

# Hst-1 (FGF-4) Antisense Oligonucleotides Block Murine Limb Development

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**Abstract.** The initiation of limb development depends on the site specific proliferation of the mesenchyme by the signals from the apical ectodermal ridge (AER) in embryonic mouse. We have previously reported that the local expression of *Hst-1/Fgf-4* transcripts in AER of the mouse limb bud is developmentally regulated, expressed at 11 and 12 days post coitus (p.c.) embryo. In an effort to further understand the role of Hst-1/FGF-4 in mouse limb development, an antisense oligodeoxynucleotides (ODNs) study was performed. We first established a novel organ culture system to study

mouse limb development in vitro. This system allows mouse limb bud at 9.5–10-d p.c. embryo, when placed on a sheet of extracellular matrix in a defined medium, to differentiate into a limb at 12.5-d p.c. embryo within 4.5 d. Using this organ culture system, we have shown that exposure of 9.5–10-d p.c. embryonic limb bud explants to antisense ODNs of Hst-1/FGF-4 blocks limb development. In contrast, sense and scrambled ODNs have no inhibitory effect on limb outgrowth, suggesting that Hst-1/FGF-4 may work as a potent inducing factor for mouse limb development.

**I**N mammals, there are presently nine genes that have been classified as family members of the fibroblast growth factors (FGFs)<sup>1</sup> (Delli Bovi and Basillico, 1987; Delli Bovi et al., 1987; Burgess and Maciag, 1989; Goldfab, 1990; Miyamoto et al., 1993). FGFs are mitogenic to a large number of ectodermally and mesodermally derived cells and can act as inducers of cell differentiation (Slack et al., 1987; Folkman et al., 1989; DiMario et al., 1989). Hst-1/FGF-4 was first identified as a transforming gene from human stomach cancer (Sakamoto et al., 1986; Yoshida et al., 1987) and its transcript was identified in male germ cell tumors (Yoshida et al., 1988a) and embryonal carcinoma (EC) cells (Yoshida et al., 1988b). However, the gene is normally silent in the normal adult tissues, giving rise to the presumption that Hst-1/FGF-4 expression is important during embryonic stage. This was supported by the fact that *Fgf-4* gene is expressed during early embryonic development (Niswander and Martin, 1992). Moreover, this was further bolstered by targeted disruption of *Fgf-4* gene, which results in embryonic lethality at the gastrulation stage (Feldman et al., 1995).

In addition to its early embryonic function, Hst-1/FGF-4 may play crucial role in later stage of development. There

are many reports that Hst-1/FGF-4 may act as an inducer of limb mesenchyme proliferation. For understanding the molecular mechanisms of limb development, the developing chick limb serves as an excellent experimental system. FGF-2 and Hst-1/FGF-4 were used in in vivo studies of chick limb development. Ectopic production of human FGF-2 in the chick limb bud by using retroviral vector caused formation of patterned extra-skeletal elements (Riley et al., 1993). If the apical ectodermal ridge (AER) is removed surgically, regeneration does not follow. When ectopic human HST-1/FGF-4 was supplied to the chick limb bud after AER removal, limb development occurred normally (Niswander et al., 1993). The same effect was observed when ectopic FGF-2 was supplied to the chick apical bud mesoderm after ridge removal (Fallon et al., 1994). In the mouse system, we and others (Suzuki et al., 1992; Niswander and Martin, 1992) have reported that mouse Hst-1/FGF-4 is detected in 11- and 12-d post coitus (p.c.) embryo, where it was localized to the AER of mouse limb bud. The limb outgrowth seems controlled by two signaling molecules, HST-1/FGF-4 as a stimulator and bone morphogenetic factor 2 (BMP2) as an inhibitor (Niswander and Martin, 1993). Other members of the FGF family are also expressed in the mouse AER: FGF-8 (Ohuchi et al., 1994; Crossley and Martin, 1995) and presumably FGF-2, although FGF-2 has only been shown to be in the chick limb (Savage et al., 1993; Dono and Zeller, 1994), and they can provide the signals necessary for limb development. Many other molecules and genes such as retinoides (Bryant and Gardiner, 1992; Rutledge et al., 1994) and homeoboxes (Izpisua-Belmonte et al., 1991) are incorporated into the signaling pathways in the developing limb bud (reviewed by Tabin, 1991, 1995; Tickle, 1991). An-

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1. *Abbreviations used in this paper:* AER, apical ectodermal ridge; BMP2, bone morphogenetic factor 2; EC, embryonal carcinoma; FGF, fibroblast growth factor; ODN, oligodeoxynucleotides; P.C., post coitus; P-D, proximal-distal.

other key signal governing limb patterning is a *Sonic hedgehog* which has recently been reported to initiate expression of secondary signaling molecules, including BMP2 and HST-1/FGF-4 (Laufer et al., 1994; Niswander et al., 1994). Thereby the interest lies in elucidating which of these genes normally functions in vivo. However, the lack of a suitable in vitro culture system for mouse limb development makes understanding the molecular mechanisms of mouse limb outgrowth incomplete.

