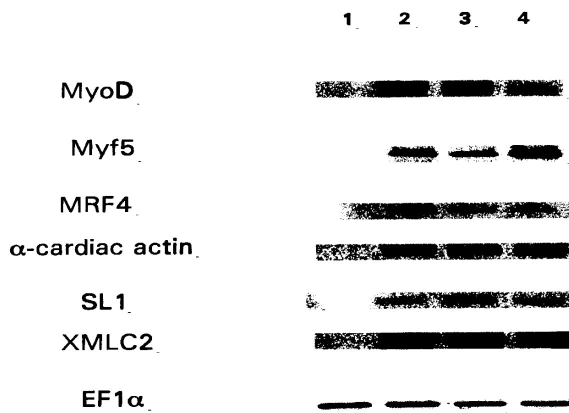

Fig. 3. Expression of muscle specific genes in animal caps treated with PDGF. *Animal caps were dissected at the blastula stage and treated with PDGF or activin or FGF. In the animal cap explant of neural fold stage equivalent, the expression of a set of dorsal mesodermal markers was assessed by RT-PCR. In the animal caps treated with PDGF the expression of the general dorsal mesodermal marker XMyoD family and α-cardiac actin was induced. Somite mesodermal markers, SL1 and cardiac mesodermal marker, XMLC2 were also expressed. However, in untreated caps the expression of any of those markers was not induced. The Ef1-α control demonstrates that a comparable amount of RNA was assayed in each set. Lane 1, non-treated control; lane 2, 50 ng/ml PDGF; lane 3, 50 ng/ml Activin; lane 4, 50 ng/ml FGF.*

XPDGFR signaling is required in the formation of somitic mesoderm

At the 2 cell stage, the antisense RNA was injected into one blastomere, while the control embryo was injected with neo pa RNA (Boehringer Mannheim). The side originated from the blastomere into which antisense RNA was injected had less expression of α-cardiac actin and showed poorer development in the somitic mesoderm than the other side (Fig. 5C,D,E). Although the function of intercellular activin and FGF existed, the expression of α-cardiac actin was reduced by the injection of PDGFRα antisense mRNA. Muscle is largely absent from the dorsal and lateral regions. The neural fold has not closed, or the neural tube was severely distorted. The control embryos injected with neo pa RNA were normal. These results provide evidence that XPDGFRα signaling is necessary for the formation of dorsal mesoderm.

Discussion

The experiments presented here demonstrate that PDGF converted ectodermal cells into mesodermal cells and induced the expression of the dorsal (*MyoD* family genes), lateral mesodermal genes (*SRF* family and *MLC2* genes) and the α-cardiac actin gene. The animal cap explants treated with PDGF showed an elongated morphology (Fig. 1B). Even though the elongation is not long as in the case of activin treated animal cap, it is a good sign of mesoderm induction because mesodermal cells go through convergent extention after gastrulation in normal development (Yamada and Modak, 1998). Although PDGF's capability for mesoderm induction may be weaker than activin, the histological analysis reveals that PDGF may be a mesoderm-inducer (Fig. 2B).

Expression of XMyoD, XMyf5, XMRF4, and the α-cardiac actin genes was induced by PDGF (Fig. 3). *XMyoD* and *XMyf5* begin to be expressed at the early gastrula stage (st. 10-11), then they are actively expressed in the dorsal (somitic) mesoderm at neurula (st. 18). When XMyoD and XMyf5 mRNA are microinjected together into *Xenopus* animal caps full myogenesis fails, but when *XMRF4* is microinjected with them it succeeds to occur (Gurdon *et al.,* 1992). The gene is expressed in the somitic mesoderm at neural fold stage and the gene product functions in late mesodermal differentiation (Jennings, 1992). Since MyoD family genes were induced by PDGF, we suggest that PDGF plays a role in the pattern formation of dorsal mesoderm. This notion is supported by the fact that PDGF induced the expression of *SL1* (Fig. 3). The *RSRF* gene, belonging to the MEF2 family, is expressed in the dorsal mesoderm of the early *Xenopus* embryo (Chambers *et al.*, 1992). The MEF2 protein family activates *XMyoD* transcription and *XMEF2* expression in myogenic cells and contributes to the activation and stabilization of XMyoD transcription during muscle cell differentiation (Wong *et al.*, 1994). SL1 (MEF2D) lies downstream of the myogenic factors in the skeletal myogenic pathway and is a dorsal mesoderm marker. SL1 regulates the cardiac muscle-specific transcription of *XMLC2* in *Xenopus* embryos. XMLC2 is a direct target for trans-activation by the SL1 protein and a marker for terminal differentiation of cardiac muscle in the *Xenopus* embryo. Its expression is detected first in stage 28/29 tadpoles in the presumptive heart region and is subsequently confined to the developing heart tube (Chambers *et al.*, 1992, 1994). In our experiment, both *SL1* and *XMLC2* were expressed in animal cap cells treated with PDGF, activin and FGF. This indicates that PDGF is involved in the formation of cardiac muscle originated from lateral mesoderm. However, the possibility of the PDGF inducing ventral mesoderm needs more research.

