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Midkine Counteracts the Activin Signal in Mesoderm Induction and Promotes Neural Formation[1]

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Midkine (MK) is a heparin-binding growth factor that has been implicated in neural survival and differentiation, fibrinolysis, and carcinogenesis. It is expressed in the nervous system during early *Xenopus* development. In the present study, we demonstrated that injection of vegetal blastomeres with *Xenopus* MK at the 8-cell stage results in incomplete invagination. In the case of dorsal vegetal injection, hypertrophic neural tissue is produced. Animal caps isolated from embryos that have been injected with *Xenopus* MK and cultured with activin do not elongate, and all mesoderm markers examined, including both head and trunk/tail ones, are greatly diminished. In contrast, head-specific neural markers, XANF-1 and Xotx2, are induced, while trunk/tail neural markers, XlHbox6 and F-spondin, are decreased. Moreover, MK showes the same effects in animal caps injected with *Xenopus* Smad2 mRNA.

Key words: activin, mesoderm induction, midkine, neural formation, pleiotrophin.

At the blastula stage of *Xenopus* development, equatorial cells develop into mesoderm in response to inductive signals produced by the underlying endoderm in vegetal blastomeres (*1, 2*). Several soluble factors have been revealed to exhibit this inductive activity. These factors include members of the TGF-β superfamily, *i.e.*, activin (*3-6*), bone morphogenic proteins (BMPs) (*7-9*), and Vg1 (*10-12*), and fibroblast growth factors (FGFs), specifically, b-FGF and e-FGF (*13-15*). Additional molecules, follistatin, noggin, chordin, and members of the Wnt family, can alter the mesoderm induction pattern, although these factors lack intrinsic mesoderm-inducing activity (*16, 17*).

Although its localization has yet to be determined, the activin protein is present in the blastulae of *Xenopus* and is a candidate for a natural mesoderm-inducing factor (*18*). In the animal cap assay, activin induces not only the mesoderm but also, secondarily, neural tissues, probably *via* inductive signals produced by the induced mesoderm. Extensive studies have demonstrated that FGF signaling is required in this activin-mediated mesoderm and neural induction (*19-22*). Many studies have been conducted to identify the cytoplasmic components of the activin signaling pathway, and Smads have been recently identified as the mediator of TGF-β superfamily signaling (*23, 24*). In *Xenopus* embryos, Smad proteins have distinct functions in response to signals for specific ligands of the TGF-β family. In activin signal transduction, Smad2 is the cytoplasmic molecule in this cascade (*25*).

Midkine (MK) cDNA was first cloned as the product of a retinoic acid-responsive gene using an embryonal carcinoma cell-differentiation system (*26*). MK is a heparin-binding growth factor with a molecular weight of 13 kDa (*26-31*). Together with pleiotrophin (PTN)/HB-GAM, it comprises a unique subfamily of heparin-binding growth factors (*26, 28, 32, 33*). It has been implicated in neuronal survival and differentiation, fibrinolysis in endothelial cells, chemotaxis in inflammation, and carcinogenesis (*30, 34-40*). PTN exhibits similar activities, although either the timing or localization of its expression appears to be different from that of MK (*41*). In mouse development, MK is expressed in the embryonic ectoderm at 7.5E, then its expression becomes increasingly restricted, especially to sites at which an epithelial-mesenchymal interaction takes place during mid-gestation (*27*). Zygotic expression of *Xenopus* MK (XMK) starts at the mid-gastrula stage in the neural anlage, and becomes increasingly marked in the central nervous system and head mesenchyme at early neurula stages (*42*).

With regard to neurotrophic activity, MK serves as a nerve cell adhesion molecule which promotes cell survival and neurite extension (*30, 43*), and, importantly, it is expressed in radial glial processes, thereby providing guidance for nerve cell migration, in the late stage of rat embryogenesis (*44*). Under these circumstances, the character of the heparin-binding of MK may play a central role in these activities, since exogenous heparin greatly decreases the trophic activity of MK (*43, 45*). On the other hand, it has been shown that MK also acts as a soluble trophic factor in the spinal cord, dorsal root ganglion and

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Abbreviations: HB-GAM, heparin-binding growth associated molecule; MK, midkine; PBS, phosphate-buffered saline; PTN, pleiotrophin.

mesencephalic neurons (*34, 46*). MK injected into the eye prevents photoreceptor cells from degenerating in response to constant light exposure (*38*). Furthermore, MK enhances fibrinolysis in endothelial cells, by acting as a soluble factor (*37*). These findings suggest that MK may utilize its own signaling through a specific receptor, which remains to be identified. Members of syndecan family (syndecan 1, N-syndecan, and ryudocan) and phosphotyrosine phosphatase ζ have been identified as MK-binding proteins on cell surface (*41, 47-49*). MK may utilize these molecules as co-receptors in combination with a specific receptor to exert its functions.