To try to directly assess the role of Hst-1/FGF-4 in limb development in vitro, we initially established a novel culture system in a serum-free medium formulation for causing mouse limb outgrowth. This culturing system not only allows limb outgrowth but also provides us with a suitable model system to explore the key factors for limb development by controlling a variety of culture conditions with or without additives such as several growth factors, inhibitors, synthetic peptides, and antisense oligodeoxynucleotides (ODNs). To investigate further the role of Hst-1/FGF-4 in limb bud outgrowth, we used an ODN strategy in cultures of 9.5–10-d p.c. embryonic mouse limb bud explants in a serum-free, chemically defined medium. We report here that inhibition of Hst-1/FGF-4 expression results in marked impairment of limb outgrowth. Our results suggest direct evidence that Hst-1/FGF-4 worked naturally as an inducer of mouse limb development.

## Materials and Methods

### Microdissection and Limb Organ Culture

Pregnant dams (ICR, 7-wk-old) were obtained from Charles River Breeding Laboratories (Yokohama, Japan) and were killed by diethylether excess on 9.5–10-d p.c. (the day on which the vaginal plug is found = 0.5-d p.c.). Embryos were aseptically dissected from uterine decidua and developmentally staged by morphology of hind limb bud form, and 9.5-d p.c. embryos (forelimb) and 10-d p.c. embryos (hindlimb) were used for the experiments. The bodies of the embryos were microsurgically cut off at the middle of the abdomen with the head and tail trimmed away. The trunk fragments were placed on a sheet of extracellular matrix (a mixture of 25  $\mu$ g/ml laminin, 50  $\mu$ g/ml fibronectin, 2  $\mu$ g/ml vitronectin, 1  $\mu$ g/ml type I and type IV collagen, 10  $\mu$ g/ml chondroitin sulfate proteoglycan, 10  $\mu$ g/ml dermatan sulfate proteoglycan, 50 ng/ml heparan sulfate proteoglycan) in a limb differentiation medium (DME;  $10^{-4}$  M 2-mercaptoethanol, 200  $\mu$ M monothioglycerol, 0.8% methylcellulose,  $10^{-7}$  M selenium, 5  $\mu$ g/ml linoleic acid, 4  $\mu$ g/ml hydrocortisone, 5  $\mu$ g/ml transferrin) and then incubated at 37°C, 5% CO<sub>2</sub>. All chemicals except proteoglycans (Becton Dickinson & Co., Mountain View, CA) were purchased from Sigma Chemical Co., St. Louis, MO. As the limb bud grows it develops asymmetries along the anterior–posterior, dorsal–ventral, and proximal–distal (P–D) axes. At the end of culture, for the indication of limb outgrowth quantitatively, P–D length was measured under a stereoscopic microscope. To determine the skeletal development of limb culture, they were fixed with 10% formalin, stained in 0.1% alcian blue in 70% alcohol at 37°C for 72 h, dehydrated in ethanol, and cleaned in methyl salicylate (Lyons et al., 1990).

### Cell Culture

NIH3T3 cells transformed by mouse *Hst-1/Fgf-4* gene (Sakamoto et al., 1986) were grown at 37°C in 5% CO<sub>2</sub> in DME containing 5% heat inactivated calf serum (GIBCO-BRL, Gaithersburg, MD). Calf serum was omitted from cell culture during the treatment of oligodeoxynucleotides.

### Synthetic Oligodeoxynucleotides

Phosphorothioate ODNs were synthesized with 18 mers against ATG translation start site (5'-d-CGGCCCGCGTTTCGCCAT-3'), donor (5'-d-AGAGTCCAGAAGACCTG-3'), and acceptor (5'-d-CCCCTGGCACT-CACCAC-3') sites for exon 2 of mouse *Hst-1/Fgf-4*, respectively. As a control, sense and scrambled ODNs were used. The most striking antisense

effects were obtained by antisense ODNs for ATG site; a sense sequence of 3'-d-GCCGGGCGCAAAGCGGTA-5'-d, and a scrambled sequence of 5'-d-AGCTGCTGCTGCCCC-3' were used as a negative control. All ODNs were repeatedly ethanol precipitated and were dissolved in double distilled water and quantitated by optical density at OD<sub>260</sub>.

