Title: Role of dipeptidyl peptidase-4 as a potentiator of activin / nodal signaling pathway

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ABSTRACT

DPP4 (dipeptidyl peptidase-4), a highly conserved transmembrane glycoprotein with an exo-peptidase activity, has been shown to contribute to glucose metabolism, immune regulation, signal transduction, and cell differentiation. Here, we show that DPP4 is involved in control of activin/nodal signaling in *Xenopus* early development. In support of this, gain of function of DPP4 augmented Smad2 phosphorylation as well as expression of target genes induced by activin or nodal signal. In addition, *Dpp4* and *Xnr1* showed synergistic effect on induction of ectopic dorsal body axis, when co-injected at suboptimal doses in early embryos. Conversely, saxagliptin, a DPP4 inhibitor repressed activin induction of Smad2 phosphorylation. Notably, overexpression of *Dpp4* disrupted specification of dorsal body axis of embryo, leading to malformed phenotypes such as *spina bifida* and a shortened and dorsally bent axis. Together, these results suggest that DPP4 functions as a potentiator of activin/nodal signaling pathway.

INTRODUCTION

The transforming growth factor β (TGF-β) superfamily of secreted proteins has more than 30 members including TGF-βs, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs), and Activin and Nodal. These members are involved in regulation of early development and tissue homeostasis of metazoans (1-3). In particular, Activin/Nodal and BMPs are responsible for induction and subsequent patterning of mesoderm and endoderm germ layers, as well as for gastrulation in vertebrate early embryos (4-6). Signaling by TGF-β and related ligands uses two types of receptors, type Ⅰ and type Ⅱ transmembrane serine-threonine kinases. Ligand binding results in formation of heterotetrameric receptor complex in which type II receptor activates type I receptor via phosphorylation of its GS region. Subsequently, the activated type I receptor triggers phosphorylation of receptor-regulated Smads (R-Smads), such as Smad 1/5/8 in BMP pathway and Smad 2/3 in Activin, Nodal and TGF-β signaling pathways. Phosphorylated R-Smads interact with Co-Smad, Smad4 and then move into the nucleus to regulate transcription of a variety of target genes (1, 7).

DPP4 (dipeptidyl peptidase-4), also known as adenosine deaminase complexing protein 2 or CD26 (cluster of differentiation 26), is a highly conserved cell surface glycoprotein belonging to the serine protease family (8). It is ubiquitously expressed on a variety of tissues including immune, endothelial and epithelial cells (9). However, it also exists in soluble form in plasma and other body fluids (10). It consists of a short cytoplasmic domain, a transmembrane region and an extracellular domain with dipeptidyl peptidase activity, which selectively removes the N-terminal dipeptides such as X-Pro and X-Ala from polypeptide substrates (11). Activity of this transmembrane exo-peptidase exerts major influence on immune regulation, signal transduction, glucose metabolism, cell migration, and cell

differentiation by controlling the activity of its substrates or by associating physically with other proteins (10). DPP4 acts indiscriminately on a broad range of substrates (12), including growth factors, chemokines, neuropeptides, and vasoactive peptides. In contrast to its crucial regulatory roles, DPP4 knockout mice show normal phenotypes, even with an ameliorated glucose metabolism and more physical activity (13). Either increase or decrease of DPP4 expression has been shown to underlie a variety of pathophysiological processes (9). Furthermore, it appears to work in the development of cancer and tumors (8, 14, 15).

In our initial attempt to identify the role of DPP4 in vertebrate early embryogenesis, it has been found that DPP4 is involved in control of TGF-β signaling. In this study, we present evidence that it specifically acts as a potentiator of activin/nodal signaling pathway.