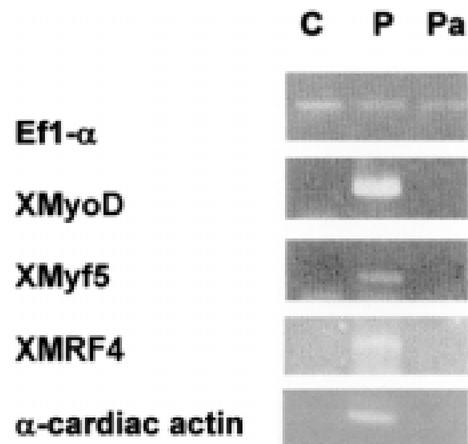

Fig. 4. Blockade of XPDGF receptor signal inhibits mesoderm induction from pre-ectodermal cells. *XPDGF receptor antisense RNA (0.7 ng) was microinjected into the animal hemisphere at single-celled stage, the embryos were reared in 10% MMR, the animal caps of the embryos were dissected at blastula stage (st. 8-9), and the animal caps were treated with PDGF. When control embryos reached to neural tube stage, RNA was extracted from the animal caps, then RT-PCR assay was performed. While PDGF treated animal caps expressed XMyoD, XMyf5, XMRF4 and α-cardiac actin genes, the animal caps in which XPDGF receptor signal were blocked did not express any of these genes, as well as untreated animal caps. Ef1-α, control marker of RT-PCR was expressed in all cases. C, non-treated control; P, PDGF-treated; Pa, XPDGFR As injected.*

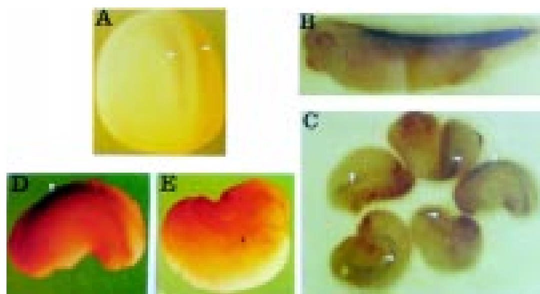

Fig. 5. XPDGF receptor antisense RNA inhibited the normal development of mesoderm. *Embryos were microinjected with XPDGFR antisense RNA (0.7 ng) at 2 cell stage, cultured until control embryo reached to stage 40, were then assayed by whole-mount in situ hybridization for the expression of α-cardiac actin. While the neo pa RNA injected control embryo showed normal appearance* **(A,B)**, *the embryos injected with XPDGFRα antisense RNA showed that axis was distorted* **(C**, *white arrow head***)**, *α-cardiac actin gene was partially expressed on the non-injected side* **(D**, *white arrow head***)**, *and neural fold was not closed* **(E**, *black arrow***)**.