### Targeted Exposure of ODNs

*Hst-1/Fgf-4* transformed NIH3T3 cells were exposed to antisense or control ODNs at 500 nM with Lipofectin (GIBCO-BRL) according to the recommended method; ODNs were mixed with Lipofectin reagent: 1:1 (wt/wt) liposome formulation of the DOTMA and DOPE, at a ratio of 50  $\mu$ l of Lipofectin/50  $\mu$ l of 1.43  $\mu$ g/ml ODNs (100  $\mu$ l/10<sup>5</sup> cells), and then incubated for 15 min at room temperature. The mixture was added to the cells and then cultured for 3 d with serum-free DME. As for limb organ culture, the same ODNs-Lipofectin mixture (5  $\mu$ l/site) was put directly on the surface of limb bud (AER region) under a stereoscopic microscope (Nikon SMZ-U) and incubated for 20 min in the incubator without culture medium. After several washings with phosphate buffered saline to remove the excess ODNs-Lipofectin mixture, the limb differentiation medium was added to the explants, and then cultured as described. As for a rescue experiment, antisense ODNs bearing limbs were cultured in the limb differentiation medium supplemented with recombinant FGF-4 proteins (R & D Systems, Minneapolis, MN) at a final concentration of 800 ng/ml.

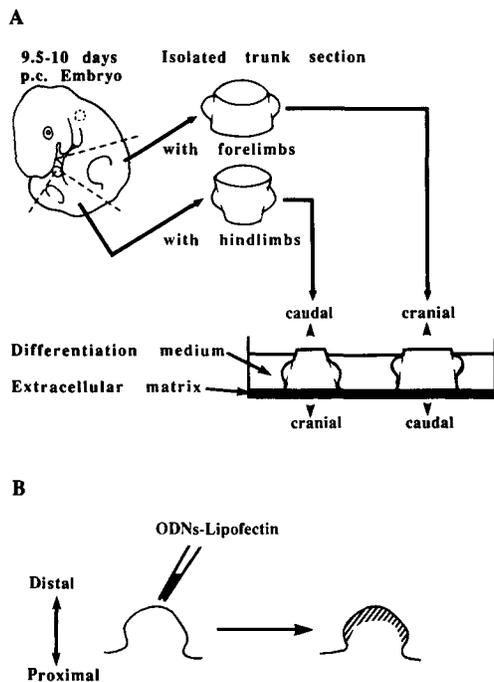
### Northern Hybridization Studies

After the exposure of ODNs, 20 pairs of limb buds from the explants were dissociated and pooled, then total RNA was extracted. About 25  $\mu$ g of each RNA sample was subjected to RNA blot analysis using the full size of mouse *Hst-1/Fgf-4* cDNA, basic FGF/*Fgf-2* cDNA, and AIGF/*Fgf-8* cDNA (Tanaka et al., 1992) as a probe. As an internal reference, the same filter was later rehybridized to a mouse  $\beta$ -actin probe.

## Results

### Establishment of a Novel Culture System for Limb Development

As a first step toward studying the possible involvement of Hst-1/FGF-4 in mouse limb development, we initially established a novel in vitro culture system in a serum-free medium formulation for causing mouse limb outgrowth as well as limb skeletal development (Fig. 1). Table I lists the ingredients and their concentrations for the three distinct media formulations used in this investigation. All media formulations used DME supplemented with 20 mM Hepes, pH 7.4, 100 U/ml penicillin G sodium, 100  $\mu$ g/ml streptomycin sulfate, and 5  $\mu$ g/ml amphotericin B as a basal medium. The original serum-free medium formulation (M1) contained six additives and was sufficient to maintain and allow limb outgrowth (data not shown). However, the trunk fragments were unstable in a liquid media and often spoiled at the tip of limbs. To overcome this problem, alternative formulations (M2: 0.4% gelatin; M3: 0.8% methylcellulose) were investigated. Although we did not show individual results, M3 formulation gave a stable result and greatly enhanced longevity of limb culture. Therefore we put the trunk fragment with limbs on a sheet of extracellular matrix and then cultured with a limb differentiation medium of M3 formulation. Under the conditions of this system, the trunc section with forelimbs (9.5-d p.c.) and hindlimbs (10-d p.c.) (Fig. 2, A and B) was cultured for 4.5 d and subsequently stained with alcian blue to reveal the skeletal pattern. The morphological pattern of cultured forelimb and hindlimb (Fig. 2, C and D) resembled that of limbs from a 12.5-d p.c. embryo that developed in vivo. Alcian blue staining showed that skeletal development also occurred normally in culture (Fig. 2, E and F). Thus, this



