Int. J. Dev. Biol. 48: 327-332 (2004)

Original Article

Activin-like signaling activates Notch signaling during mesodermal induction

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ABSTRACT Both activin-like signaling and Notch signaling play fundamental roles during early development. Activin-like signaling is involved in mesodermal induction and can induce a broad range of mesodermal genes and tissues from prospective ectodermal cells (animal caps). On the other hand, Notch signaling plays important roles when multipotent precursor cells achieve a specific cell fate. However, the relationship between these two signal pathways is not well understood. Here, we show that activin A induces *Delta-1, Delta-2* and *Notch* expression and then activates Notch signaling in animal caps. Also, *in vivo,* ectopic activin-like signaling induced the ectopic expression of *Delta-1* and *Delta-2,* whereas inhibition of activin-like signaling abolished the expression of *Delta-1* and *Delta-2*. Furthermore, we show that *MyoD,* which is myogenic gene induced by activin A, can induce *Delta-1* expression. However, *MyoD* had no effect on *Notch* expression, and inhibited *Delta-2* expression. These results indicated that activin A induces *Delta-1, Delta-2* and *Notch* by different cascades. We conclude that Notch signaling is activated when activin-like signaling induces various tissues from homogenous undifferentiated cells.

KEY WORDS: *Xenopus laevis, activin, Notch signaling, MyoD, mesoderm induction*

Introduction

Activin A, a member of the TGF-β family, which has a strong mesoderm-inducing activity (Asashima *et al.,* 1990), can induce a broad range of mesodermal genes and tissues from amphibian prospective ectodermal cells (animal caps) in a concentration-dependent manner (Ariizumi *et al.,* 1991a; Ariizumi *et al.,* 1991b; Green and Smith, 1990). At low concentrations, activin A induces the formation of ventral and posterior mesodermal tissues such as blood cells, coelomic epithelium and mesenchyme. At intermediate concentrations, activin A induces muscle, and at high concentrations, it induces dorsal and anterior mesoderm tissues such as notochord, and yolk-loaded endoderm. In addition, activin A in combination with retinoic acid induces pronephros (Brennan *et al.,* 1999; Moriya *et al.,* 1993; Osafune *et al.,* 2002) and anterior endoderm tissues such as pancreas (Moriya *et al.,* 2000), liver, and intestine. Furthermore, activin A can also induce beating heart muscle (Ariizumi *et al.,* 1996) and jaw cartilage (Furue *et al.,* 2002) by the sandwich-culture method. *Notch* encodes a large transmembrane protein that serves as a receptor for the Delta, Serrate, and Lag-2 (DSL) family of ligands. Binding of a DSL ligand to the extracellular domain of Notch causes cleavage of the receptor's intracellular domain, which is then released from the cell membrane (De Strooper *et al.,* 1999; Mumm *et al.,* 2000). The intracellular domain of Notch translocates into the nucleus, where it can interact with members of the CBF-1, Suppressor of Hairless, Lag-1 (CSL) family. The complex of DSL and the intracellular domain of Notch subsequently activates the expression of target genes, such as *ESR*, *Hairy*, and *Hey* (Bailey and Posakony, 1995; Lecourtois and Schweisguth, 1995; Leimeister *et al.,* 1999; Tannahill *et al.,* 1995). Notch signaling is involved in multiple developmental processes and controls the cell-fate decision by regulating local cell-cell communication (Artavanis-Tsakonas *et al.,* 1999). Notch signaling main-

Abbreviations used in this paper: ALK4 dn, dominant negative activin receptor-like kinase 4; AR1, activin type II receptor; DSL, Delta, Serrate and Lag2 ligand family; ODC, ornithine decarboxylase.

