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Fibroblast growth factor enhances expression of TGFβ-stimulated-clone-22 gene in osteoblast-like cells

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Transforming growth factor-β1 (TGFβ1)-stimulated clone 22 (TSC-22) is a primary response gene isolated by subtractive screening of genes expressed in murine osteoblastic cells treated with TGFβ1, which is one of the cytokines abundantly stored in bone. Fibroblast growth factor (FGF) is also stored in bone matrix and acts as a potent autocrine/paracrine modulator of osteoblastic function. In this report, we investigated FGF effects on the expression of TSC-22 gene in a murine osteoblast-like cell line, MC3T3E-1. Treatment with recombinant basic FGF enhanced TSC-22 mRNA level in these cells within 1 h. This effect peaked at 2 h with several fold enhancement, after which the mRNA abundance returned to the basal level by 24 h. The FGF effect was dose-dependent, starting at 0.2 ng/ml, peaking at 2 ng/ml, and then declining at 20 ng/ml. The FGF effect on TSC-22 mRNA was blocked by actinomycin D, indicating the involvement of transcriptional events. The FGF enhancement of TSC-22 mRNA expression was partially blocked by genistein. Additive effect was observed upon cotreatment with saturating concentrations of FGF and TGFβ, suggesting the presence of at least two independent pathways for the two cytokines in the regulation of TSC-22 gene expression. These results indicate that the TSC-22 gene is one of the targets of FGF action in osteoblasts.

Keywords: transforming growth factor-β1 (TGFβ1)-stimulated clone 22 (TSC-22); fibroblast growth factor (FGF); osteoblast

Introduction

Osteoblastic proliferation and differentiation are under the control of both systemic and local regulators. These regulators exert their effects via binding to their cognate receptors, which is followed by a cascade of events in signal transduction, and finally modulate expression of genes involved in differentiated cell function. Gene regulation could be controlled in several steps including transcriptional, translational and post-translational events. Among these, transcriptional control is the major event in the regulation of many genes. As is the case in many other types of cells, expression of genes in osteoblastic cells are under the control of several classes of transcription factors (Ruther *et al.*, 1987; Kerner *et al.*, 1989; Kawaguchi *et al.*, 1992; Tamura & Noda, 1994; Turner *et al.*, 1994). Regulation of the osteoblastic functions by extracellular signals such as hormones and cytokines would be at least in part mediated by the functions of these transcription factors.

Transforming growth factor-β[1] (TGFβ1)-stimulated-clone-22 (TSC-22) is a recently identified novel primary response gene isolated by subtractive screening of genes expressed in murine osteoblastic (MC3T3E-1) cells treated with TGFβ1 (Shibanuma *et al.*, 1992). TSC-22 gene expression was also induced in these cells by phorbol 12-myristate 13-acetate, serum, cholera toxin, or dexamethasone, but not appreciably by epidermal growth factor. The nucleotide sequences of TSC-22 cDNA showed no homology with those of any previously known genes. The open reading frame and *in vitro* translation product indicated that the gene encodes a polypeptide of 143 amino acids with a molecular weight of 18 kDa that contains a putative leucine-zipper motif, which has been identified as a dimerization motif for transcription factors such as Jun and Fos. Its mRNA is expressed abundantly in bone, thymus, liver and less in other tissues (Shibanuma *et al.*, 1991). Recently, a rat homologue of TSC-22 was also identified from FSH treated Sertori cells (Hamil & Hall, 1994).

Basic fibroblast growth factor (FGF) is a member of FGF family and has been shown to be a potent growth stimulator and a differentiation modulator in many kinds of cells (Gospodarowicz *et al.*, 1987). FGF is stored in bone matrix and is known to be a potent autocrine/paracrine modulator of proliferation and differentiation in osteoblasts (Globus *et al.*, 1989).