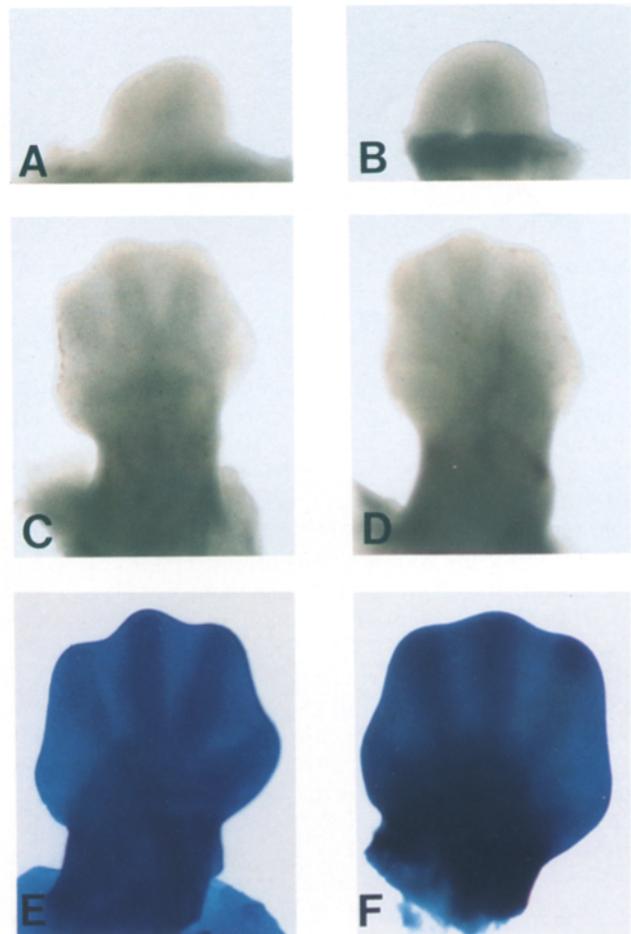
**Figure 1.** Schematic representation of a novel culture system for limb development. (A) The body of the 9.5–10-d p.c. mouse embryo was cut off (broken lines indicate position of cuts: the head and tail trimmed away) and was placed on a sheet of extracellular matrix (a mixture of laminin, 25  $\mu\text{g/ml}$ ; fibronectin, 50  $\mu\text{g/ml}$ ; vitronectin, 2  $\mu\text{g/ml}$ ; type I and type IV collagen, 1  $\mu\text{g/ml}$ ; chondroitin sulfate proteoglycan, 10  $\mu\text{g/ml}$ ; dermatan sulfate proteoglycan, 10  $\mu\text{g/ml}$ ; heparan sulfate proteoglycan, 50  $\text{ng/ml}$ ) in the limb differentiation medium (DME; 2-mercaptoethanol,  $10^{-4}$  M; monothioglycerol, 200  $\mu\text{M}$ ; methylcellulose, 0.8%; selenium,  $10^{-7}$  M; linoleic acid, 5  $\mu\text{g/ml}$ ; hydrocortisone, 4  $\mu\text{g/ml}$ ; transferin, 5  $\mu\text{g/ml}$ ), and then incubated at 37°C, 5%  $\text{CO}_2$ . (B) Targeted administration of ODNs with liposome: The ODNs-Lipofectin (GIBCO-BRL) mixture (5  $\mu\text{l/site}$ ) was put directly on the surface of limb bud and incubated for 20 min in the incubator without culture medium. The limb differentiation medium was added to the explants and then cultured as described.

culturing system allows limb outgrowth normally as in vivo.

### Effects of Antisense ODNs on Transformed Phenotype of NIH3T3 Cells

To determine the inhibitory effect of antisense ODNs against *Hst-1* expression, we examined the efficiency of the ODNs as to whether treating cultured NIH3T3 cells transformed via *Hst-1/Fgf-4* gene with antisense ODNs would make them revert to a normal morphology. Synthetic three types of phosphorothioate antisense ODNs against ATG translation start site, donor and acceptor site for exon 2 of mouse *Hst-1/Fgf-4* were used at 250 nM with liposome (Lipofectin) in cultures of cells for 3 d with serum-free DME, followed by evaluation of the cell morphology. The most striking effects were obtained by antisense ODNs against ATG site (60–70% in repeated experiments); no significant effect was observed on the other two antisense ODNs (1.5–2.0% reversion relative to untreated cells). The greatest response ( $\geq 90\%$  reversion) was attained when antisense ODNs against ATG site were administered with Lipofectin at a dose of 500 nM (Fig. 3).

## forelimb                      hindlimb



**Figure 2.** Limb development in vitro. Right forelimb bud from the mouse embryo at 9.5-d p.c. (A) and hindlimb bud from the mouse embryo at 10-d p.c. (B). Cultured right forelimb (C) and hindlimb (D) in the limb differentiation medium for 4.5 d as described above. They were fixed in 10% formalin, stained in 0.1% Alcian blue in 70% acid alcohol at 30°C for 72 h, dehydrated in ethanol, and cleaned in methyl salicylate: right forelimb (E) and hindlimb (F).