RESULTS

DPP4 enhances downstream events induced by activin/nodal signal but not by BMP4 signal

To unravel biological functions of DPP4 in *Xenopus* early development, we initially sought to investigate its activities in stem cell-like ectodermal tissue of early embryo. For this, we overexpressed *Dpp4* mRNA in that tissue and analyzed its effects on downstream events induced in response to key cellular signalings, such as Activin/Nodal, BMP, FGF and Wnt signaling pathway. Overexpression of *Dpp4* did not affect ERK phosphorylation induced by FGF signal and β-catenin stabilization by Wnt signal, as well as expression of target genes by both of them (data not shown). Of note, treatment with activin protein or injection of *Xnr1* mRNA induced ectopically phosphorylation of Smad2, a downstream mediator of activin/nodal pathway, in ectodermal tissue (Fig. 1A, lanes 1, 2, 5 and 6), which could be

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further enhanced by expression of increasing doses of *Dpp4* (Fig. 1A, lanes 3, 4, 7 and 8). In contrast, when co-injected, *Dpp4* had no effect on phosphorylation of Smad1 induced in BMP4-stimulated explant tissues (Fig. 1B).

Using RT-PCR analysis, we have also investigated whether the enhancing effects of DPP4 on activin/nodal signaling may be exerted at the transcriptional levels. As shown in Fig. 1C, ectopic expressions of mesodermal markers *Chordin* and *Xbra* were induced by stimulation with activin or Xnr1 in animal explants (lanes 3, 4, 7 and 8), and their expression levels were up-regulated gradually by co-injection of increasing amount of *Dpp4* (lanes 5, 6, 9 and 10). In contrast, co-injection of *Dpp4* did not influence increased levels of expression of target genes such as *Xbra* and *Xvent2* induced by BMP4 in animal caps (Fig. 1D). These results are in line with the effects of DPP4 on levels of phosphorylated R-Smads in activin/nodal or BMP4-activated animal cap cells. Overall, we conclude that DPP4 acts as an enhancer of activin/nodal signaling, but not of BMP signaling.

DPP4 synergizes with nodal signal in inducing an ectopic dorsal axis of embryo

Injection of *Xnr1* mRNA in the ventral region of embryos at the optimal levels can induce ectopically a secondary dorsal trunk with no head (16), whereas it causes no extra axis when injected at below optimal levels, i.e. suboptimal levels. Next, this secondary axis assay was used to test whether DPP4 could augment suboptimal levels of nodal signal, to levels sufficient to induce ectopic dorsal axis. As shown in Fig. 1E, single ventral injection of a range of concentration of *Dpp4* could cause no secondary dorsal trunk. Likewise, *Xnr1* did not induce any ectopic dorsal axis when injected at suboptimal levels. By contrast, the ectopic dorsal trunks were induced efficiently in embryos co-injected ventrally with *Dpp4* and *Xnr1* at the same doses. Thus, this synergism in inducing an ectopic dorsal axis supports that DPP4

may function to potentiate activin/nodal signaling pathway.

A DPP4 inhibitor suppresses Smad2 phosphorylation induced by activin signal

We next examined whether DPP4 activity is critical for activin/nodal signaling by using DPP4 inhibitors, including alogliptin, sitagliptin, vildagliptin and saxagliptin. Interestingly, stimulation with activin protein induced high levels of phosphorylated Smad2 in HEK293T cells (Fig. 2, lanes 1-6), which could be disturbed efficiently by treatment with saxagliptin but not with alogliptin, sitagliptin or vildagliptin (lanes 7-10). Since DPP4 inhibitors have unique drug-specific effects depending on the cell type (17), it is possible that only saxagliptin could interfere with DPP4 potentiation of activin signaling in HEK293T cells. Collectively, these data indicate that DPP4 activity is essential for activin/nodal signaling.

Spatio-temporal expression pattern of *Dpp4*

To investigate whether DPP4 enhancement of activin/nodal signaling could be observed at the organismal level during *Xenopus* early embryogenesis, we first examined the temporal expression pattern of *Dpp4* by RT-PCR analysis, using cDNAs from whole embryos harvested at several embryonic stages. As shown in Fig.3A, maternal mRNA messages of *Dpp4* were abundant, but its zygotic transcription was barely detectable. *In situ* hybridization was also performed to determine the spatial expression pattern of *Dpp4* in *Xenopus* early embryos. Of note, its expression was strong in the animal and marginal regions of early gastrulae and absent in the vegetal region (Fig. 3B). Consistently, this regional distribution of *Dpp4* transcripts was also confirmed by RT-PCR experiment (Fig. 3C). Given that activin/nodal signal is responsible for mesoderm formation in the marginal zone at the blastula and gastrula stages (18), both the spatial and temporal expression patterns of *Dpp4*

transcripts suggest that it may function as a maternal factor to regulate formation of primitive germ layers.