To confirm that PDGF induces mesoderm, the signals of XPDGFR's signals were blocked by the microinjection of XPDGFR antisense RNA. The activation of PDGF receptor tyrosine kinase is known to depend on the dimerization of this receptor in response to ligand binding and this dimerization is thought to be mediated principally by the extracellular domain of the receptor (Ullrich and Schlessinger, 1990). To block ligand-receptor binding, *Xenopus* PDGFRα antisense (XDPGFRαAS) RNA complementary to the extracellular domain of the receptor was synthesized and capped with 5'7meGppp5'G to minimize degradation in cytoplasm. The results of the microinjection of XPDGFR antisense RNA suggest that the blockade of PDGFR inhibited PDGF mesoderm-inducing ability from pre-ectodermal cells in *Xenopus*. This was identical with the previous results-mesoderm induction by PDGF. The results indicate that the blockade of PDGFR signaling would result in abnormal mesodermal patterning in the whole embryo. As shown in Figure 5, in embryos where the PDGFR signaling was blocked, many abnormal features were obvious. Most of the injected embryos had unclosed neural folds (Fig. 5E and Table 2). In some embryos, the neural fold was opened toward the posterior side. In other embryos, the cement gland was developed but the tail was not. Furthermore, in that case axial formation was abnormal and the arrangement of somites was in a ring form due to abnormal gastrulation (Atalios *et al.,* 1995). The expression of the α-cardiac actin gene shows some interesting phases. Abnormal somites developed in the region originated from the XPDGFRα antisense RNA-injected side of the embryo. The size of the somites was about half, compared with that of a normal embryo and the distance of the somites was wider than that of somites in a normal embryo. In those embryos, the axis was distorted, thus arrangement of the somites was crooked (Fig. 5C) but that of somites in control embryo was in a straight line along the anterior-posterior axis (Fig. 5A,B). This result reveals that PDGF relates to mesoderm formation, which requires the expression of MyoD family genes. As the embryo in which the signal of PDGF receptor was blocked still has the other resident signals such as activin and FGF receptors, muscle formation cannot be interrupted completely. Nonetheless, the reason why the extent of malformation was severe might be that PDGF compensates for the role of TGF-β. TGF-β would only be mitogenic for cells which express PDGF receptors (Leof *et al*., 1985), thus pattern formation was significantly abnormal. Further information on the role of PDGF receptor α during normal patterning of the somites has been obtained from studies in mouse embryos. Mice carrying a targeted null mutation had a deficiency in myotome formation (Soriano, 1997). Unclosing of the neural fold is known to be due to the virtual absence of presumptive sclerotomal cells (Schatteman *et al*., 1992). This reflects that the initial migration and proliferation of sclerotomal precursors requires PDGFR expression. Our results suggest that PDGF mediates mesoderm induction and plays an important role in early development of *Xenopus laevis*. This PDGF study is expected to contribute to understanding the complexities of the mechanism of mesoderm induction in *Xenopus laevis*.

Materials and Methods

Embryos

Xenopus embryos were fertilized and chemically dejellied using 2% cysteine-HCl, pH 7.8, then maintained in 10% Marc's modified Ringer's (0.1xMMR) containing 50 units/ml of antibiotics mixture at 20-24°C until they had reached the appropriate stages for injection or analysis. Microinjection was performed in a solution of 3% Ficoll in 0.1xMMR using bevelled glass capillary needles. The number of embryos used and the amount of RNA injected are shown in Table 2. For the animal cap assay, the vitelline membrane of the embryos was removed manually, then animal caps were explanted at stage 8 using a tungsten needle and maintained in 75% MMR/0.1% bovine serum albumin until control embryos had reached

TABLE 1

OLIGONUCLEOTIDE PRIMERS USED IN THIS STUDY FOR RT-PCR ASSAY

Markers Sequences	Annealing Temperature (° C)	Reference
XMyoD	60.9	
U 5'-CCT TCC CGA CCC CCG ACG ACT TC-3'		Hopwood *et al*., 1989
D 5'-CCT TGG GGA GCC TTT GGT TGG GG-3'		
XMyf5	54.3	
U 5'-AGC CCT CAA TGG TCT GGA AG-3'		Hopwood *et al*., 1991
D 5'-GCG GAA GGG AGT CAG TGC TA-3'		
XMRF4	55.7	
U 5'-TGT GCG GAT GGG GTT TTA TT-3'		Jennings, 1992
D 5'-TGG TCC CTC TCC TGC TGG TT-3'		
α-cardiac actin	55.0	
U 5'-GCT GAC AGA ATG CAG AAG-3'		Mohun *et al*., 1986
D 5'-TGT GTG AGG AGG TCC CGT CA-3'		
SL1	55.0	
U 5'-CTC ACA AGG GGG AAA AGG GTT-3'		Chambers *et al*., 1992
D 5'-AAC TGC CGG GAG AAC TGA ACG-3'		
XMLC2	55.0	
U 5'-GTG GGC ATG TCG GAG AAA-3'		Chambers *et al*., 1994
D 5'-CCA CAC ATC CAG CCA CCC-3'		
XPDGFRα	55.0	
U 5'-GTT ACG GTC CAT GAT GCC TG-3'		Jones *et al*., 1993
D 5'-GCC GCT GTT GTT TTC TTC AC-3'		
EF1-α	55.0	
U 5'-CAG ATT GGT GCT GGA TAT GC-3'		Krieg *et al*., 1989
D 5'-GAT CCT CAG TAG TTC CGT CA-3'		