Contrarily, the same sense and scrambled ODNs against ATG site used as control revealed no effect. None of the synthetic ODNs at 500 nM increased the positivity of stained cells with trypan blue exclusion test during the course of the experiment, suggesting that the inhibitory effects of antisense ODNs were not due to cytotoxicity of the synthetic ODNs. The flat revertant induced by antisense ODNs returned to the original spindle shape after 5 d in culture unless there was a further supply of fresh ODNs into the culture medium (data not shown).

### Antisense Inhibition of *Hst-1/Fgf-4* mRNA of the Cultured Limbs

The presence of *Hst-1/Fgf-4* mRNA in the explants of forelimb bud at 9.5-d p.c. embryo was detected 1.5–2.5 d after the in vitro culture and diminished thereafter (data not shown). To ascertain whether treatment with *Hst-1/FGF-4* antisense ODNs caused a decrease in the amount

**Table I. Three Distinct Media Formulations for Mouse Limb Development**

Supplement	Medium concentration		
	M1	M2	M3
2-mercaptoethanol	10 <sup>-4</sup> M	10 <sup>-4</sup> M	10 <sup>-4</sup> M
Monothioglycerol	200 μM	200 μM	200 μM
Selenium	10 <sup>-7</sup> M	10 <sup>-7</sup> M	10 <sup>-7</sup> M
Linoleic acid	5 μg/ml	5 μg/ml	5 μg/ml
Hydrocortisone	4 μg/ml	4 μg/ml	4 μg/ml
Transferrin	5 μg/ml	5 μg/ml	5 μg/ml
Gelatin	–	0.4%	–
Methylcellulose	–	–	0.8 %

All media formulations used DME supplemented with Hepes, pH 7.4, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 5 μg/ml amphotericin B as a basal medium.

of mRNA of *Hst-1/Fgf-4* in the mouse limb bud quantitatively, total RNAs were prepared from a pool of limb buds treated with ODNs for 2.5 d, and then RNA blot hybridization was performed. An extremely small amount of *Hst-1/FGF-4* transcript was detected in RNA isolated from limb buds exposed to *Hst-1/FGF-4* antisense ODNs, a >90% reduction compared to untreated control (Fig. 4); in contrast, the expression of endogenous β-actin was clearly detectable without reduction, indicating the selective reduction of *Hst-1/FGF-4* transcripts. Other control ODNs showed no apparent effect on the level of *Hst-1/FGF-4* transcripts. In addition, we tested the amount of transcripts from a molecule closely related to *Hst-1/FGF-4* such as *FGF-2* and *FGF-8*, which are also expressed in the limbs of early embryonic stage. As shown in Fig. 4, no apparent inhibitory effect on *FGF-2* and *FGF-8* transcripts was detected. Overall, these results suggest that antisense ODNs against ATG translation start site of *Hst-1/FGF-4* significantly alter the degree of *Hst-1/FGF-4* expression in a sequence-dependent manner in the limb bud cultures without any cytotoxic effects.

#### *Hst-1/FGF-4* Antisense ODNs Block Limb Development In Vitro

By using this novel organ culture system, we treated the 9.5–10-d p.c. embryo with antisense ODNs complemen-

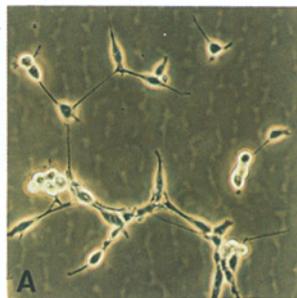
tary to mouse *Hst-1/Fgf-4* gene for 4.5 d. This treatment resulted in the blocking of forelimb and hindlimb outgrowth (Fig. 5, A and B). On the contrary, sense ODNs have no inhibitory effects (Fig. 5, C and D). Measurements of the P–D length of antisense ODNs treated forelimbs and hindlimbs showed that the embryonic stage was slightly advanced, most like the 10.5-d p.c. embryonal limbs in vivo and then their growth was stopped as summarized in Table II.

In a separate experiment, *Hst-1/FGF-4* antisense ODN-treated limbs were cultured in limb differentiation medium supplemented with recombinant human FGF-4 proteins (800 ng/ml). As shown in Table II, exogenous FGF-4 protein rescued outgrowth (P–D length) of limbs from the inhibitory effect of antisense ODNs (~80% rescued in P–D length relative to antisense ODNs-treated forelimbs and hindlimbs). Furthermore, to verify that inhibition of limb development by antisense ODNs does not correspond to a cytotoxic effect of ODNs, limb culture was examined by trypan blue exclusion test 2.0 and 4.5 d after the addition of antisense ODNs and compared to untreated limb culture. Although data is not shown, specific cell death was not detected in antisense ODNs-treated limbs at day 2.0 nor day 4.5 in culture. These results indicated direct evidence that *Hst-1/FGF-4* expression is critical for the induction of mouse limb outgrowth.