Herein, we investigated the role of MK in *Xenopus* development. Injection of dorso-vegetal blastomeres with XMK mRNA results in not only incomplete invagination but also the production of hypertrophic head neural tissue. MK inhibits the expression of both head and trunk/tail mesoderm markers in the animal cap assay. In contrast, neural induction shows segregation between the head and trunk/tail: head neural markers are induced, while trunk/tail neural markers are suppressed. Furthermore, MK shows the same effect in animal caps injected with Smad2, a downstream signal molecule of activin. Since XMK expression starts at the mid-gastrula stage (stage 11) in the neuroectoderm, these findings suggest that XMK plays an important role in neural formation in early *Xenopus* development.

MATERIALS AND METHODS

Embryos—Eggs were obtained by injecting *Xenopus laevis* with human chorionic gonadotropin (Gestron; Denka Seiyaku, Kawasaki). Both males and females were injected with 800 U of gonadotropin. Embryos were chemically dejellied using a 3% cysteine hydrochloride solution in Steinberg's solution [58 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.85 mM $MgSO_4$, 4.6 mM Tris HCl pH 7.4, 0.1 g/liter kanamycin sulfate], washed thoroughly, transferred to fresh Steinberg's solution, and incubated at 20°C. They were then transferred to 10% Steinberg's solution before the gastrulation stage (stage 9) (*50*).

RNA Injection—cDNA fragments including the entire open reading frame were subcloned into the pSP64T vector. The pSP64T vector, Xglobin (Xβm), and Xmad2 cDNAs were generously provided by Dr. D.A. Melton. The capped XMK, Xglobin, and Xmad2 mRNAs were synthesized *in vitro* as described previously (*51*). XMK or Xglobin mRNA was injected into two cells of dorsal-vegetal or ventral-vegetal blastomeres at the 8-cell stage in 5% Ficoll-Steinberg's solution, and at stage 9, the embryos were transferred to 10% Steinberg's solution and cultured for 4 d. For the animal cap assay, *in vitro* synthesized XMK or Xglobin, with or without Xmad2 mRNA, was injected into each blastomere at the 2-cell stage. The results were reproducible for three lots of XMK mRNA prepared independently.

Animal Cap Assay—Embryos injected with *in vitro* synthesized XMK or Xglobin, with or without Xmad2 mRNA, were dissected at stage 9, then cultured in Steinberg's solution containing 0.1% bovine serum albumin (BSA) with or without activin A. Human activin A was a generous gift from Dr. Eto (*52*). For RT-PCR analysis, the animal caps were cultured for 3 h or 2 d. For histological analysis, the animal caps were cultured for 3 d.

RT-PCR—Total RNA was extracted from *Xenopus* embryos by the acid guanidium thiocyanate-phenol-chloroform (AGPC) method with some modifications (*53*). RT-PCR reactions were carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) according to the standard method (*54*) in a 20-μl reaction mixture containing cDNA primed with oligo(dT) from total RNA as a template. The RT-PCR fragments were subcloned and their sequences were determined with an automatic DNA sequencing analyzer (ABI). Xbra (*55*), chordin (*17*), cerberus (*56*), s-actin (*57*), N-CAM (*58*), XANF-1 (*59*), Xotx2 (*60*), XAG1 (*61, 62*), XlHbox 6 (*63*), F-spondin (*64*), and EF1-α (*65*) were analyzed, as previously described (*56, 59, 65-68*). In addition, we performed RT-PCR using the following primers: Mix.1 (*69*), 5′-AATGTCTCAAGGCAGAGG-3′ and 5′-TGTCACTGACACCAGAA-3′.

Histological Analysis—Animal caps and embryos were fixed in Bouin's fluid (picric acid:formaldehyde:acetic acid, 15:5:1) for 3 h, dehydrated in an ethanol series, then embedded in paraffin. The paraffin blocks were sectioned at a thickness of 6 μm and stained with hematoxylin/eosin.

