Microinjection of activin into the right side of *Xenopus* neurula embryos induces complete inversion of the left-right axis of the heart and visceral organs

(*Xenopus*神経胚の右側面へのactivinの微量注射は、心臓及び内臓器官の左右軸の完全な逆転を誘起する)

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Vertebrate neurula embryos have distinct morphological polarity along the dorso-ventral axis and antero-posterior axis, and several genes are expressed unilaterally on one side of bilateral neurula embryos[1,2,3,4,5].The polarity along left-right axis is considered to have been determined to some extent before neurula stage. Experimental manipulations have shown that left-right (L-R) axis of neurula embryos can be randomized in up to 50% of treated embryos, but no more than 50% in previous experiments[2,5,6]. However, we discovered that L-R axis was inverted in nearly 100% of injected embryos using right-side microinjection of activin molecules into the lateral hypodermic space of *Xenopus* neurula embryos. Left-side injection had no such effect. We report the details of the L-R inversion by injection of activin, and discuss the implication of our results for L-R specification of vertebrate embryo.

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Activin, a member of TGF superfamily signaling peptides, is one of the candidates for endogenous L-R asymmetrical morphogenetic signals in
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vertebrate embryos of various classes[1,2,7,8]. In chick embryos, mRNAs of the activin βB subunit and activin type IIa receptor are expressed asymmetrically on the right side of the primitive streak[1,2]. In activin type IIb receptor null mice, alternation of the L-R patterning of many internal organs was reported[8].

Previously, we exposed *Xenopus* neurula embryos to saline containing 0.2-2.5μg/ml activin A, and found that reversal of the embryonic L-R axis occurred in 16% of the treated embryos (21 out of 133; unpublished data). We observed the L-R inversion of both heart and digestive organ or of heart-alone. But we could not obtain the embryos with inverted gut-alone with the exposure experiment. Considering L-R asymmetrical administration, then we injected unilaterally 5-25nl of bovine activin A solution (at concentrations not more than 10μg/ml) into the sub-epidermal space of *Xenopus* neurulae facing lateral plate mesoderm (Table 1; total number of embryos was 1618). In order to check the position where activin was injected, Nile blue was dissolved in activin solution at a concentration of 1% (w/v, Fig.1a).

The results were clear and drastic (see Table 1). With *right*-sided injection, all the early neurulae just after gastrulation movement (stage 13-14, according to the table by Nieuwkoop and Faber, 1967[9]), mid-neurulae during forming neural groove (stage 15-16) and late neurulae with several somites just before neural tube closure (stage 17-18) showed 100%-nearly 100% reversal of the visceral axis called *situs inversus* with 50-250pg of activin (n=362/378). With higher doses of activin (50-250pg), more than 80% of embryos in *situs inversus* showed absolute reversal of the whole visceral organs named *situs inversus totalis* (stage13-14, n=54/67; stage15-16, n=111/118; stage17-18, 156/177; Fig.1b). We observed that less than half of stage13-14 neurula embryos caused alternation of L-R axis by right-sided injection of lower dose of activin (0.001-0.5pg). The embryos after neural

tube closure (stage19-20) inverted their L-R axis in less than 40% of the injected samples (n=63/165). Through injection to early tail bud stage embryos (stage21-22), the incidence of L-R inversion was about 25% (n=25/108, Table 1). The incidence of L-R inversion of stage23-24 embryos was about the same as that of uninjected control embryos (1%, n=1/96), suggesting that activin could not alter the L-R axis of stage 23-24embryos. We injected of activin (0-0.01pg) into right-sided at early neurula stage and did not find L-R reversal embryos. The survival ratio of the injected 4-day embryos was over 90%. Here we should note that, though presumptive heart region was distant from the injection point, the heart situs was always inverted by activin at an optimal dose.

By injection of 10pg of activin into the *left* flank of neurula embryos, the occurrence of L-R inversion was less than 1% (n=1/140, Table 1) as observed in normal untreated siblings (2%; n=17/820). When the same dose of activin molecules (1-50pg) were microinjected into both sides of neurula embryos (stage13-18), the incidence of L-R inversion was 61% on average (n=69/113, Table 2). Left-injected activin seems to have interfered with the complete L-R reversal by right-injected activin, thus decreasing the incidence ($P<0.001$). Right-sided microinjection of a mixture of an effective dose of activin (1-10pg) and the twenty-fold dose of follistatin stopped the reversal of L-R orientation (11%; n=10/90 of stage 13-14 embryos). This result proves that injected activin surely acted through the activin receptor-mediated signal transduction pathway (Table 3).

We investigated whether other growth factors can induce the inversion of L-R axis of neurulae as well as activin. We injected into right-side or left-side of neurula embryos with follistatin, which inhibits the effects of activin, LIF (Leukemia inhibitory factor), HGF (Hepatocyte growth factor), midkine

and NGF (Nerve growth factor). These growth factors had little or no effects to the L-R axis (see Table 4).