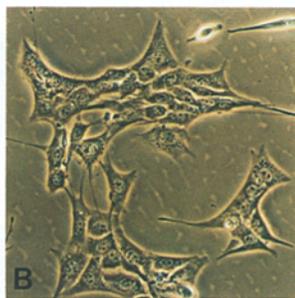
#### Discussion

Although we originally identified the *Hst-1/Fgf-4* as a transforming oncogene on transfection assay of NIH3T3 cells (Sakamoto et al., 1986), we have subsequently noted that mouse *Hst-1/FGF-4* is also expressed on cells of AER in developing mouse limb bud (Suzuki et al., 1992). *Hst-1/FGF-4* expression reaches a peak 11–12-d p.c. in the embryonal limbs and then disappears as limb development progresses 16-d p.c. in forelimb bud. Several genes are expressed in the mouse AER other than *Hst-1/Fgf-4*, including *Fgf-2* and *Fgf-8*. However, it has not yet been possible to determine which of these genes normally functions in vivo. In addition to the fact that there are no classical mu-

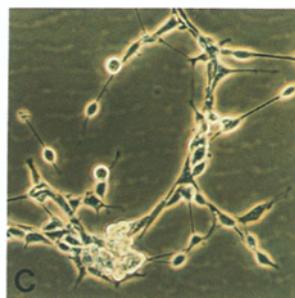
***Hst-1* transformant**



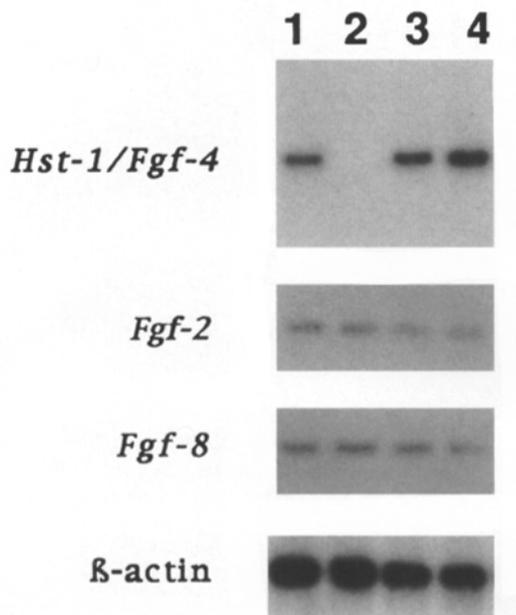
**+ antisense**



**+ sense**



**Figure 3.** Effect of antisense ODNs on *Hst-1/Fgf-4* transformed NIH3T3 cells. NIH3T3 cells transformed by mouse *Hst-1/Fgf-4* gene (A) showed malignant phenotype with spindle shape. They were exposed to antisense (B) or control sense (C) ODNs at 500 nM with Lipofectin according to the recommended method and then cultured for 3 d with 5% heat-inactivated calf serum-DME. *Hst-1/FGF-4* antisense ODNs: 5'-d- CGGCCCGCGTTTCGCCAT-3'; control sense ODNs: 5'-d-ATGGC-GAAACGCGGGCCG-3'; control scrambled ODNs: 5'-d-AGC-TGCTGCTGCTGCC-3'.



**Figure 4.** Expression of FGFs mRNA of the limbs after exposure to antisense ODNs. ODNs were administered to the limb bud as described in Fig. 1 B at a final concentration of 500 nM for 2.5 d in culture. At the end of the incubation period, 20 pairs of limb buds were pooled and then total mRNAs were extracted. About 25  $\mu$ g of each RNA sample was subjected to RNA blot analyses using full size of mouse *Hst-1/Fgf-4* cDNA, *Fgf-2* cDNA, and *Fgf-8* cDNA as a probe. Lane 1, untreated forelimb buds; lane 2, Hst-1/FGF-4 antisense ODNs treated forelimb buds; lane 3, control sense ODNs treated forelimb buds; lane 4, control scrambled ODNs treated forelimb buds. Treatment with ODNs did not affect the expression of  $\beta$ -actin transcripts.