Overexpression of *Dpp4* disrupts formation of dorsal axial structures

To determine whether DPP4 could affect germ layer specification, we next overexpressed *Dpp4* mRNA into the animal, marginal or vegetal regions of *Xenopus* cleavage stage embryos, and observed resulting morphological phenotypes at the tadpole stages. In particular, when injected in the dorsal marginal region, *Dpp4* caused a failure in gastrulation movements, which led to *spina bifida* and a reduced and dorsally kinked body axis (Fig. 4A). These malformed phenotypes are reminiscent of those of embryos exposed to increased levels of activin/nodal signals (19, 20), substantiating that DPP4 acts to up-regulate these signals. However, ventral injection of *Dpp4* generated no morphological alterations (Fig. 4A). We also checked the effects of overexpression of *Dpp4* on endogenous expression of mesodermal marker in early gastrulae. Notably, expression of a dorsal mesodermal marker *Goosecoid* was expanded in the LacZ staining-positive area of embryo overexpressing *Dpp4* (Fig. 4B), compared with that in the control embryo injected with *LacZ* alone. Together, these results indicate that gain-of-function of DPP4 up-regulates expression of target genes of activin/nodal signal such as *Goosecoid*, thereby disrupting axial structures of *Xenopus* embryo.

DISCUSSION

It has been shown that DPP4 knockout mice are phenotypically normal, though it plays critical roles in immune regulation, cell migration, cell differentiation, glucose metabolism,

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and signal transduction (10). In this study, we have thus re-investigated the function of DPP4 at the organismal level using *Xenopus* early embryos with the result that it may act as a potentiator of activin/nodal signal to control germ layer specification. As shown by developmental RT-PCR analysis, strong expression of *Dpp4* during the blastula and gastrula stages of embryos suggests its possible role as a maternal factor in early induction of germ layers. Given that in *Xenopus*, mesoderm is induced in the marginal zone of blastulae by signals from its vegetal region (21), spatial expression of *Dpp4* in early gastrulae also indicates its probable function in mesoderm formation. In support of this, overexpression of *Dpp4* caused morphological changes in embryos, including *spina bifida*, exo-gastrulation, and a shortened and kinked body axis, which recapitulate defective phenotypes of embryos experiencing up-regulated levels of mesoderm-inducing signal, nodal (22). Knockdown of *Xenopus* Lefty, a secreted antagonist of nodal signaling pathway, causes expansion of mesendoderm, thereby leading to exo-gastrulation (19). In addition, depletion of maternal Zic2 transcription factor, which impedes the expression of nodal-related genes during anteroposterior patterning, results in the same phenotypes as enhanced nodal signaling (20). Since gain-of-function of DPP4 causes the same embryonic defects as excess Nodal signal, it seems likely that DPP4 acts as a positive regulator to control nodal signaling. Consistently, overexpression of *Dpp4* enhanced phosphorylation of Smad2 and transcription of target genes induced by activin or nodal signal. In contrast, DPP4 did not affect phosphorylation of Smad1 and expression of target genes induced by BMP4 signal. Furthermore, ventral injection of *Dpp4* produced no morphological alteration, though BMP4 signal is highly active in the ventral region of embryo. Taking together, we conclude that DPP4 functions to potentiate only activin/nodal signal, but not BMP signal during formation of primitive germ layers.

DPP4 has been known to exist not only as a transmembrane glycoprotein but also in a secreted soluble form (10). This feature of DPP4 suggests that it may act either as a cell surface protein or as an extracellular matrix component to control activin/nodal signaling. Given the enhancing effects of DPP4 on Smad2 phosphorylation induced by activin or nodal ligand, it is possible that DPP4 facilitates access of the ligand to its cognate receptor and co-receptor, by interacting with the former on cell surface or outside the cell. Activin, nodal and BMP ligands share type II receptors for signaling, such as activin receptor type 2A (ACVR2A) and ACVR2B (2). Activin and nodal, but not BMP ligands, share type I receptors including ALK2, ALK4, and ALK7. As DPP4 augments activin as well as nodal signaling pathways but not BMP4 signaling, it may also promote assembly of the receptor complex, triggered by ligand binding, by associating with type I receptors shared by activin and nodal. In addition, it cannot be excluded that DPP4 may function as an exo-peptidase to modulate activity of substrates for regulation of activin/nodal signaling. Substrates may include activin/nodal ligands, their receptors, or extracellular enzymes responsible for ligand processing.