Our experiments revealed that determination of L-R orientation can be completely re-specified still in late neurula embryos with asymmetrical administration of activin molecules. This suggests that left and right halves of neurulae are still equivalent in the potency of L-R handed differentiation. How does activin invert L-R orientation completely? If the right-injected activin simply provides the 'left' character for right-side cells, it will lead to a struggle for left-side identity between the original left tissue and *left-characterized* right tissue. This situation seems to cause the randomization of L-R axis or isomerism (bilateral abnormality of the asymmetrical organs) of the visceral organs. Our results suggest that some kind of inhibitory signal across the midline structures is transmitted from the activin-injected right tissues to left tissues to realize the 100% inversion of L-R orientation[10]. Without hypothesizing such a communication, it is difficult to explain the occurrence of 100% *situs inversus*.

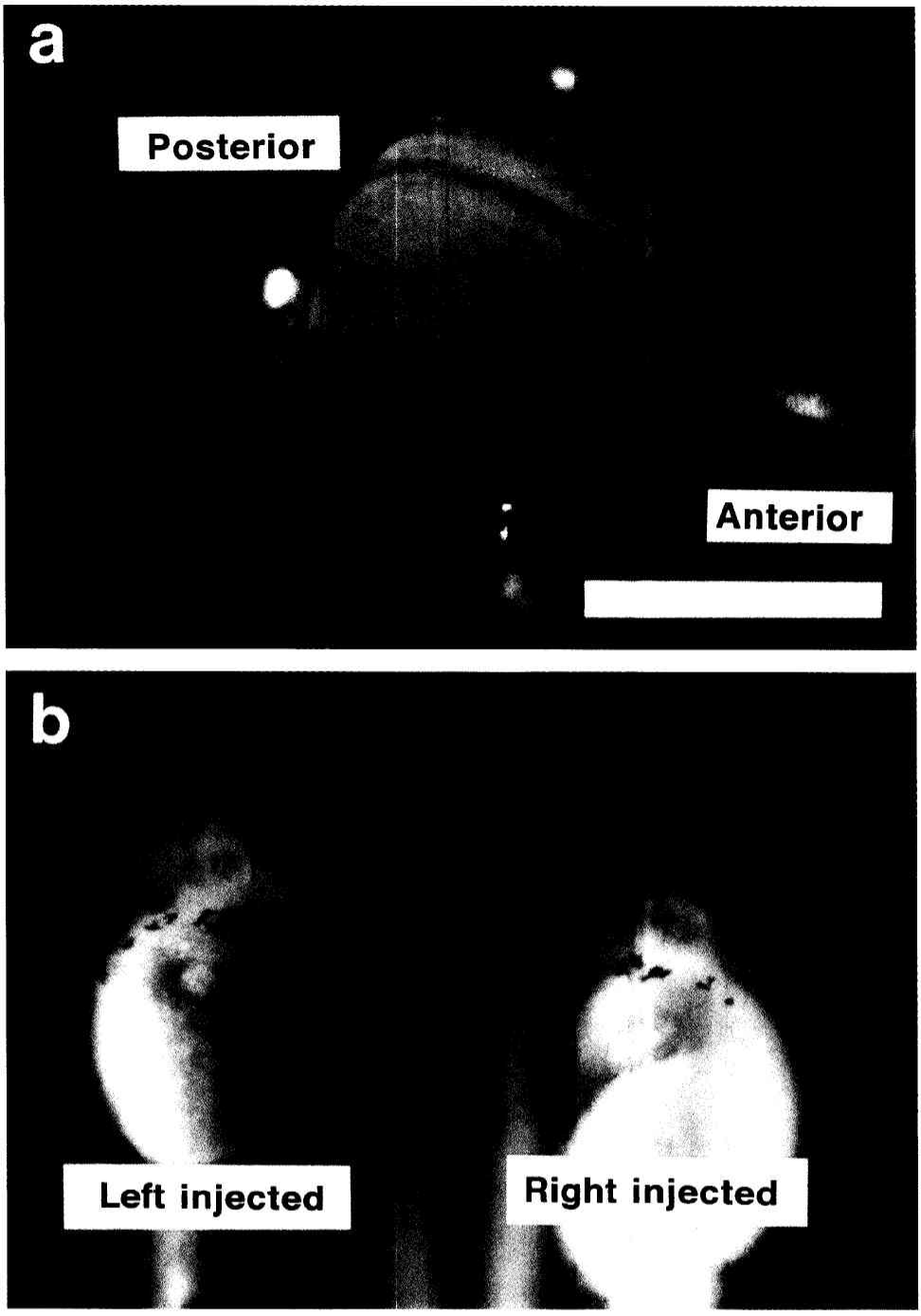

Fig.1 Right-sided microinjection of activin, causing inversion of L-R orientation in neurula embryos

a) The scene of right-sided hypodermic microinjection of activin.
Activin A together with 1% Nile Blue (vital dye) was microinjected into the lateral sub-epidermal space of *Xenopus* neurula embryos (stage16). Scale bar, 1mm.