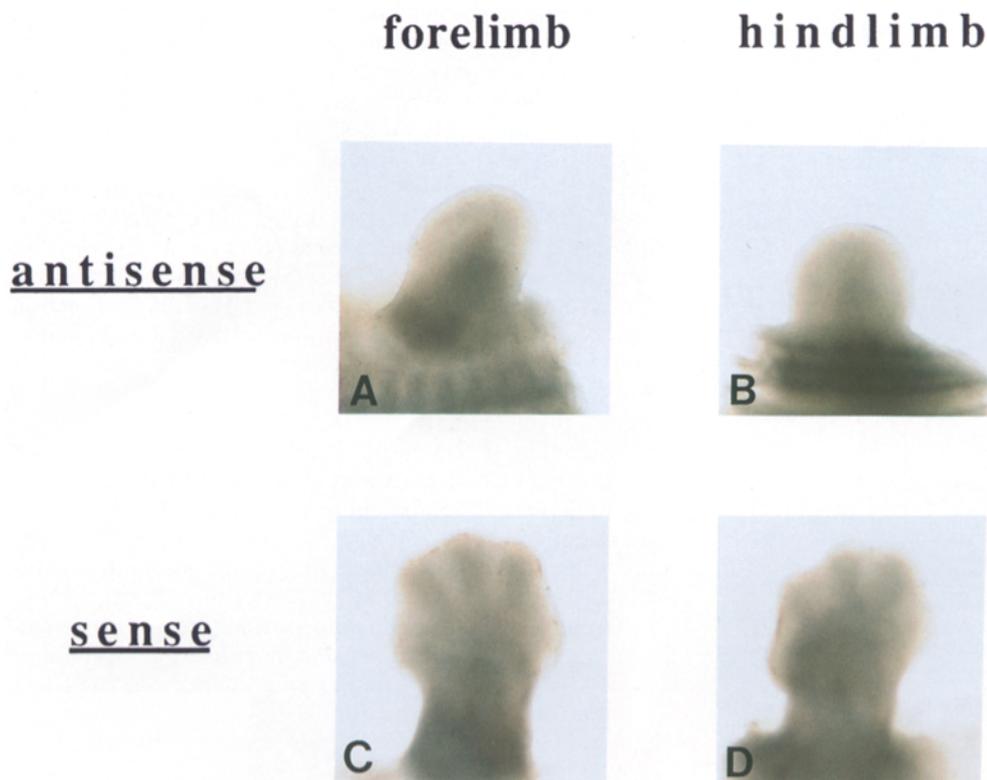
tations of *Fgf-4*, targeted disruption of mouse *Fgf-4* gene results in early embryonic lethality (Feldman et al., 1995), indicating that FGF-4 knock-out mice could not provide any insights into the role of Hst-1/FGF-4 on limb development. Thus its role in mouse limb development can not be studied by conventional genetic methods.

Our results provide direct evidence that specific expression of Hst-1/FGF-4 is required during limb development. We found that antisense Hst-1/FGF-4 ODNs treatment of embryonic mouse limb bud resulted in a retardation of limb outgrowth, which was accompanied by a specific destruction of Hst-1/FGF-4 translation. The expression of other FGF family members such as *Fgf-2* and *Fgf-8* were not affected by Hst-1/FGF-4 antisense ODNs. Therefore, among FGF genes, it is conceivable that *Hst-1/Fgf-4* plays a major role as a positive signal for mesenchyme proliferation of the mouse limb.

One of the critical problems for studying the signals that direct limb outgrowth is a lack of a suitable in vitro system except for the chick developing limb system in ovo. Here we established a novel in vitro culture system in which mouse limb bud at 9.5–10-d p.c. embryo placed on a sheet of extracellular matrix in a defined medium differentiates into a limb at 12.5-d p.c. embryo within 4.5 d. Our culturing system is superior to the ordinary method in that it enables further and more nearly normal limb development as in vivo. Our culture system reported here may provide a good clue toward understanding the molecular basis of

limb development including tissue-specific differentiation, control of cell growth, and cell to cell communication by controlling a variety of culture conditions with or without additives such as several growth factors, inhibitors, synthetic peptides, and ODNs. Recently, it has been suggested that BMP2 (Wang et al., 1988; Wozney et al., 1988) keeps the mesenchyme undifferentiated (Lyons et al., 1990; Niswander and Martin, 1993) and controls patterning in the developing chick limb (Francis et al., 1994). In addition, it has been shown that N-cadherin is expressed in limb mesenchyme and perturbation of N-cadherin function significantly inhibits both limb mesenchymal aggregation and chondrogenesis in chick (Oberlender and Tuan, 1994). Moreover, recent progress suggests that *Sonic hedgehog* signals regulate expression of both *BMP2* and *Fgf-4* gene (Laufer et al., 1994). In this connection, it may be of some interest to see whether those molecules have a regulatory effect on limb development in our system. Another interest in limb development is the apoptotic process which enables digit formation. When we kept the forelimb explant (9.5-d p.c.) in culture for 10–12 d with one change of limb differentiation media containing serum (10% horse serum/10% fetal calf serum) it became most like the forelimb of the 14.5-d p.c. embryo (unpublished data). The digit formation was observed as normal, suggesting that our system will be useful in studying molecular aspects of apoptosis on limb development.