FGF effects on osteoblastic cells are not confined to its regulation of extracellular matrix proteins, but its effects have been observed in the case of expression of genes encoding cytokines such as TGFβ. FGF enhances expression of TGFβ in osteoblastic cells (Noda & Vogel, 1989), while TGFβ enhances FGF expression in these cells as well (Globus *et al.*, 1988). On the surface of osteoblastic cells, several types of receptors for FGF have been shown to be expressed. FGF binds not only to its cognate receptors but was also suggested to bind to other proteoglycan containing membrane proteins such as betaglycan. TGFβ, as an active form, binds to betaglycan at its core protein and FGF binds to betaglycan at its sugar side chains (Andres *et al.*, 1992). These observations suggest close relationship between FGF and TGFβ in regulation of osteoblastic function.

The aim of this paper was to examine the effects of FGF on the expression of TSC-22 gene in osteoblast-like (MC3T3E-1) cells. We found that FGF enhanced the expression of TSC-22 mRNA several fold within 1 h and this effect was mediated at least in part via transcriptional events. Furthermore, we also observed that the pathway through which FGF action was mediated was independent from that for TGFβ. Our results indicated that TSC-22 gene would be a common target of both of the two potent modulators of osteoblastic function.

Results

Time course of FGF effect on TSC-22 gene expression

We first examined the time course of the effect of basic FGF treatment on TSC-22 gene expression in MC3T3E-1 cells. After reaching confluency, the cells were cultured in 0.1% FBS one day before the initiation of the FGF treatment. Treatment with 10 ng/ml FGF enhanced TSC-22 mRNA level within 1 h and this effect peaked at 2 h with several fold enhancement, after which the TSC-22 mRNA level returned to the basal level by 24 h (Figure 1A,B). The level of β-actin mRNA served as control.

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Dose-dependence of FGF effect on TSC-22 gene expression

Dose dependency of the FGF effect on TSC-22 mRNA level was then examined in these MC3T3E-1 cells. The cells were treated for 2 h with FGF at various concentrations ranging from 0.002 to 20 ng/ml. The FGF effect on TSC-22 mRNA expression was observed starting at 0.2 ng/ml, maximal at 2 ng/ml, and biphasic with some decline at 20 ng/ml (Figure 2A,B).

Modulation by actinomycin D of the FGF effect on TSC-22 gene expression

To examine the mode of FGF action in regulation of TSC-22 gene expression, the cells were treated for 2 h with FGF in the presence or absence of an inhibitor for transcription. The inhibitor was added to the medium 15 min before FGF treatment. As shown in Figure 3, Actinomycin D treatment (2 μg/ml) alone suppressed the basal level by about 50%, and FGF effect was blocked in the presence of this inhibitor, not proved but suggesting the involvement of transcriptional events at least in part in mediating the FGF effect on TSC-22 gene expression.

Additive effects of FGF and TGFβ on TSC-22 gene expression

Since TSC-22 has been cloned as a gene expressed in response to TGFβ1 treatment, we examined whether FGF may share any pathway(s) with TGFβ1 in regulation of TSC-22 gene expression. When the cells were treated with saturating concentrations of FGF (2 ng/ml) and TGFβ1 (4 ng/ml) for 2 h, additive effect was observed, indicating the presence of at least two independent pathways for the two cytokines in enhancing TSC-22 gene expression (Figure 4).

Tyrosine kinase inhibitor partially blocks FGF effect on TSC-22 gene expression

To examine the pathway through which FGF exerts its effect on TSC-22 gene expression, the cells were treated for 2 h with FGF in the presence or absence of protein kinase inhibitors, H7 and genistein. Each inhibitor was added to the medium 15 min before FGF treatment. The FGF effect on TSC-22 mRNA expression was partially blocked by genistein (100 ng/ml) (the averaged ratio of [genistein + FGF] over [genistein alone] from the two independent experiments was