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0214-6282/2004/$25.00

Printed in Spain
www.ijdb.ehu.es

tains precursor cells in an undifferentiated state, and allows cells within a multipotent precursor population to achieve a specific cell fate during development (Hitoshi *et al.,* 2002). Furthermore, Notch signaling regulates the cytodifferentiation of myogenesis, pronephric duct, pronephric tubules, pancreatic cells, and heart (Apelqvist *et al.,* 1999; Delfini *et al.,* 2000; McCright *et al.,* 2001; McLaughlin *et al.,* 2000; Nofziger *et al.,* 1999; Rones *et al.,* 2000).

We are interested in the tissues whose differentiation is regulated by Notch signaling because activin A predominantly induces the same range of tissues in animal caps. In *Xenopus* embryos, *Delta-1* and *Delta-2* are expressed from gastrulation onward in the ring around the blastopore. *Notch* expression is higher in dorsal mesoderm, although *Notch* expression is relatively uniform throughout the embryo (Coffman *et al.,* 1990). These expression regions of members of Notch-signaling pathway genes seem to be overlapping the region where activin-like signaling induces mesoderm. From these findings, we speculated that the activin-like signaling and Notch signaling pathways interact at the stage during which activin-like signaling induced various tissues from homogenous undifferentiated cells in early *Xenopus* development, in particular during mesoderm induction. In this study, we demonstrate that activin A can induce Notch-signal pathway genes in a concentration-dependent manner and activate Notch signaling in animal caps. Also *in vivo*, activin-like signaling controls *Delta-1* and *Delta-2* expression. Furthermore, we show that activin A induced *Delta-1*, *Delta-2* and *Notch* by a different cascade.

Results

Activin A induces the transcription of Notch-signaling pathway genes and activates Notch signaling

We speculated that when activin A induces various tissues from homogenous undifferentiated cells, Notch signaling is activated and plays some roles. To ascertain whether activin A induced Notch signaling, we examined the time course of the precise expression levels of *Delta-1*, *Delta-2*, *Notch*, and *ESR-1* in animal caps after the treatment with activin A using real-time RT-PCR. Animal caps were dissected from stage-9 embryos and treated with 0, 0.1, 1.0, 10, and 100 ng/ml activin A for 1 hour. The expression levels of *Delta-1* (Fig. 1A), *Delta-2* (Fig. 1B), and *Notch* (Fig. 1C) remained unchanged for 9 hours after the treatment with 0.1 ng/ml activin A. The expression levels of these genes increased with time from 3 hours after the treatment with more than 1.0 ng/ml activin A. The expression of *Delta-1* and *Delta-2* in the animal caps treated with 1.0 ng/ml activin A increased approximately 6- and 8-fold at 9 hours, respectively, compared to the expression in untreated animal caps. Activin A at 1 and 10 ng/ml were more efficient in inducing *Delta-1* and *Delta-*