It is of note that among DPP4 inhibitors tested in this study, only saxagliptin disturbed activin induction of Smad2 phosphorylation but not alogliptin, sitagliptin and vildagliptin. While these inhibitors act commonly by inhibiting the catalytic activity of the DPP4 enzyme, they display unique drug-specific effects, depending upon the cell type (17). Unlike alogliptin and sitagliptin, saxagliptin and vildagliptin inhibit DPP4 by forming covalent enzyme-inhibitor complexes characterized by slow rates of binding and dissociation, which results in their relatively prolonged duration of activities (23). In addition, the half maximal inhibitory concentration (IC_{50}) of saxagliptin is lower than that of vildagliptin, albeit in the low nanomolar range. These features of saxagliptin may contribute to its unique inhibition of

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activin signaling in HEK294T cells as shown in our data. However, more experiments are necessary to elucidate the precise mechanism underlying inhibition of activin signaling by saxagliptin.

In summary, we have found that DPP4 functions to enhance activin/nodal signaling for mesoderm formation in *Xenopus* early embryos. Future studies are warranted to identify activin/nodal signaling components with which DPP4 interacts, and to check the requirement of its exo-peptidase activity for this signaling, to gain deep insight into the molecular mechanism by which DPP4 potentiates activin/nodal signaling pathway.

MATERIALS AND METHODS

Embryo and *in vitro* fertilization

Xenopus laevis were obtained from NASCO & Korean *Xenopus* Resource Center for Research. Unfertilized eggs were *in vitro* fertilized using macerated testis, dejellied in 2% cysteine buffer (pH 8.0), and cultured in 0.33 x Modified Ringer (MR) until stage 8 and then transferred to 0.1 x MR with gentamycin. Developmental stages of embryos were determined according to Nieuwkoop and Faber's normal table of development (24).

DNA constructs and RNA synthesis

The complete coding region of *Xenopus laevis Dpp4* was amplified by PCR and inserted in the sites of pCS2+ vector (*Dpp4*-CS2). DNA constructs used in this study are as follows: *Xnr1*-CS2+ (21) and *BMP4*-CS2+ (25). Capped mRNAs were synthesized by *in vitro* transcription of *NotI*-linearized DNA constructs using mMESSAGE mMACHINE RNA

synthesis kit (Ambion) and SP6 RNA polymerase.

Animal cap assay

Animal pole cells from *Xenopus* blastula stage embryos are pluripotent, and can differentiate into a variety of cell types depending on the signals they are exposed to. Thus, the stem cell-like animal cap explants are dissected at stage 8-8.5 from embryos animally injected with specific RNAs, which encode secreted signaling molecules or transcriptional regulators, cultured to desired stages in 1 x MR containing bovine serum albumin (BSA, 10 μg/ml) and gentamicin (50 μg/ml), and then subjected to further experiments.

RT-PCR (reverse transcriptase-polymerase chain reaction)

Total RNA was isolated from whole embryos and explant tissues using TRI Reagent (Sigma). For RT-PCR, the method of Wilson and Melton (26) was broadly followed except that DNA amplification was performed non-radioactively. Approximately 5 μg of DNase I-treated total RNA was reverse transcribed using a random hexamer and M-MLV reverse transcriptase (Promega). PCR reactions were performed in a standard 50 μl PCR reaction with *Taq* polymerase (TaKaRa). Primers were: *Dpp4*, (forward) 5'-GTGGGAATTGGGAAGTAACC-3', (reverse) 5'-GTGGGCATCTGAATCTCATC-3'; *Xbra*, (forward) 5'-GCTGGAAGTAT GTGAATGGAG-3', (reverse) 5'-TTAAGTGCTGTAATCTCTTCA-3'; *Chordin*, (forward) 5'-CCTCCAATCCAAGACTCCAGCAG- 3', (reverse), 5'-GGAGGAGGAGGAGCTTTGG GACAAG-3'; *Vg1*, (forward) 5'-ATGCCTATTGCTTCTATTTGC-3', (reverse) 5'-GGTTTA CGATGGTTTCACTCA-3'; *ODC*, (forward) 5'-GTCAATGATGGAGTGTATGGATC-3', (reverse), 5'-TCCATTCCGCTCTCCTGAGCAC-3'; *Xvent2*, (forward) 5'-TGAGACTTGGG CACTGTCTG-3', (reverse), 5'-CCTCTGTTGAATGGCTTGCT-3'; *EF1-α*, (forward) 5'-