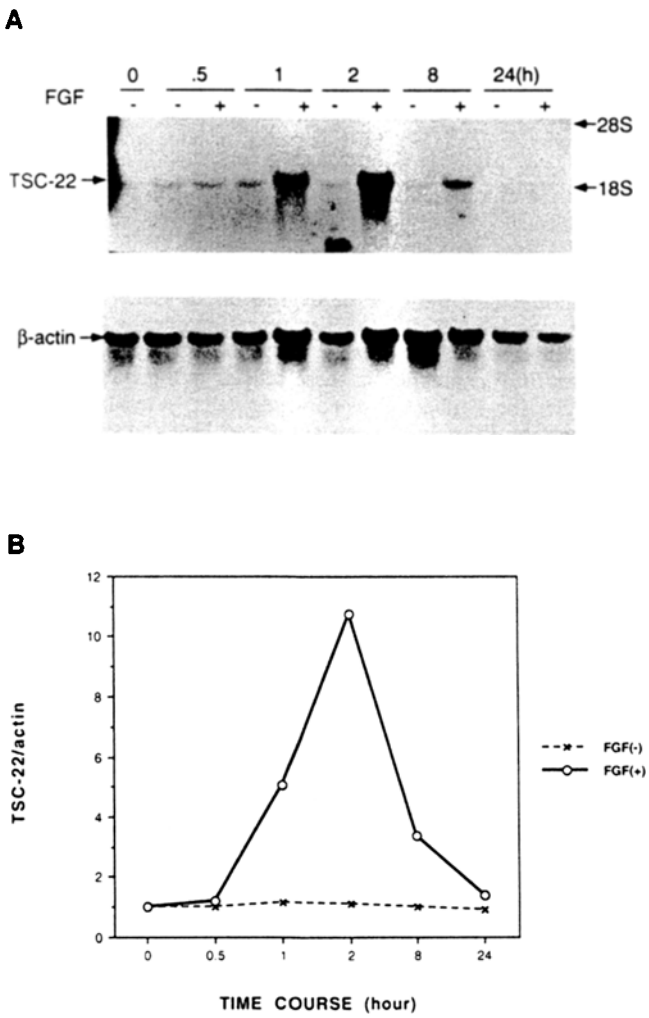

Figure 1 Time course of FGF effect on TSC-22 gene expression. Confluent MC3T3E-1 cells were cultured in the absence or presence of FGF (2 ng ml) for the indicated periods of time (h). (**A**) Total RNA was extracted according to AGPC method and Northern blot analysis was conducted as described in Materials and methods. Each lane was loaded with 10 μg of total RNA. β-actin mRNA level is served as control. The positions of TSC-22, 18S and 28S ribosomal RNA are indicated. The data represents one of two independent experiments with similar time dependency with some variation in the peak levels. (**B**) Quantification of the northern blot autoradiogram shown in **A**