RESULTS

MK Blocks Invagination—*In vitro* synthesized capped XMK mRNA or β-globin mRNA was injected into dorsal or vegetal blastomeres at the 8-cell stage. In the late gastrula stage (stage 12), the invagination was almost complete in normal and β-globin-injected embryos (Fig. 1a). In contrast, embryos injected with XMK as ventral vegetal blastomeres showed suppression of invagination in the ventral area (Fig. 1b). Incomplete invagination in both the

TABLE I. **Effects of ectopic XMK expression.**

		Injected number	Normal	Abnormal gastrulation		
				Deformed head	Spina bifida	Short tail
Dorsal vegetal	XMK (1 ng)	52	1 (1.9)[a]	50 (96.2)	1 (1.9)	0 (0.0)
	XMK (500 pg)	26	1 (3.8)	24 (92.4)	1 (3.8)	0 (0.0)
	XMK (100 pg)	25	25 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Xglobin (1 ng)	31	31 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Ventral vegetal	XMK (1 ng)	50	0 (0.0)	0 (0.0)	13 (26.0)	37 (74.0)
	XMK (500 pg)	28	10 (35.7)	0 (0.0)	2 (7.2)	16 (57.1)
	XMK (100 pg)	21	7 (33.3)	0 (0.0)	5 (23.8)	9 (42.9)
	Xglobin (1 ng)	31	31 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)
Uninjected		80	80 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)

[a] The numbers in parentheses are the percentage of incidence.

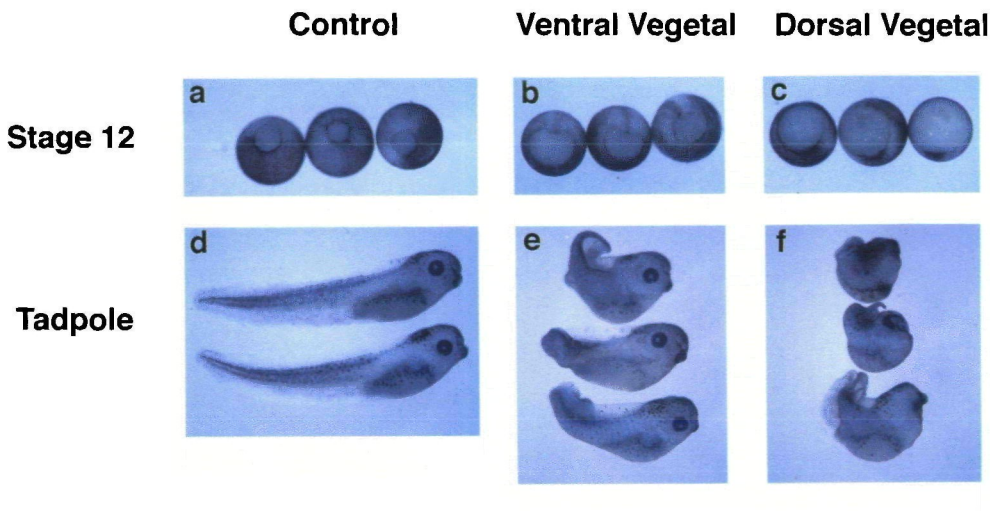

Fig. 1. **MK inhibits invagination.** Either ventral vegetal or dorsal vegetal blastomeres at the 8-cell stage were injected with XMK mRNA at 1 ng/embryo. Vegetal views of the embryos at stage 12 are shown: the upper and lower sides represent the dorsal and ventral sides, respectively. Embryos injected with β-globin mRNA developed normally, like those shown in a and d. a, control embryo at stage 12; b, ventral vegetal injected embryo at stage 12; c, dorsal vegetal injected embryo at stage 12; d, control tadpole; e, ventral vegetal injected embryo at tadpole stage; f, dorsal vegetal injected embryo at tadpole stage.

dorsal and ventral areas was seen in embryos injected with XMK as dorsal vegetal blastomeres, probably due to inhibition of the initiation of invagination (Fig. 1c). These late gastrula phenotypes were consistent with those in the tadpole stage. Ventral vegetal injection of XMK produced embryos with aberrant trunks and tails (a short tail and spina bifida) but normal heads, while dorsal vegetal injection affected the development of the whole body, including the head structure (Fig. 1, e and f, and Table I). Histological analyses showed that hypertrophic head neural tissues were produced in embryos with dorsal vegetal injection of XMK (Fig. 2, b and d). In summary, XMK blocked invagination, and the most likely target appeared to be mesoderm induction, which prompted us to perform the animal cap assay.