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CCTGAACCACCCAGGCCAGATTGGTG-3', (reverse) 5'-GAGGGTAGTCAGAGAAGC TCTCCACG-3'.

In situ hybridization

Whole-mount *in situ* hybridization was performed as described in (27). Anti-sense digoxigenin RNA probes were *in vitro* synthesized using template cDNA encoding *Dpp4* and *Gsc* (28). For anti-sense *Dpp4* probe, *Dpp4-pGEM T* vector was linearized with *NcoI* and transcribed with SP6 RNA polymerase. BM purple (Roche) was used as substrate for alkaline phosphatase.

Western blotting analysis

For Western blot analysis, animal cap explants or HEK293T cells were homogenized in Triton X-100 lysis buffer (1% Triton X-100, 50 mM NaCl, 1mM EDTA, 50 mM Tris-Cl (pH 7.6), 10 mM NaF, 1mM Na_3VO_4, 1mM PMSF, 20 μg/ml Aprotinin, 20 μg/ml Leupeptin). Equal amounts of protein were separated by 10 or 8% SDS–PAGE electrophoresis. Western blots were performed, according to standard protocol with P-Smad1 (Cell signaling, 1: 1,000), Smad1 (Cell signaling, 1:1,000), P-Smad2 (cell signaling, 1: 1,000) and Smad2 (Cell signaling, 1: 1000) primary antibodies.

ACKNOWLEDGMENTS

CONFLICTS OF INTEREST

The authors have no conflicting interests.

FIGURE LEGENDS

Figure 1. Dpp4 enhances activin/nodal signaling but not BMP4 signaling. (A-D) Four-cell stage embryos were injected in the animal pole region as indicated with increasing doses of *Dpp4* (10, 1000 pg) alone or with *Xnr1* (50 pg) or *BMP4* (100 pg) mRNAs, and then animal cap explants were excised at stage 8.5 from injected or uninjected embryos, cultured to stage 10.5 for RT-PCR analysis (C and D) or 11 for Western blotting (A and B) in the presence or absence of activin protein (5 ng/ml) as shown. Smad1, Smad2 and *ODC* serve as loading controls. Co AC, uninjected control animal caps. (-), no injection of *Dpp4*. WE, stage 10.5 whole embryo. –RT, control in the absence of reverse transcriptase. (E) One blastomere of four-cell stage embryos was injected in the ventral marginal region with *Dpp4* (50 pg) and/or *Xnr1* (5 pg) mRNAs as indicated, and injected embryos were cultured until uninjected sibling embryos reached tadpole stages. Arrows denote the induced secondary dorsal axis. Embryos are shown in lateral views with anterior to the left.

Figure 2. Saxagliptin, a DPP4 inhibitor represses Smad2 phosphorylation induced by activin. HEK293T cells were pre-treated for 1 hour with DPP4 inhibitors as indicated and subsequently incubated for 3 hours with or without activin (20 ng/ml), and harvested for Western blotting. Smad2 is a loading control.

Figure 3. Spatio-temporal expression pattern of *Dpp4* in *Xenopus* early embryogenesis. (A) Temporal expression pattern of *Dpp4* analyzed by RT-PCR. St., a developmental stage. *ODC* serves as a loading control. –RT, stage 27 control whole embryos in the absence of reverse transcriptase. (B and C) Whole mount *in situ* hybridization (B) and RT-PCR (C)

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showing spatial expression pattern of *Dpp4* transcripts at stage 10.25. Embryos in (B) are shown in lateral (upper panel) or dorso-vegetal (lower panel) view with animal to the top. For (C), respective regional explants were dissected from stage 10.25 whole embryos. D, dorsal; V, ventral; VP, vegetal pole; MZ, marginal zone; AP, animal pole; DMZ, dorsal marginal zone; VMZ, ventral marginal zone. *Chordin* is a dorsal mesodermal marker, *Vg1* is a member of TGF-β family expressed vegetally, and *EF1-α* is a loading control. –RT, stage 10.25 control whole embryos in the absence of reverse transcriptase.