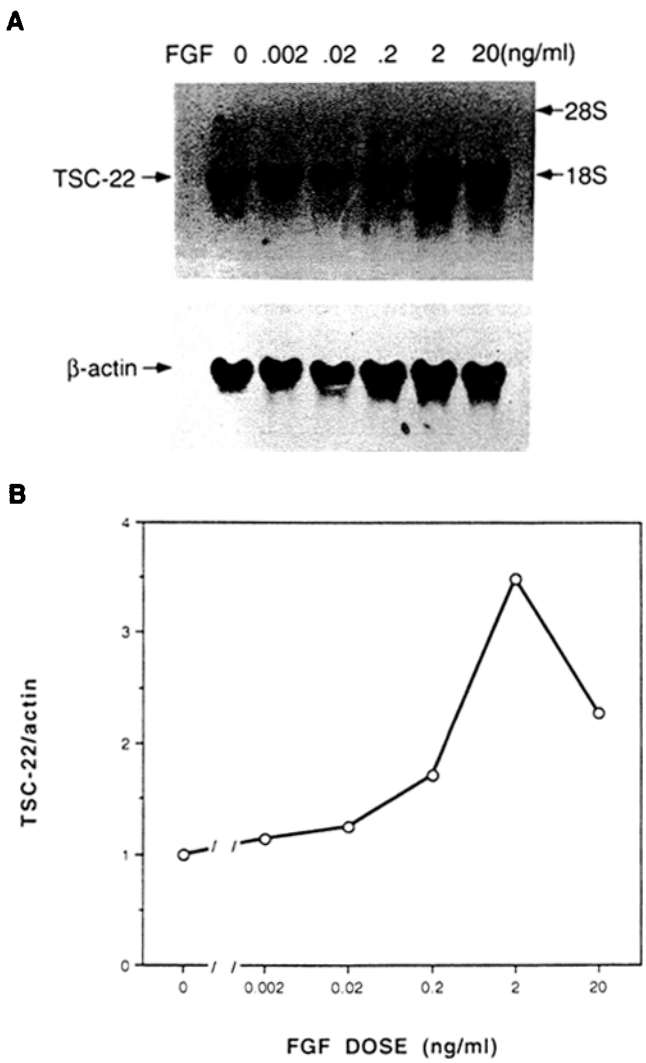

Figure 2 Dose-dependence of FGF effect on TSC-22 gene expression. Confluent MC3T3-E1 cells were treated for 2 h with the indicated concentrations of FGF (ng/ml) and Northern blot analysis was conducted as described in Materials and methods. (**A**) Each lane was loaded with 20 μg of total RNA. β-actin mRNA level is served as control. The positions of TSC-22, 18S and 28S ribosomal RNA are indicated. The data represents one of two independent experiments with similar results. (**B**) Quantification of the northern blot autoradiogram shown in **A**

1.25) but not by H7 (50 μM) (the averaged ratio of [H7 + FGF] over [H7 alone] from the two independent experiments was 2.42), suggesting the involvement of tyrosine kinases at least in part in mediating the FGF effect on TSC-22 gene expression (Figure 5).

Discussion

In this report, we showed that FGF potently enhanced the expression of TSC-22 gene in murine osteoblastic MC3T3E-1 cells. This effect peaked at 2 h after the initiation of the treatment. The pattern of this time-dependent enhancement was similar to that of TGFβ effect on the expression of this gene. Therefore, it appeared that TSC-22 gene expression may be subjected to a similar type of regulation by these two cytokines. However, cotreatment experiments indicated that at least a part of the pathway through which FGF exerted its effects was independent from that of TGFβ. Therefore, although the response with regard to TSC-22 gene expression was similar, the post-receptor mechanisms through which the TSC-22 gene expression was activated would be diverse between these two cytokines.

Examination of the mode of FGF actions on the regulation of TSC-22 gene expression suggested that FGF effects would be at least in part mediated through transcriptional control. Our observation predict that the promoter region of TSC-22 gene may have at least two diverse response elements, one for FGF and the other for TGFβ respectively. Other transcriptional regulators including calciotropic steroid hormone receptors could also act on the promoter via their own cognate sites.

Although the downstream targets of TSC-22 is not yet clear at this point, it is intriguing that not only TGFβ but also FGF which is implicated in regulation of calcium metabolism modulates the expression of the TSC-22 gene in osteoblastic cells. FGF effect on TSC-22 mRNA expression in osteoblastic MC3T3-E1 cells was partially blocked by genistein, suggesting the tyrosine kinase involvement in the post-receptor events. H7 at 50 μM did not block the FGF action. Higher doses were not examined since the basal TSC-22 mRNA levels was already reduced by the treatment with 50 μM H7 *per se*. It is also still to be examined whether our observation could be found in the case of primary cultures of osteoblastic cells.