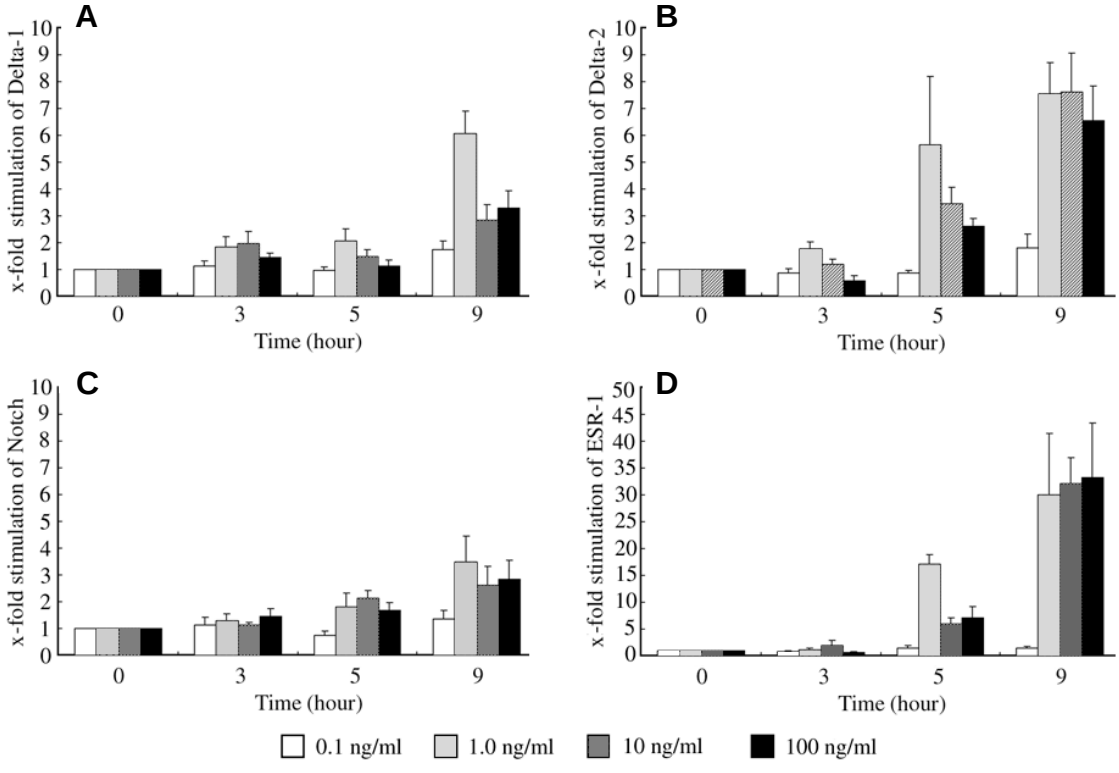

Fig. 1. Time course analysis of *Delta-1, Delta-2, Notch* and *ESR-1* mRNA expression induced by activin A by real-time RT-PCR. *RNA samples were derived from stage-9 animal caps which were treated with 0.1, 1.0, 10, and 100 ng/ml activin A for 1 hour, and then cultured for 3, 5 and 9 hours. The expression levels of* Delta-1 **(A)**, Delta-2 **(B)**, Notch **(C)** and ESR-1 **(D)** *mRNA were quantified by real-time RT-PCR. The relative "stimulation fold" was calculated as the individual expression in activin A-treated animal caps relative to that in untreated animal caps at each developmental stage. The efficiency of cDNA synthesis from mRNA was assessed on the basis of real-time RT-PCR for* ODC. *The results represent the mean from three or four independent experiments, and error bars indicate the SEM.*