Recently, several antisense ODNs studies have reported inhibition of tooth (Kronmiller et al., 1991; Diekwisch et al., 1993), kidney (Sariola et al., 1991), heart (Runyan, 1991), muscle (Biro et al., 1993), and lung (Souza et al., 1994). In general, a rigid control is required in antisense experiments. At first, the toxicity of individual batches of ODNs was tested on the cultured cells before they were put on the limb culture and it was shown that every batch of ODNs has no cytotoxic effect up to 10  $\mu$ M. Actually, as shown in Fig. 4, the expression of the house-keeping actin gene in the limb bud was not affected by antisense ODNs. Second, although data are not shown, the inhibition of the transformed phenotype by addition of antisense ODNs was reversible, indicating that the phenotypic change of cells was not due to the cytotoxic effect of ODNs. Third, we designed phosphorothioate modified ODNs of 18 mers targeted against sequences adjacent to the ATG initiation codon of mouse *Hst-1/Fgf-4* mRNA. Many other investigators have suggested that antisense ODNs against sequences adjacent to initiation codons are most effective in inhibiting translation (Shakin-Esheleman and Liebhaber, 1988; Malcom, 1992). Actually, in our experiments, antisense ODNs targeted to sequences other than the ATG site proved less efficient. Fourth, a recent paper has indicated that phosphorothioate ODNs may bind directly to FGF-2 and possibly FGF-4, and that this may inhibit their function (Guvakova et al., 1995). As shown in Fig. 5 and Table II, control sense and scrambled phosphorothioate ODNs did not show any inhibitory effect on limb development. In addition, although we did not mention the results, we examined the inhibitory effect of non-phosphorothioate Hst-1/FGF-4 antisense ODNs against ATG start site. The transforming phenotype of *Hst-1/Fgf-4* transformed NIH3T3 cells was efficiently inhibited at 0.1–2  $\mu$ M by non-phosphorothioate antisense ODNs, suggesting that the in-



**Figure 5.** Hst-1/FGF-4 antisense ODNs block limb development in vitro. ODNs treatment was performed as described in Fig. 4. and then treated limbs were cultured for 4.5 d in vitro. Antisense ODNs treated right forelimb (A) and hindlimb (B); control sense ODNs treated right forelimb (C) and hindlimb (D). Although data are not shown, control scrambled ODNs result in no inhibitory effects on limb outgrowth.

hibitory effect of antisense ODNs may not be due to the structure nor chemical modification of oligos such as phosphorothioate linkages. Fifth, the fact that Hst-1/FGF-4 antisense ODNs did not alter the expression of FGF-2 and FGF-8 on mouse limb bud ensures that the inhibition of limb development by Hst-1/FGF-4 antisense ODNs is a sequence-specific effect. Lastly, inhibition of limb outgrowth by antisense ODNs was rescued when exogenous recombinant FGF-4 was supplied to the limb culture, indicating that antisense inhibition is not a non-specific cytotoxic effect. Therefore our antisense experiments are adequate to make a conclusion that Hst-1/FGF-4 is critical for limb outgrowth.

Our ODNs-transfer method is different from the widely used methods of simply adding ODNs into the culture medium in that we administered ODNs with a form of cationic liposome complexes. These complexes are well ab-

sorbed into the cultured explants without any great diffusion when placed on the desired small area and then are highly incorporated into the cells. Therefore, one could introduce ODNs in a site-specific manner. Furthermore, the liposome transfer method makes it possible to use nanomolar amounts of ODNs which were almost 100 less as compared to not using liposome. This means that we can see the effects of antisense ODNs with less cytotoxicity.

In summary, the results of our studies indicate that the *Hst-1/Fgf-4* gene product is essential for embryonic limb outgrowth in mice. Similar antisense strategies can be possibly used to clarify the functions of many other growth factors/cytokines and their receptors on embryonic limb development by using our novel mouse limb culture system.

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**Table II.** Effects of Hst-1 Antisense ODNs on P-D Length of Cultured Limbs

ODNs	Length of limb in mm (mean $\pm$ SD)	
	Forelimbs	Hindlimbs
None	1.304 $\pm$ 0.05	1.608 $\pm$ 0.06
Antisense	0.580 $\pm$ 0.10	0.702 $\pm$ 0.10
Sense	1.312 $\pm$ 0.05	1.588 $\pm$ 0.05
Scrambled	1.289 $\pm$ 0.10	1.600 $\pm$ 0.10
Antisense + FGF-4*	1.012 $\pm$ 0.25	1.204 $\pm$ 0.15

Trunc fragments with limbs (9.5-10-d p.c. embryo) were treated with ODNs as described and cultured on matrix gel for 4.5 d in M3 limb differentiation medium. Measurements of the P-D length were made for each pair of limbs. Number of forelimb or hindlimb pairs analyzed = 30.

\*Recombinant FGF-4 protein was added to the culture medium (800 ng/ml) of antisense ODN-treated limbs and cultured for 4.5 d. Number of forelimb or hindlimb pairs analyzed = 6.

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