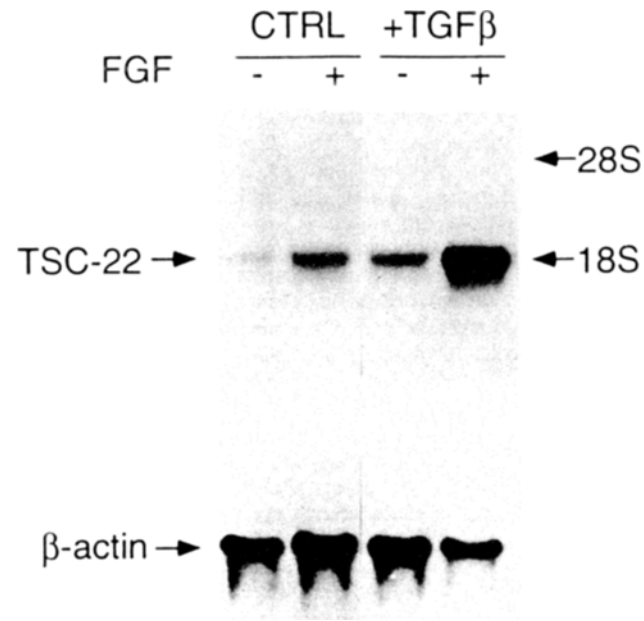

Figure 4 Additive effects of FGF and TFGβ on TSC-22 gene expression. Confluent MC3T3E-1 cells were treated for 2 h with vehicle alone [CTRL] or TGFβ1 (4 ng/ml) in the presence or absence of 2 ng/ml FGF. Northern blot analysis was carried out as described in Materials and methods. β-actin mRNA level is served as control. The positions of TSC-22, 18S and 28S ribosomal RNA are indicated. The data represents one of two independent experiments with similar results.

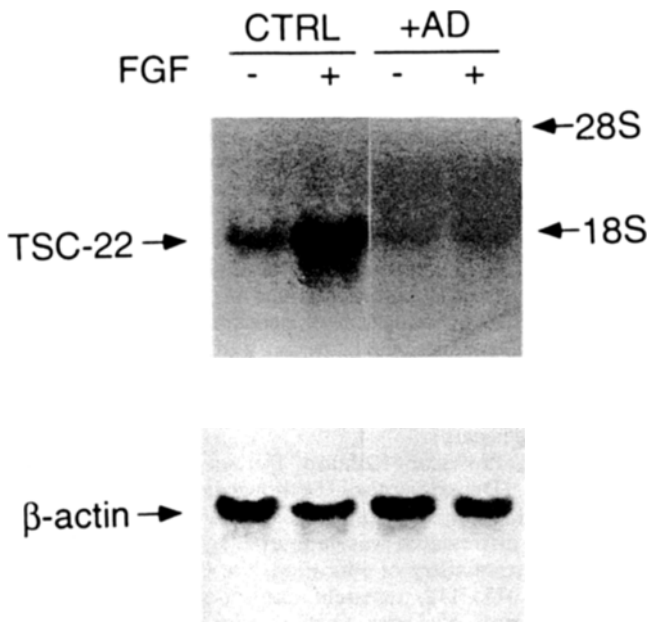

Figure 3 Modulation by actinomycin D of the FGF effect on TSC-22 gene expression. Confluent MC3T3E-1 cells were treated for 2 h with FGF (2 ng/ml) in the presence or absence of 2 μg/ml actinomycin D[AD]. Northern blot analysis was carried out as described in Materials and method. β-actin mRNA level is served as control. The positions of TSC-22, 18S and 28S ribosomal RNA are indicated. CTRL; vehicle alone. The data represents one of two independent experiments with similar results.