b) Inversion of L-R axis by right-sided injection of neurula embryo. Both heart and gut situs were inverted by injection of 5nl of 2μg/ml activin solution at stage 15. Left-side injection of activin resulted in normal situs. 5nl of 2μg/ml activin solution was microinjected in the left flank of the embryo at stage15. Scale bar, 1mm.

Right

stage	neurula stage			tail bud stage		
	13-14	15-16	17-18	19-20	21-22	23-24
dose (pg)	Upper, %; Lower, inverted/survived					
250	**95** 18/19	**96** 22/23	**93** 71/76	**27** 6/22	**25** 6/24	**0** 0/16
50	**100** 49/49	**96** 96/100	**95** 106/111	**37** 17/46	**25** 5/20	**3** 1/32
10	**96** 100/104	**90** 149/166	**80** 78/98	**41** 40/97	**22** 14/64	**0** 0/48
1-2	**87** 60/69					
0.1-0.5	**52** 34/66					
0.01	**0** 0/55					
0.001	**0** 0/65					
0	**5** 2/42					

Left

stage	neurula stage		
	13-14	15-16	17-18
dose (pg)	Upper, %; Lower, inverted/survived		
250	- -	**5** 1/21	- -
50	- -	**0** 0/45	- -
10	**2** 1/45	**0** 0/71	**0** 0/24

Table 1 Inversion of the L-R axis by right-sided or left-sided injection of activin

Upper; Stage- and dose-dependency of the effect of *right*-injected activin.
At all neurula stages, more than 95% of the embryos showed inverted situs of visceral organs by injection of 50-250pg of activin. Injection at early tail bud stage (stage19-22) was less effective. Activin could not invert L-R axis of the stage 23-24 embryos. Dose (0-250pg)-dependent change of the incidence of L-R inversion was also revealed, assuring that activin really induced L-R reversal of the internal organs. Situs of the heart and the gut were scored at stages 41-42.
Lower; Left-sided injection of activin. Incidence of L-R inversion was about the same as that of untreated embryos.

Right+Left

stage	neurula stage		
	13-14	15-16	17-18
dose (pg)	Upper, %; Lower, inverted/survived		
50	- -	**68** 13/19	**60** 15/25
10	**56** 28/50	- -	- -
1	**68** 13/19	- -	- -

Table 2 Inversion of the L-R axis by both-sided injection of activin

Microinjection of the same amount of activin molecules into both sides of neurula embryos (1-50pg) was operated. The ratio of L-R inversion was less than 70%. Statistical test (2x2 contingency table test) reveals that the incidence of double injection was lower than that of right-sided injection of activin (50+50pg vs. 50pg activin) at 0.1% significance level.

Right, **activin-only vs. activin+follistatin(1:20)**

stage13-14	follistatin	
activin	free	x20
10 pg	**96** 100/104	**0** 0/27
1 pg	**89** 32/36	**10** 6/63

Upper, %
Lower, inverted/survived

Table 3 Coinjection of activin and follistatin

We investigated the effect of follistatin to the incidence of activin-induced situs inversus. The two were mixed in the ratio of 1:20 (dose of activin, 1-10pg; follistatin, 20-200pg). Follistatin blocked activin signaling pathway, to reduce greatly the incidence of L-R inversion.

follistatin

stage13-14	Right	Left
500 pg	**0** 0/13	**0** 0/21
50 pg	**0** 0/43	**0** 0/24
10 pg	**0** 0/21	**0** 0/12

LIF, **50pg**

stage	13-14	15-16
Right	**6** 2/35	**5** 1/20
Left	**0** 0/36	**4** 1/28

HGF, **50pg**

stage	13-14	15-16
Right	**0** 0/24	- -
Left	**9** 5/58	**16** 6/37

midkine, **25pg**

stage	13-14	15-16
Right	- -	**4** 1/24
Left	- -	**4** 1/23

NGF, **25pg**

stage	13-14	15-16	17-18
Right	**0** 0/30	- -	**3** 1/36
Left	**5** 1/22	- -	**0** 0/26

Upper, %

Lower, inverted/survived

Table 4 Inversion of the L-R axis by other growth factors

We injected follistatin-alone, LIF, HGF, midkine and NGF into right-side or left-side of neurula embryos. In consequence, we hardly observed L-R inverted embryos by injection of the growth factors except activin. These growth factors seemed to have little or no effects on L-R orientation.

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