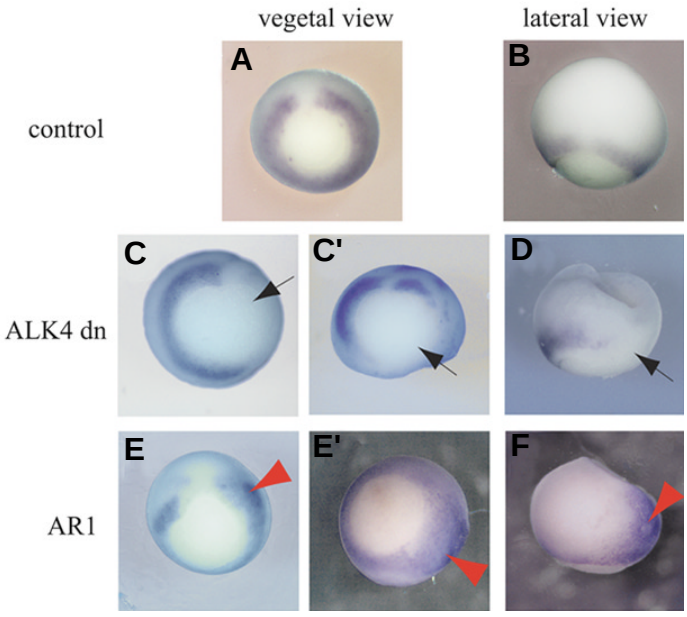

Fig. 2. The effect of Activin-like signaling on *Delta-2* expression *in vivo*. *The expression pattern of* Delta-2 *at stage 11 was examined by whole mount* in situ *hybridization.* **(A,B)** *Uninjected control embryos.* **(C,C',D)** *Embryos were injected with 500 pg of* ALK4 dn *mRNA into one blastomere at the 2- or 4-cell stage dorsally (C) or ventrally (C'). The injection of* ALK4 dn *mRNA suppressed the expression of* Delta-2 *on the injection side (black arrow).* **(E,E',F)** *Embryos were injected with 200 pg of* ARI *mRNA into one blastomere at the 2- or 4-cell stage dorsally (E) or ventrally (E'). The injection of* ARI *mRNA increased the expression of* Delta-2 *on the injection side (red arrowhead). Vegetal views, dorsal at top (A,C,C',E,E'). Lateral views, animal pole at top (B,D,F).*

2 expression than treatment with activin A at 100 ng/ml. These results indicate that there is an optimal activin A concentration for the induction of *Delta-1* and *Delta-2*. *Notch* exhibited the same temporal expression pattern as *Delta-1* and *Delta-2*. However, *Notch* expression in animal caps treated with 1.0 ng/ml activin A increased only about 3-fold at 9 hours compared with untreated animal caps. The expression of *ESR-1*, a target gene of Notch signaling, increased at 5 hours in the animal caps treated with more than 1.0 ng/ml activin A, and increased more than 30-fold at 9 hours (Fig. 1D). The increase of *ESR-1* expression ensured that activin A could trigger Notch signaling. Together, these results indicate that concentrations of activin A higher than 1.0 ng/ml induce the expression of Notch-signaling member genes, and activate Notch signaling in animal caps.

Activin-like signaling controls Delta-1 *and* Delta- 2 *expression in embryos*

The animal cap assay is a useful method to evaluate the effects and capacity of inductive signals *in vitro*, because its results likely mimic the inductive events occurring *in vivo*. To clarify whether activin-like signaling activates Notch signaling *in vivo* as it did in the animal caps, we investigated the expression of *Delta-2* in embryos injected with wild-type activin type II receptor [AR1] (Hemmati-Brivanlou *et al.,* 1992) or truncated (dominant-negative) activin type I receptor [ALK4-dn] (Chang *et al.,* 1997). In stage-11 embryos, *Delta-2* was expressed in a ring around the blastopore, with a gap in the most dorsal region (Fig. 2 A,B). The unilateral injection of ALK4-dn dorsally (Fig. 2C) or ventrally (Fig. 2C') at the 4-cell stage completely abrogated *Delta-2* expression on the injected side. In contrast, the unilateral injection of AR1 dorsally (Fig. 2E) or ventrally (Fig. 2E') at the 4-cell stage caused an increase in *Delta-2* expression on the injected side, and its expression region expanded laterally (Fig. 2F). Similarly, *Delta-1* expression was blocked by the injection of ALK4-dn and increased by the injection of AR1 (data not shown). These results indicate that activin-like signaling induces *Delta-1* and *Delta-2* expression also *in vivo*, as seen in the animal caps. Furthermore, both dorsal and ventral expression of *Delta-1* and *Delta-2* are under the control of Activin-like signaling.

MyoD *induces* Delta-1 *expression but not* Delta-2 *expression*

We showed that *Delta-1*, *Delta-2*, and *Notch* were expressed by 3 hours after activin A treatment, indicating that activin A may indirectly induce the expression of these genes. Wittenberger *et al.* reported that activin A induces *MyoD* expression and it in turn induces *Delta-1* expression (Wittenberger *et al.,* 1999). Then, we next examined whether *MyoD* can induced *Delta-2* and *Notch* expressions in addition to *Delta-1*. Animal caps were dissected from *MyoD*-injected stage-9 embryos and expression levels of *Delta-1*, *Delta-2*, *Notch*, and *ESR-1* were measured using real-time RT-PCR (Fig. 3A). The expressions of *Delta-1* and *ESR-1* in *MyoD*-injected animal caps increased approximately 8-fold compared to the expression in untreated animal caps. However, the expressions of *Notch* and *Delta-2* in MyoD-injected animal caps were almost the same or slightly reduced compared to the expressions in untreated animal caps (*Notch*, 1.72-fold, *Delta-2*, 0.65-fold). These results indicated that *MyoD* induced *Delta-1* expression but did not induce *Delta-2* and *Notch* expressions in animal caps.