MK Blocks Mesoderm Induction by Activin and Modulates Neural Formation—XMK mRNA and β-globin mRNA were injected into the animal poles at the two-cell stage, and the animal caps were cultured in the presence or absence of activin. Without activin treatment, there were no morphological differences between untreated, XMK-injected, and β-globin-injected animal caps (Fig. 3, a, b, and d). Activin (10 ng/ml, 1 h) promoted elongation of the animal caps (Fig. 3c), while XMK injected at 0.1 and 1.0 ng/embryo (Fig. 3, e and g), but not β-globin (Fig. 3, f and h), inhibited this elongation. Histologically, activin induced muscle and neural tissues (Fig. 4a), while XMK inhibited the generation of muscle tissue induced by activin (Figs. 4c and 5). Interestingly, even if the mesoderm induction by activin was suppressed in XMK-injected animal caps, XMK induced neural tissue in cooperation with activin (Fig. 4c). Thus, neural tissue was prominent in animal caps which had been injected with XMK and treated with activin (Fig. 4c). In addition, XMK alone induced the cement gland (Figs. 4d and 5).

In an attempt to clarify the molecular mechanism by which XMK modulates the inductive activity of activin, we performed RT-PCR for a variety of markers. Comparison of XMK/activin treatment with β-globin/activin treatment revealed that mesodermal markers (cerberus, chordin, mix1, s-actin, and Xbra) were diminished or moderately suppressed by XMK (Fig. 6). In contrast, there was segregation between the head and trunk in neural induction. Trunk/tail neural markers (XlHbox6 and F-spondin) were again suppressed by XMK, while a head neural marker (XANF-1) was induced (Fig. 6), probably reflecting the phenomenon seen in the embryos injected with XMK as dorsoventral blastomeres: the formation of hypertrophic head neural tissue (Fig. 2, b and d). N-CAM (a general neural marker) was unchanged (Fig. 6b). Expression of XAG1, a marker of the cement gland, was induced by XMK alone (Fig. 6b), this being consistent with the histology of

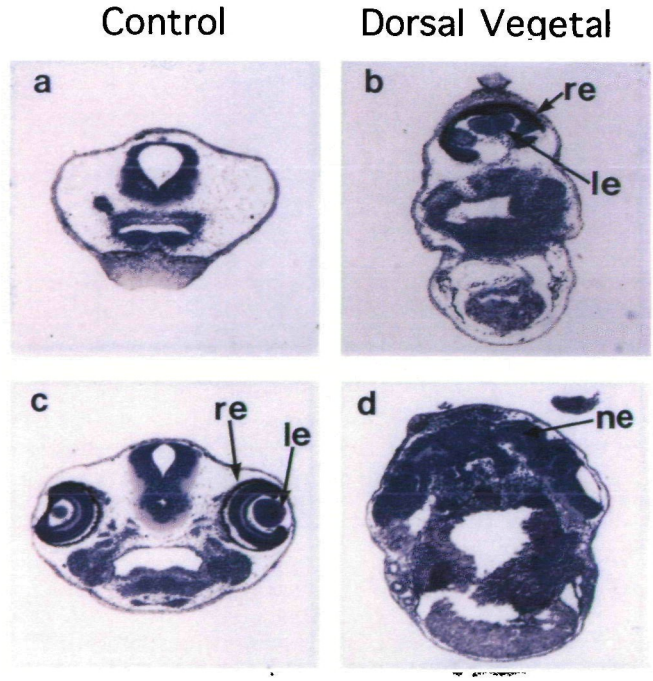

Fig. 2. **Histology of embryos injected with XMK as dorsal vegetal blastomeres.** A tadpole-stage embryo equivalent to that shown in Fig. 1f is compared with a normal embryo. The XMK-injected embryo has a single eye, and exhibits reciprocal orientation of the retina and lens (b). Note that the XMK-injected embryo also exhibits hypertrophic head neural tissue (d). a and b, and c and d show sections at the same level, respectively. le, lens; ne, neural tissue; re, retina.