U and D refer to upstream and downstream primers. Most primers were designed by us. Some primers were designed after published papers.

TABLE 2
DEFECTS IN NEURAL FOLD CLOSURE BY THE INJECTION OF XPDGFR ANTISENSE RNA

	XPDGFRα As RNA injected Number of embryos	**neo pa RNA** injected Number of embryos
	Survival ratio	
	alive : dead	**alive : dead**
Expt.1	57 : 3	23 : 0
Expt.2	81 : 0	36 : 0
Expt.3	80 : 0	30 : 0
	*** Neural fold**	
	not closed : closed	**not closed : closed**
Expt.1	57 : 0	0 : 23
Expt.2	81 : 0	0 : 36
Expt.3	80 : 0	0 : 30

Embryos were injected with 0.7ng (expt. 1, 2, 3) of XPDGFRα antisense RNA at 2cell stage and scored at the time when control embryos reached stage 40.

* In that experiment, the embryos injected with Myf5 antisense used as injection control showed the same result that was identical with control embyo.

the required stage. Growth factors (50 ng/ml PDGF-AA, Boehringer Mannheim, 50 ng/ml FGF2, Gibco-BRL, 50 ng/ml activin, kindly provided by Asashima, Tokyo university) were added directly to the culture. The stage followed the normal table of *Xenopus laevis* by Nieuwkoop and Faber (1967).

RT-PCR assay

Total RNA was prepared from animal cap explants using UltraspecTM-II kit (KDR). Cellular DNA was digested by treatment with DNase (Boehringer Mannheim) for 30 min at 37°C. Reverse transcription was carried out on RNA from 15 animal cap equivalents using AMV reverse transcriptase (20 units, Promega) at 40°C for 60 min in a 20 μl reaction mixture containing 50 mM Tris pH 8.0, 30 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 1 mM of dNTP, 50 units RNAsin (Promega), 0.1 mg/ml BSA and 1.6 μl oligo(dT) (IDT). Two μl of RT sample was used per PCR reaction. PCR reactions were carried out in a 25 μl reaction volume and carried out as described previously by Hemmati-Brivanlou *et al.,* 1992. The primers used for this study are listed in Table 1.

In vitro transcription

To generate XPDGFRα antisense RNA, PCR generated partial cDNA (406-636) of XPDGFRα was cloned, transcribed, and capped with 5'7 meGppp5'G. The quality of the synthetic RNA was assessed by 6% polyacrylamide gel electrophoresis and the concentration volume was determined spectrophotometrically.

Whole-mount in situ hybridization

Partial length α-cardiac actin antisense and sense probes were generated in the presence of dUTP-digoxygenin by PCR. For the antisense probe, the ratio of antisense primer versus sense primer was 50: 1 and the ratio for sense probe was 1: 50 (Boehringer Mannheim). Whole-mount *in situ* hybridization of albino embryos was carried out as described by Harland (1991). A mixture of 4-nitro blue tetrazolium chloride (NBT) and X-phosphate was used as substrate for the color reaction. The animal cap explants were sectioned, then the results were examined using Olympus IMT-2 inverted microscopes.

Acknowledgments

This paper was supported by NON DIRECTED RESEARCH, Korea research Foundation, 1996-98. We are extremely grateful to Drs. G. Malascinski and M. Asashima for critical readings of earlier versions of this manuscript and Yoon Chul-Jung for his help with the embryo section.

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Received: April 1999
Accepted for publication: June 1999