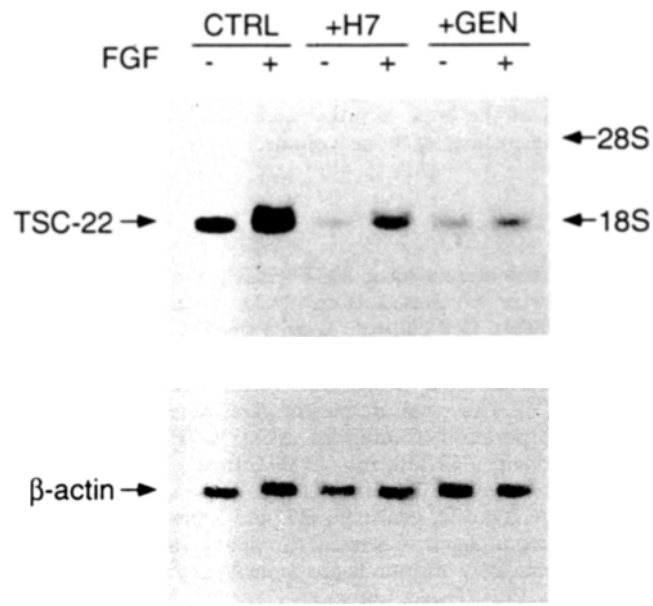

Figure 5 Modulation by H7 or genistein of the FGF effect on TSC-22 gene expression. Confluent MC3T3E-1 cells were treated with vihcle (CTRL) or FGF (2 ng/ml) for 2 h in the presence or absence of H7 (50 μM) or genistein [GEN] (100 ng/ml). Northern blot analysis was carried out as described in Materials and methods. β-actin mRNA level is served as control. The data represents one of two independent experiments with similar results. The positions of TSC-22, 18S and 28S ribosomal RNA are indicated.

Osteoblastic functions are regulated by the transcription factors, including Sp-1, Fos, Jun, NF-κB, homeobox proteins, certain steroid receptor families and possibly ISGF3, HLH proteins and so on (Bohmann *et al.*, 1987; Angel *et al.*, 1988; Chiu *et al.*, 1988; Hazel *et al.*, 1988; Leonardo & Baltimore, 1989; Murre *et al.*, 1989; Levy & Dernell, 1990). At least for a subset of the phenotype-related genes expressed in osteoblasts, their promoter regions have been shown to contain response elements to which transcription factors have been shown to bind and activate the expression of the genes (Schmidt *et al.*, 1986; Kerner *et al.*, 1989; Morrison *et al.*, 1989; Demay *et al.*, 1990; Markose *et al.*, 1990; Pavlin *et al.*, 1992). FGF enhancement of TSC-22 gene expression would be a part of the regulatory network which is governing the osteoblastic function under the influence of local and systemic regulatory molecules.

In the case of osteoblastic cells, FGF has been shown to enhance proliferation of primary calvaria derived osteoblast-enriched cells, rat osteoblastic osteosarcoma ROS17/2.8 cells, bovine bone derived cells and primary human bone derived osteoblastic cells (Canalis *et al.*, 1988; Globus *et al.*, 1988; McCarthy *et al.*, 1989; Kaye *et al.*, 1990). FGF in its basic or acidic form is stored in bone matrix at approximately 100 μg/kg dry weight bone matrix, which is roughly similar to the abundance of insulin-like growth factor-I (IGF-I) and platelet derived growth factor (PDGF) (Hauschka *et al.*, 1986). In contrast to these several cytokines which stimulate or promote both proliferation and differentiation of osteoblasts such as IGF-I, FGF exhibits a unique way of regulation of the differentiation-related gene expression in osteoblastic cells. FGF suppresses expression of type I procollagen, alkaline phosphatase, IGF-I, IGF-II, and IGF binding protein gene in both primary cultures of osteoblasts and several osteoblastic cell lines (Rodan *et al.*, 1989; Hurley *et al.*, 1993 and 1995). On the other hand FGF promotes expression of osteopontin in osteoblastic cells. The roles of FGF in regulation of bone metabolism have been suggested based on *in vivo* experiments, in which FGF expression has been detected in callus formed after fractures (Jingushi *et al.*, 1992). Local injections of FGF have been shown to promote osteoid or bone formation in animals (Jingushi *et al.*, 1991). These observations suggest that FGF is at least one of the most potent modulators of osteoblastic functions. As observed in the tissues other than bone, the local regulation of cellular function is taking place in concert with the systemic regulation *in vivo*. It is still to be determined what the role(s) of TSC-22 gene product would be, especially in bone, in the regulation of the bone turnover and hence the maintenance of the homeostasis of bone volume.