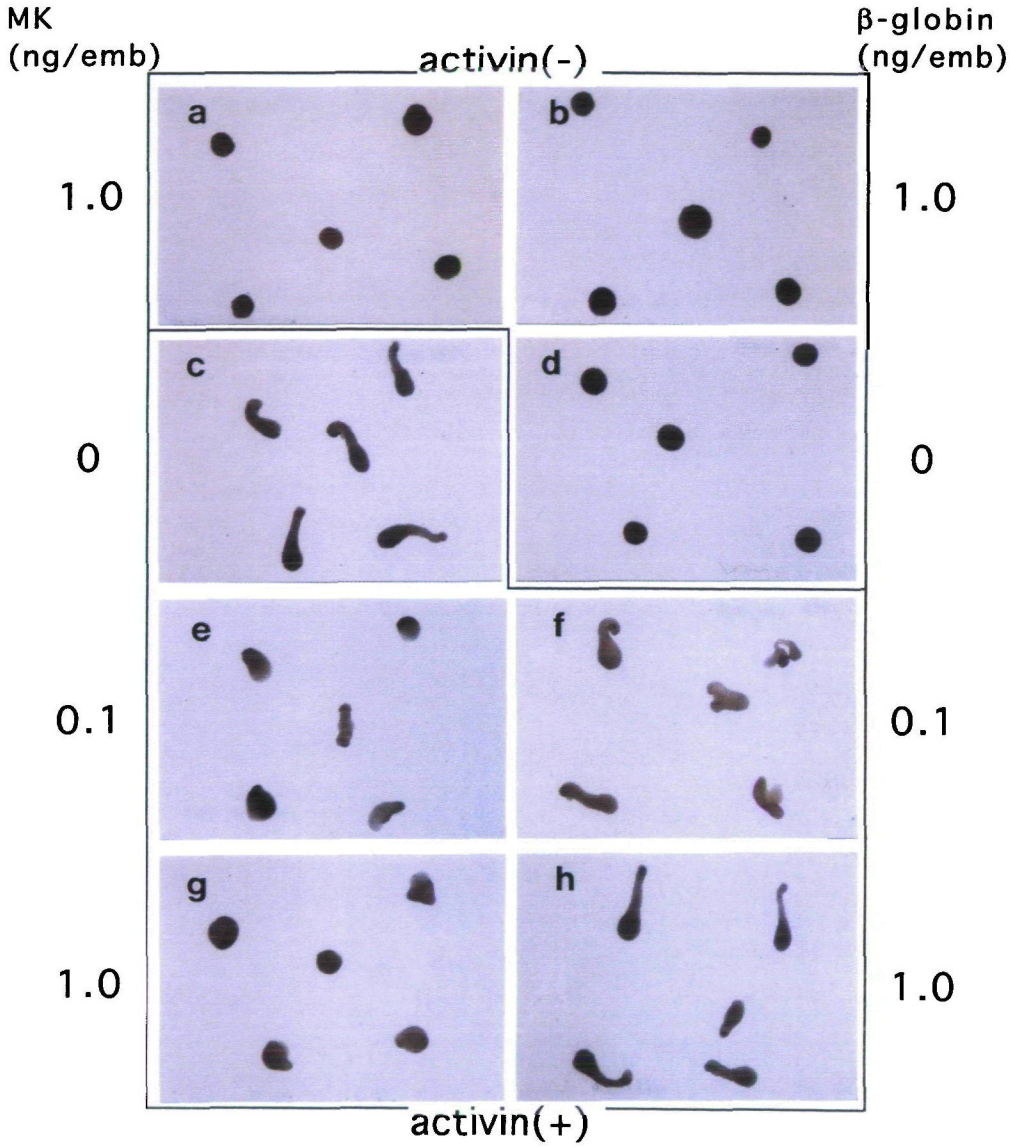

Fig. 3. **MK inhibits the elongation of animal caps treated with activin.** Animal caps injected with XMK (a) are indistinguishable from ones injected with β-globin (b), or untreated caps (d). Animal caps treated with activin (10 ng/ml, 1 h) elongate due to mesoderm induction (c). XMK preinjection inhibits this elongation (e, g), while β-globin does not (f, h).