In order to examine whether *MyoD* modulates *Delta-1* and *Delta-2* expressions *in vivo*, we investigated the expression of *Delta-1* and *Delta-2* in stage-11 embryos injected with *MyoD*.

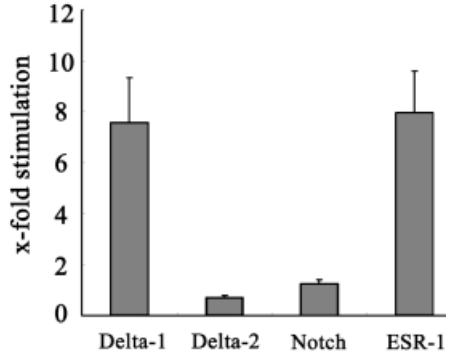

Fig. 3. The expression levels of *Delta-1, Delta-2, Notch* and *ESR-1* in *MyoD*-injected animal caps. *Animal caps were dissected from stage 9 embryos which were injected with 300 pg of* MyoD *mRNA into animal pole at 2-cell stage, and then cultured for 3 hours. The expression levels of* Delta-1, Delta-2, Notch *and* ESR-1 *mRNA were quantified by real-time RT-PCR. The relative stimulation fold was calculated as the individual expression in* MyoD*-injected animal caps relative to that in untreated animal caps. The efficiency of cDNA synthesis from mRNA was assessed on the basis of real-time RT-PCR for* ODC. *The results represent the mean from three independent experiments, and error bars indicate the SEM.*

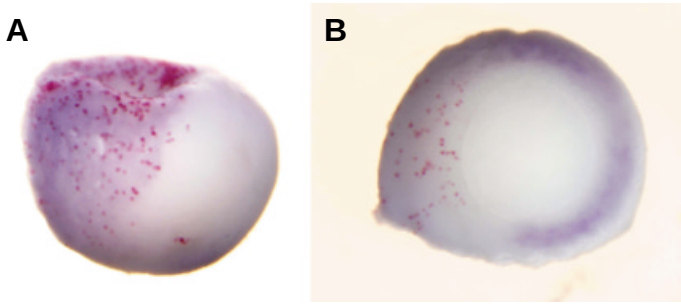

Fig. 4. The effects of *MyoD* injection on *Delta-1* and *Delta-2* expression *in vivo*. *Embryos were injected with 1.0 ng* MyoD *mRNA together with 100 pg of* lacZ *mRNA as lineage tracer into one blastomere at the 2-cell stage. The expression pattern of* Delta-1 **(A)** *(lateral view) and* Delta-2 **(B)** *(vegetal view) at stage 11 was examined by whole mount* in situ *hybridization.*

MyoD injection increased *Delta-1* expression, and ectopic *Delta-1* expression was seen in *MyoD*-injected area (Fig. 4A). In contrast, *Delta-2* expression was decreased in *MyoD*-injected area (Fig. 4B). These results indicated that *MyoD* induces *Delta-1* expression but inhibits *Delta-2* expression *in vivo*.