Figure 4. Embryonic morphological phenotypes caused by overexpression of *Dpp4*. (A) Four-cell stage embryos were injected in the dorsal or ventral marginal regions with *Dpp4* (500 pg) mRNA and cultured to tadpole stages. Embryos are shown in lateral views with anterior to the left. Uninjected, uninjected control embryos. (B) Four-cell stage embryos were injected in the dorsal marginal region with *LacZ* (50 pg) alone or with *Dpp4* (1 ng) mRNA, cultured to stage 10.25, and fixed for LacZ staining (red) and subsequent *in situ* hybridization against *Goosecoid (Gsc)*. Arrows indicate expression of *Gsc* in the dorsal lip. Embryos are shown in dorso-vegetal views with anterior to the top. Control, an embryo injected with *LacZ* alone.

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goosecoid. Cell 67, 1111-1120

Fig. 1. Figure 1
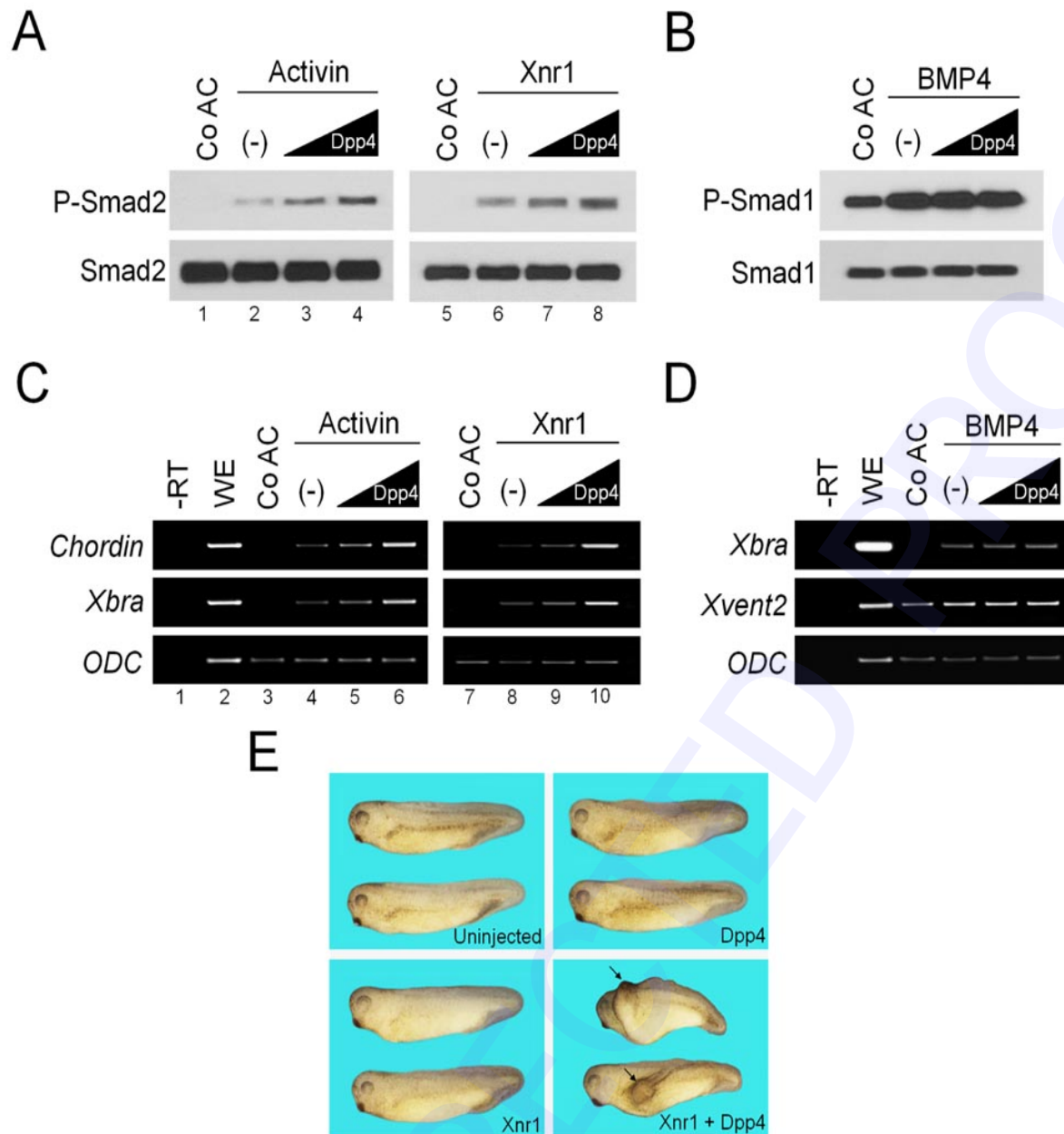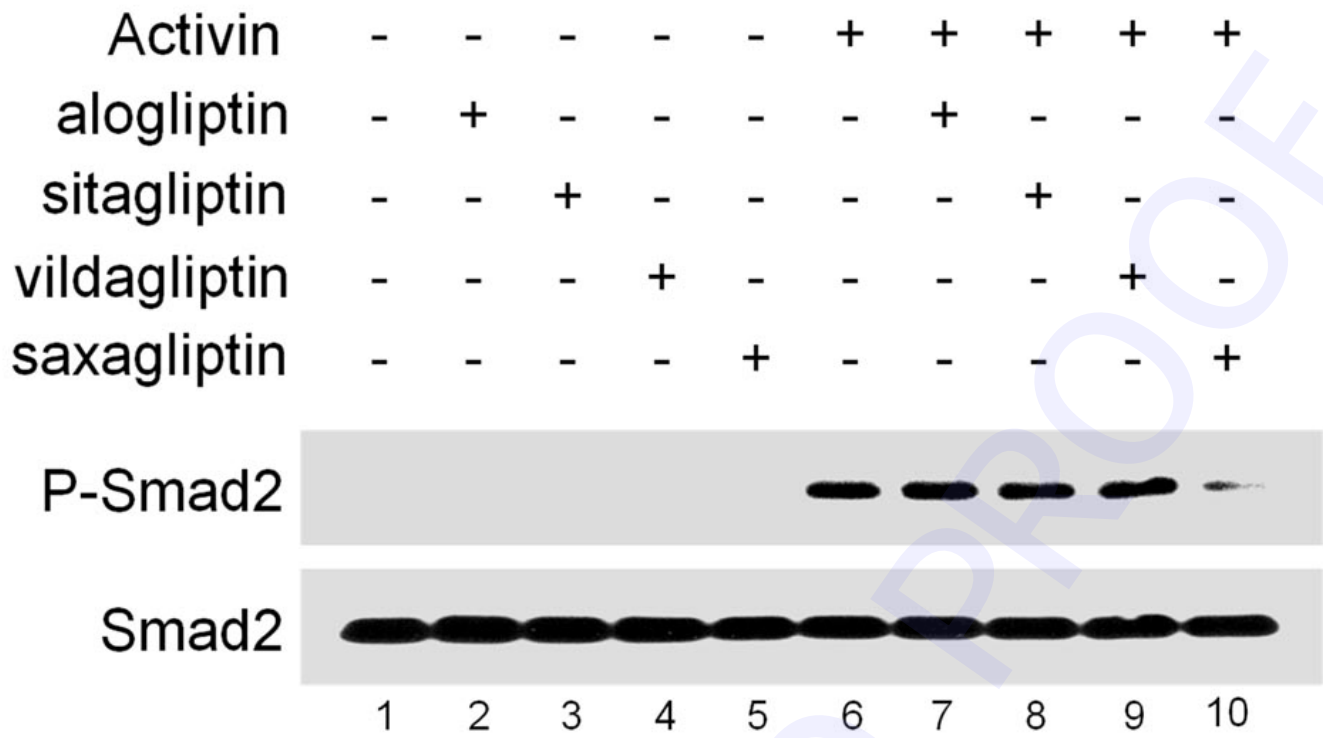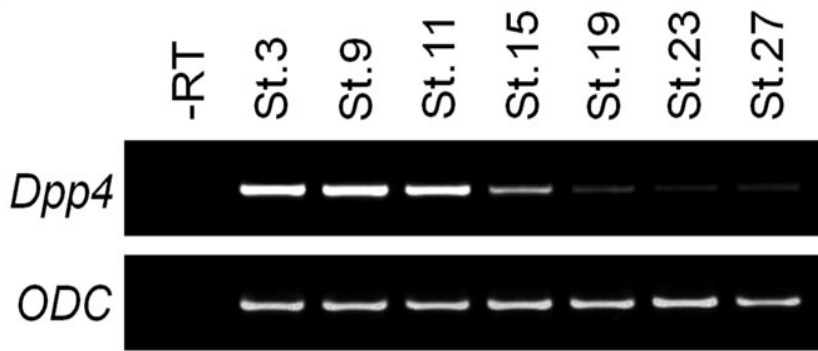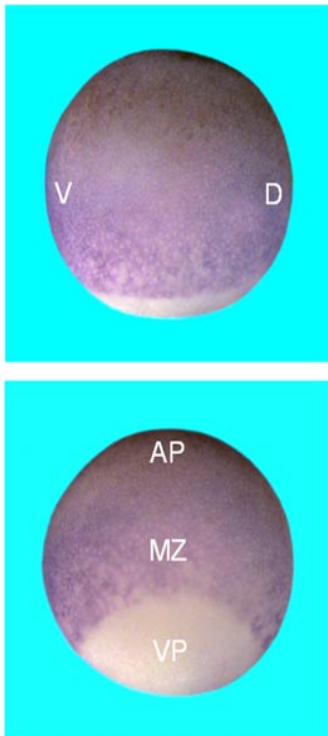
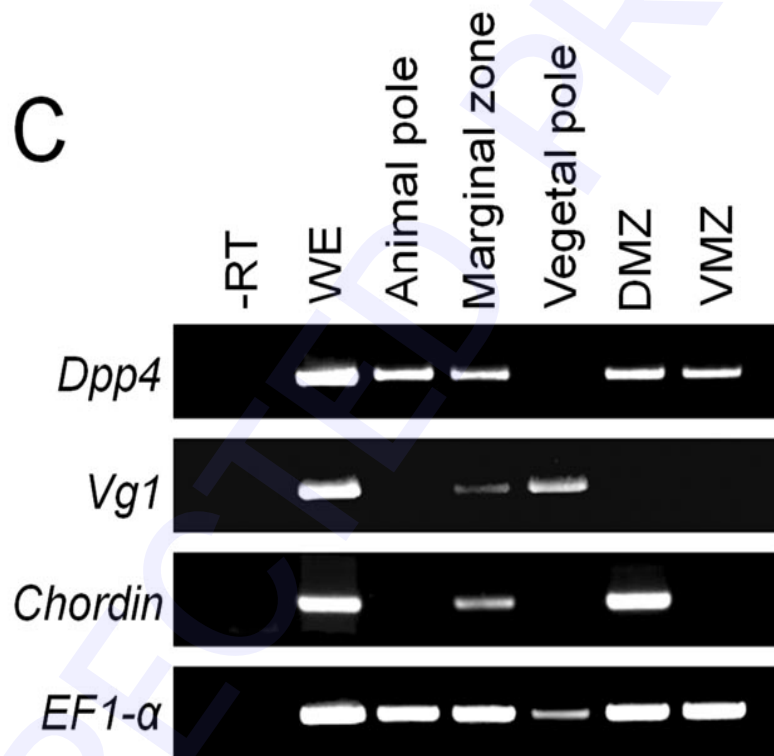| Activin | - | - | - | - | - | + | + | + | + | + |
alogliptin	-	+	-	-	-	-	+	-	-	-
sitagliptin	-	-	+	-	-	-	-	+	-	-
vildagliptin	-	-	-	+	-	-	-	-	+	-
saxagliptin	-	-	-	-	+	-	-	-	-	+

P-Smad2

Smad2

1 2 3 4 5 6 7 8 9 10

Fig. 2. Figure 2

Fig. 3. Figure 3

A

B

Fig. 4. Figure 4