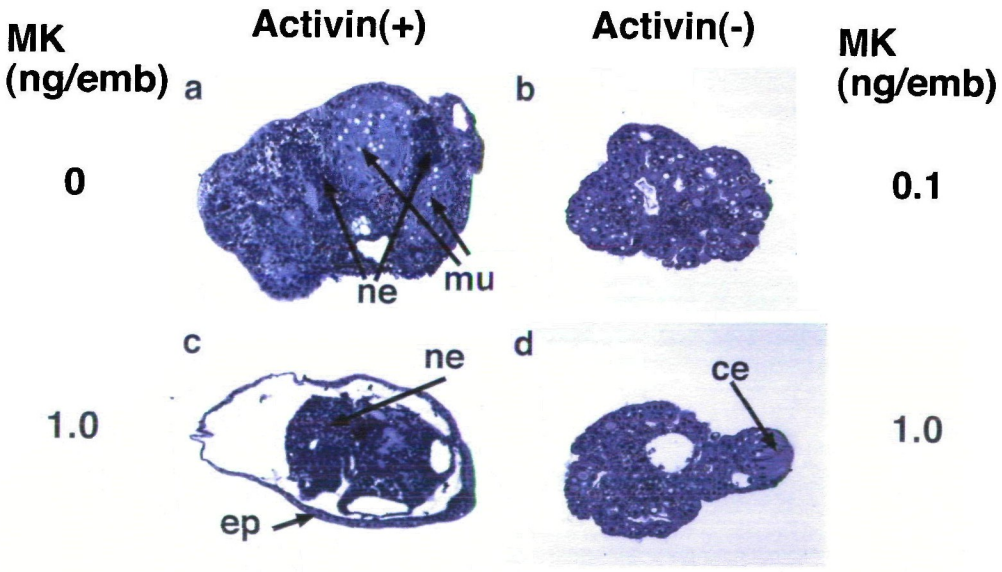

Fig. 4. **Histology of animal caps.** Activin induces muscle and neural tissues in animal caps (a). XMK preinjection decreases muscle formation, inducing neural tissues instead (c). Without activin treatment, animal caps show an atypical epidermis, which is also observed in animal caps injected with the lowest dose of XMK (0.1 ng/embryo) (b). However, injection of a medium or high dose of XMK induces the mature cement gland (d). ce, cement gland; ep, epidermis; mu, muscle; ne, neural tissue.

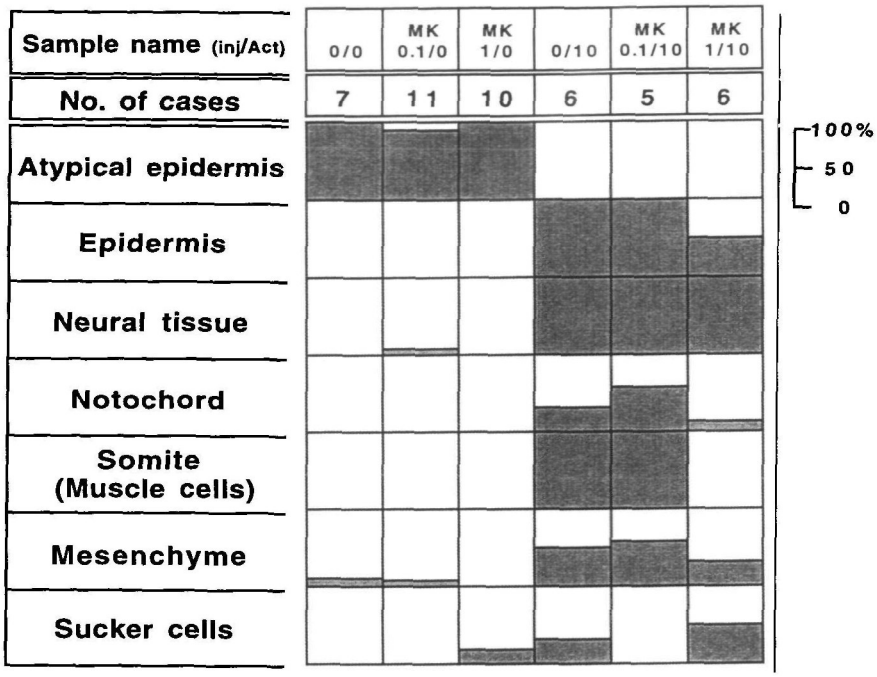

Fig. 5. **Summary of tissues appearing in animal caps.** In the uppermost row, the injected amounts of XMK are expressed as ng/embryo and activin treatment is expressed as ng/ml. Each column, shading, shows the incidence (percent) of positive tissue. Note that, with activin treatment, XMK inhibits the induction of the epidermis, notochord and somites, whereas the incidence of neural tissue induction does not change. XMK injection alone induces sucker cells (the cement gland).