Discussion

Activin and Notch signaling are both indispensable for proper early development. Activin-like signaling plays important roles in the induction of dorsal mesoderm during early embryogenesis. On the other hand, Notch signaling plays important roles when multipotent precursor cells achieve a specific cell fate. We speculated that during activin A induces various tissues from homogenous undifferentiated cells, Notch signaling is activated and plays some roles. Our results revealed that, in animal caps, activin A induced the expression of *Delta-1*, *Delta-2*, and *Notch*, followed by the expression of *ESR-1*. The delay of *ESR-1* expression compared with *Delta-1*, *Delta-2*, and *Notch* suggested that activin A first induced *Delta-1*, *Delta-2*, and *Notch* expression, which activated Notch signaling, and then *ESR-1* expression was induced (Fig. 5). Our real-time RT-PCR analysis showed that there is an optimal concentration of activin A for inducing *Delta-1* and *Delta-2*. Activin A at intermediate concentrations (1 and 10 ng/ml) was more efficient in inducing *Delta-1* and *Delta-2* expression than the lower- and higher-range concentrations (0.1 and 100 ng/ml, respectively). In stage-11 embryos, *Delta-1* and *Delta-2* are expressed in the marginal zone, in a ring around the blastopore. These findings suggest that the dose-dependent induction may restrict expression area of *Delta-1* and *Delta-2 in vivo*. Concentration of activin-like signaling in the marginal zone may be at the appropriate level to induce *Delta-1* and *Delta-2* expression. We also demonstrated that activin-like signaling activates Notch signaling *in vivo* as it did in the animal caps. Injection of ALK4-dn completely abolished the expression of *Delta-1* and *Delta-2*. These results imply that activin-like signaling is absolutely necessary and that no other signaling pathway can substitute for activin-like signaling to induce *Delta-1* and *Delta-2* expression in the pre-involuted mesoderm. *Delta-1*, *Delta-2* and *Notch* were expressed by 3 hours after activin A treatment, indicating that activin A may indirectly induce the expression of these genes. Wittenberger *et al.* reported that *MyoD* induces *Delta-1* expression in *Xenopus* embryos, triggering the activation of Notch signaling (Wittenberger *et al.,* 1999). We demonstrated in this study that in addition to *Delta-1*, activin A also induces the expression of *Delta-2* and *Notch*. Furthermore, we demonstrated that *MyoD* can induce *Delta-1* expression but can not induce *Delta-2* and *Notch* expression. *Notch* expression was neither induced nor inhibited by MyoD injection in stage-11 embryos (data not shown). To our surprise, *MyoD* inhibited *Delta-2* expression in animal caps and *in vivo*. These results indicated that activin A induced *Delta-1*, *Delta-2* and *Notch* by different cascade. Activin A may induce some protein besides *MyoD*, and it in turn may induce *Delta-2* and *Notch* expression (Fig. 5). We further speculated that the inhibition of *MyoD* might alter *Delta-1* expression. Unfortunately, however, an antisense morpholino oligonucleotide against *Xenopus MyoD(b)* (MyoD MO) did not inhibit activin A-induced *Delta-1* expression in animal caps and *Delta-1* expression shown in marginal zone (data not shown). MyoD MO may not inhibit MyoD functions completely because *Xenopus laevis* is psuedotetraploid species, having four copies of most genes. Otherwise, *Delta-1* may be induced not only by *MyoD* but also by some proteins induced by activin A, and they may compensate for the lack of *MyoD* function to induce *Delta-1* (Fig. 5, dotted arrow).

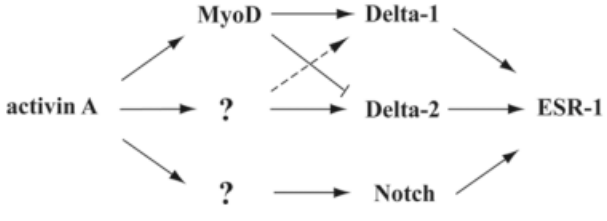

Fig. 5. Proposed model for the activation of Notch signaling induced by activin A.

In conclusion, we observed that activin A induced *Delta-1*, *Delta-2* and *Notch* expression and activated Notch signaling. Furthermore, our results suggest that activin A induces *Delta-1*, *Delta-2* and *Notch* by different cascades. Not only *MyoD* but also other activin A-induced proteins are involved in the activation of Notch signaling. We conclude that Notch signaling is activated when activin-like signaling induces various tissues from homogenous undifferentiated cells.