Materials and methods

Recombinant human basic FGF (FGF) and human TGFβ (TGFβ[1]) were purchased from R&D Systems, Inc. (Minneapolis, MN). Cell culture reagents (media, serum, trypsin) were purchased from Gibco Life Technologies, Inc. (Grand Island,, NY). Tissue culture plastic wares were obtained from Costar Corporation (Cambridge, MA). [α-^{32}P]-dCTP, with a specific activity 111 TBq/mM (3000 Ci/mM), was purchased from NEN Research Products (Tokyo, Japan). Guanidinium thiocyanate, agarose, genistein, H7, and actinomycin D, were obtained from Sigma Chemical Company (St Louis, MO). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell culture

Murine osteoblastic MC3T3E1 cells were kindly provided by Dr H. Kodama (Oh-U University, Japan) and were grown to confluence in α-modification of Eagle's medium (α-MEM) supplemented with 5% fetal bovine serum (FBS). The cells were subcultured every week. For experiments, the cells were plated at 2000 cells cm^{-2} and were grown for four days to confluence. The medium was changed to α-MEM supplemented with 0.1% FBS one day before the initiation of the treatment. All the cells used in this study were cultured in a humidified atmosphere of 5% CO_2 at 37°C.

RNA extraction

Total cellular RNA was extracted according to AGPC (acid guanidinium thiocyanate-phenol-chloroform) method (Chomczynski & Sacchi, 1987). Briefly, after discarding the medium, the cells were rinsed with PBS three times, and were lysed in 2 ml of solution D (4 M guanidinium thiocyanate/0.1 M β-mercaptoethanol). After shearing DNA by passing through 23-gauge needles, the lysates were collected from each dish and transferred to polypropylene tubes. After addition of 0.2 ml of 2 M sodium acetate, 2 ml water-saturated-phenol (pH 4.0), and 0.4 ml of chloroform:isoamyl alcohol (49:1), the lysates were mixed by shaking vigorously for 10 s, cooled on ice for 15 min, and centrifuged at 10 000 × *g* for 20 min at 4°C to separate aqueous phase, which were then mixed with 2 ml isopropanol, and kept at −20°C for 2 h followed by centrifugation at 10 000 × *g* for 20 min. The resulting RNA pellets were rinsed with 75% ethanol, vacuum dried (15 min), and dissolved in 30 μl TE (10 mM Tris Cl, pH 7.6/1 mM EDTA, pH 8.0).

Northern blot analysis

Total RNAs (10 or 20 μg) were electrophoresed in 1% agarose gel containing 0.22 M formaldehyde in 1 × MOPS (20 mM 3-(N-morphilino)propanesulfonic acid/15 mM sodium acetate/1 mM EDTA). Direct staining with ethidium bromide (EtBr) was performed to monitor RNA integrity as well as uniformity. Overnight transfer of the RNAs to Hybond N nylon filters (Amersham Inc, Arlington Heights, IL) were accomplished in 1 × TAE (0.04 M Tris-acetate/0.001 M EDTA). The filters were prehybridized overnight in 50% formamide/5 × Denharolt's solution/5 × SSC/0.1% sodium dodecyl sulfate (SDS) 50 μg/ml herring sperm DNA at room temperature. Murine TSC22 cDNA insert (1,800 base pairs, *Eco*RI/*Eco*RI fragment) was gel purified and radiolabeled with [α-^{32}P]-dCTP according to the method described by Feinberg & Vogelstein (1983). The specific activities of the probe were approximately 10^8 c.p.m./μg DNA. Overnight hybridization was performed at 42°C in a fresh buffer containing all the same ingredients in the prehybridization buffer in addition to the radiolabeled probe (approximately 10^6 c.p.m./ml). Blots were washed three times for 5 min each time at room temperature in 1 × SSC/0.5% SDS, and once for 20 min at 65°C in 0.2 × SSC/0.5% SDS, and were exposed to X-ray films for several days at −80°C using intensifying screens. Autoradiograms of the Northern blots were quantified by using a laser densitometer FAST SCAN (Molecular Dynamics, CA).

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Note added in proof

While this paper was proofed, Treisman *et al.*, reported that 'shortsighted' is a drosophila homologue of TSC22 and is involved in dpp signaling.

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