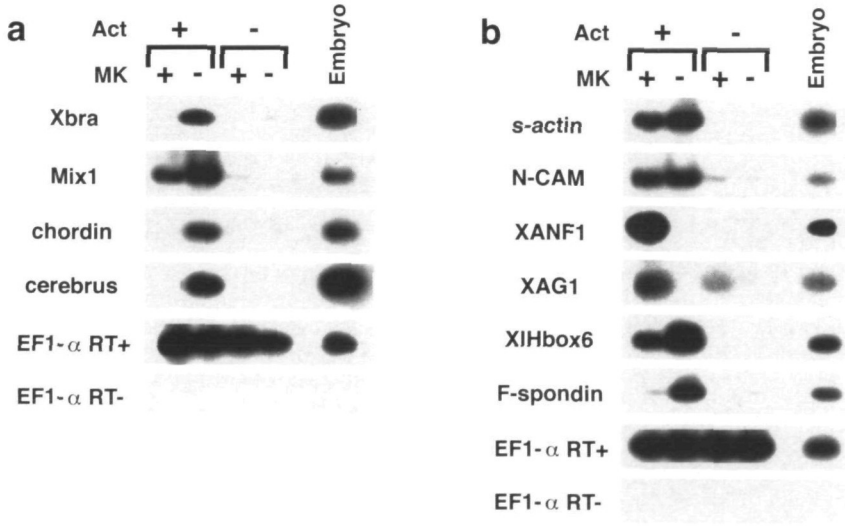

Fig. 6. **Changes in the expression of mesoderm and neural markers.** The expression of the panel of marker genes was analyzed by RT-PCR. Early and late gene expression was detected in animal caps cultured for 3 h (a) and 2 d (b), respectively. Animal caps were taken from embryos injected with MK (MK+) or β-globin (MK−), then cultured in medium containing 10 ng/ml of activin (Act+) or not (Act−) for 1 h. XMK suppressed all mesodermal markers (Xbra, Mix1, chordin, cerebrus, and S-actin) and trunk/tail neural markers (XlHbox6 and F-spondin) in animal caps treated with activin, whereas it induced a head neural marker (XANF-1). In addition, XMK injection alone induced XAG1 (a marker of the cement gland). EF1-α is an internal positive control and RT− is a negative control.

the animal cap assay (Figs. 4d and 5). Next, we co-injected MK and Smad2, a downstream signal transduction component for activin signaling. MK/Smad2 co-injected animal caps showed the inhibition of elongation (data not shown). RT-PCR analysis was then performed in the same way as for activin-treated animal caps. Mesodermal marker (s-actin) was suppressed by XMK, while head neural marker (Xotx2) expression was enhanced (Fig. 7). Interestingly, a general neural marker, N-CAM, which was not expressed in β-globin/Smad2 co-injected explants, was induced in MK/Smad2 co-injected animal caps (Fig. 7).

DISCUSSION

In the present study, we investigated the role of XMK in *Xenopus* embryos. XMK modulated the expression of both neural and mesodermal markers in the presence of activin signal in the animal cap assay. The reliability of these data was confirmed as follows. The same dose of β-globin did not affect expression of the markers, and EF1-α (an internal positive control) expression was not affected by XMK. The results were reproducible for three lots of XMK mRNA prepared independently. Furthermore, the modes of expression of the markers were essentially the same in both the cases of activin treatment and Smad2 (the intracellular signal transducer for activin) mRNA injection. The results of animal cap assays were also consistent with the phenomenon observed in whole embryos injected with XMK as dorsal or ventral vegetal blastmeres at the 8-cell stage, *i.e.*, incomplete invagination was seen.

XMK greatly diminished mesodermal markers induced by the activin signal in the animal cap assay. This is consistent with the phenotype of whole embryos injected with XMK mRNA at the 8-cell stage, *i.e.*, incomplete