Materials and Methods

Embryo manipulation

Embryos were collected from *Xenopus laevis* females and artificially fertilized as previously described (Chan *et al.,* 2000). The staging of embryos was according to the method of Nieukoop and Faber (Nieuwkoop and Faber, 1956). Embryos were dejellied by treatment with 4.5% cysteine hydrochloride in Steinberg's solution (pH 8.0). Embryos were injected with mRNA in a volume of 10 nl at the two- or four-cell stage in 5% Ficoll. Animal caps were dissected in 1X Steinberg's solution (pH 7.4) and treated with 0.1 to 100 ng/ml Activin A.

In vitro *transcription*

Recombinant plasmids of pCS2-Notch ΔE (Onuma *et al.,* 2002), pSP6T-tALK4 (Chang *et al.,* 1997), p64T-XAR1-WT (Hemmati-Brivanlou *et al.,* 1992), and pSP64T-MyoD(b) (Hopwood and Gurdon, 1990) were used as templates for the capped mRNAs. The capped mRNAs were synthesized

in vitro using the mMASSAGE mMACHINE T7 and SP6 kit according to the manufacture's instructions (Ambion, Austin, TX). Digoxigenin-labeled antisense RNA probes were *in vitro*-transcribed with T7 and SP6 polymerase from template cDNA, for *Delta-1, Delta2*, *and Notch* (kindly provided from Dr. Kinoshita) using an RNA labeling kit according to the manufacturer's instructions (Roche Molecular Biochemicals).

In situ *hybridization*

Whole mount *in situ* hybridizations were performed according to the method of Harland (Harland, 1991) with an automatic *in situ* detection machine (InsituPro, ABIMED), except that the chromogenic reaction was done using BM purple as the substrate (Roche Molecular Biochemicals) and the RNase treatment was omitted.

Real-time RT-PCR

Total RNA was extracted from 6 to 10 animal caps by ISOGEN (Nippon Gene) according to the RNA extraction kit instructions. Random primed reverse-transcription was performed using 500 ng total RNA as a template. Real-time PCR was performed on an ABI PRISM 7700 (Applied Biosystems) using SYBR Green PCR Master Mix (QIAGEN) according to the QuantiTect SYBR Green Kit instructions. The following primers were used (F, forward; R, reverse):

Delta-1	F: 5'-AAGCCCAGGTACCCTTCTGT-3'
	R: 5'-GGCAGAGTCTGGTCGTCTTC-3'
Delta-2	F: 5'-TCTCACCTTGTCAGTGCCAG-3'
	R: 5'-AAAATGTTGCAGGATTTGCC-3'
Notch	F: 5'-CCAACCGTGACATCACAGAC-3'
	R: 5'-GGAGCAGATGGGAGGTGATA-3'
ESR-1	F: 5'-TGGCAAAACTGGAACAGGAT-3'
	R: 5'-TGGGATACAACAGGGAGCTT-3'
ODC	F: 5'-CAGCTAGCTGTGGTGTGG-3'
	R: 5'-CAACATGGAAACTCACACC-3'

Acknowledgments

We thank Dr. Y. Eto (Central Research Laboratories of Ajinomoto Co. Inc.) for providing us with human recombinant activin A, and C. Chang (The Rockefeller University, New York), A. Hemmati-Brivanlou (Harvard University, Cambridge), P. Bourillot (Wellcome/CRC Institute, Cambridge), J. Gurdon (Wellcome/CRC Institute, Cambridge) and M. Kinoshita (Kwansei Gakuin University, Hyogo) for their kind gift of plasmids. This work was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan, and the Solution Oriented Research for Science and Technology Project of the Japan Science and Technology Corporation.

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Received: March 2004
Reviewed by Referees: April 2004
Modified by Authors and Accepted for Publication: May 2004
Edited by: George M. Malacinski