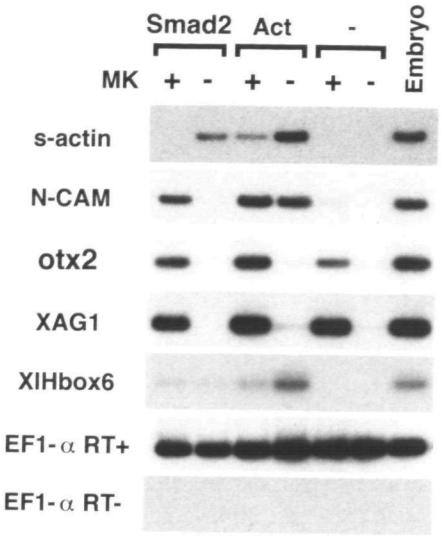

Fig. 7. **Changes in the expression of markers in co-injection of MK and Smad2.** The animal caps injected with MK (MK+) or β-globin (MK−) mRNA were cultured in medium containing 10 ng/ml of activin (Act+) or not (−) for 1 h. Smad2 (Smad2) was also co-injected with MK or β-globin. After culture for 2 d, the expression of the panel of marker genes was examined by RT-PCR. XMK suppressed mesodermal marker (s-actin) in both animal caps injected with Smad2 and treated with activin, whereas it induced head neural marker (Xotx2). In addition, XMK induced N-CAM (a general neural marker) in animal caps injected with Smad2. XMK injection alone induced XAG1 (a marker of the cement gland). EF1-α is an internal positive control and RT− is a negative control.

invagination was observed. But the physiological significance of the inhibition of mesoderm induction by XMK is not clear, since zygotic expression of XMK starts at the mid-gastrula stage (*42*), and mesoderm induction in *Xenopus* is thought to take place during the blastula stage (*2, 70, 71*). However, we should point out that two possibilities remain: (1) A low level of maternal XMK could be present in the blastula stage, and (2) *Xenopus* PTN could be present in the blastula stage. MK and PTN comprise a family of heparin-binding growth factors, and they have comparable biological functions (*30, 32, 33, 35*). Further studies are needed to clarify this point. In addition to the present findings, there are two other lines of evidence indicating reciprocal actions of MK and members of the TGF-β superfamily: fibrinolysis induction by MK and its suppression by TGF-β (*37*); and induction of the translucent area between the epithelium and mesenchyme during tooth development by BMP-2, and its suppression by MK (*72*). These data, together with the present results, raise the possibility that MK may cross-talk with members of the TGF-β superfamily.

On the other hand, XMK induced head neural markers, XANF-1 and Xotx2, in the presence of activin. When animal caps are cultured in a medium containing activin, neural tissue is secondarily induced, owing to primarily induced mesodermal tissue, resulting in expression of N-CAM, making it difficult to determine the effect of XMK injection on the expression of this general neural marker. In contrast, Smad2 mRNA injection does not induce N-CAM, probably because all cells injected have a mesodermal fate. In such a condition, XMK induced N-CAM in addition to head neural markers. Thus, the results indicate that XMK induces head neural markers in the presence of activin-type signaling. These findings are also relevant as to the formation of the hypertrophic head neural tissue induced by XMK injected as dorsal vegetal blastomeres at the 8-cell stage. Since XMK transcripts are localized in the neuroectoderm from the mid-gastrula stage (*42*), the present data suggest that XMK plays an important role in neural formation.

Concerning the physiological significance of MK in neural formation, an important question to be answered is what signal makes the neuroectoderm responsive to MK, since, unlike noggin, chordin and follistatin, XMK alone was not able to fully induce the neural markers in the animal cap assay. The fact that MK interacts with activin may be physiologically important, because activin or an activin-type signal has been reported to exert its biological activity during gastulation as well as later phases in chick development (*73*). In *Xenopus* development, the biological significance of activin and its receptor during the neurula and later stages has also been suggested by their expression modes. Activin βA and βB mRNA expression starts at stages 8-12 and shows an even distribution (*74*). During the neurula stage, the expression of activin and one activin receptor (XAR1) is mostly restricted to the dorsal side of the embryo. XAR1 mRNA is localized to the developing neural plate (*75*), while activin mRNA is mostly found in the underlying somitogenic mesoderm and possibly the notochord (*74*). XMK mRNA is localized to the neural plate (*42*). Later, during the tailbud stage, the expression of all three mRNAs (XMK, activin, and XAR1) overlaps in the brain, eye anlagen and otic vesicles (*42, 74, 75*). Thus, MK may interact with the activin signal *in vivo* during *Xenopus* development. In addition to activin, other signals remain to be identified, which could interact *in vivo* with XMK in the neuroectoderm, leading to the promotion of neural formation.

XMK alone induced the mature cement gland as well as XAG1, a marker of the cement gland, and Xotx2 expression, but not N-CAM or XANF-1. Xotx2, which we used here as a head neural marker, has also been reported to trigger the expression of cement gland differentiation genes, *e.g.*, XAG1 (*76*). Taken together, the present findings suggest that MK has a potential to determine the third ectodermal fate, the cement gland. Since the precise *in vivo* mechanism to distinguish between the cement gland and the most anterior neuroectoderm is still unclear, MK may provide a good tool to clarify this point.

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