

agarose gels and stained with ethidium bromide along with a 100 bp-ladder molecular size marker.

Alkaline phosphatase (ALPase) activity

The effect of rCCN2/CTGF on ALPase activity was assayed according to the procedure of Majeska *et.al.* [32]. The cells were homogenized in 0.5 ml of 0.9% NaCl and 0.2% Triton X-100 with a Polytron at 4°C, and centrifuged for 15 min at 12,000 × g. ALPase activity in the supernatant was measured by using *p*-nitro phenyl phosphate (*p*-NP) as a substrate. The supernatant was mixed with 0.5 M Tris-HCL buffer (pH9.0) containing 0.5 mM *p*-NP and 0.5 mM MgCl₂. The sample was then incubated at 37°C for 30 min, and the reaction was stopped by the addition of a 0.25 volume of 1N NaOH. Hydrolysis of *p*-NP was monitored as the change in absorbance at 415 nm with a spectrometer (Amersham Bioscience Corp.). The protein concentration was determined by using the BCA protein assay system (Pierce, Rockford, IL). The activity was defined in units, 1 unit being the enzyme activity hydrolyzing 1 nmol of *p*-NP per 1 mg protein in 30 min.

Collagen synthesis

The effect of rCCN2/CTGF on collagen synthesis was assayed by measuring the incorporation of [³H] proline (Amersham, 1.7 TBq/nmol) in collagenase-digestable proteins [33-35]. When MPL cells reached confluence, they were labeled with [³H] proline (37 MBq/ml) for 12 hrs and then collected into 1 ml of 50 mM Tris (pH7.2) containing 0.2% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF). Half of the solubilized homogenate was digested with purified bacterial collagenase for 4 hrs at 37°C, and the other half was incubated with the vehicle (control). The radioactivity in the suspended protein was measured with a liquid scintillation counter.

In situ hybridization

For generation of the antisense probe, a 120-bp fragment of murine *ccn2/ctgf* cDNA corresponding to a non-coding region was subcloned into the pGEM-T plasmid (Promega, Madison, WI, USA), and riboprobes were synthesized by using a DIG RNA labelling kit (Roche, Mannheim, Germany) or ³⁵S-UTP(30TBq/nmol, Amersham Biosciences Corp.) following the manufacturer's instructions. Sense probes were used as negative controls. After *in vitro* transcription, the labeling reaction mixture was treated with DNase I, and the probes were precipitated with ethanol, and then dissolved in 0.1% diethyl pyrocarbonate (DEPC)-treated double distilled water containing 0.01% dithiothreitol.

MPL cells cultured on glass slides were washed with PBS and fixed with 4% paraformaldehyde (PFA)- 0.1% sodium-cacodylate at 4°C for 2 hrs. The samples were

Table I: Expression profiles of the phenotypic markers in MPL versus osteoblastic cells

Cells	Basal <i>osf-2/cbfα1</i>		Induction by CCN2/CTGF		
	ALP	<i>opn</i>	<i>ocn</i>	<i>periostin</i>	
MPL	-	+	-	-	+
MC3T3-EI*	+	+	+	+	N.D.

*Nishida *et al.* [24]

washed with PBS, and digestion with 1 µg/ml proteinase K was then performed at room temperature for 10 min. After having been washed with PBS, the samples were treated with 2 mg/ml of glycine-PBS at room temperature for 10 min, and immersed in 0.1 M triethanolamine-HCl (pH 8.0). Thereafter acetic acid-anhydride (0.25% concentration) was added dropwise over a 5-min period, and incubation was then continued for another 15 min. The slides were rinsed with 4 × standard saline-citrate (SSC) for 10 min twice, and immersed in pre-hybridization buffer (3 × SSC and 50% formamide) at 70°C for 30 min for blocking.

Hybridization was performed with a hybridization mixture (50% formamide, 3 × SSC, 10% dextran sulfate, 1 µg/ml of tRNA, 1 µg/ml of sonicated salmon sperm DNA, and 1 µg/ml BSA) containing 0.4 µg/ml riboprobes at 55°C for 16 hrs in a humidified box. The slides were washed 3 times for 20 min each time with 2 × SSC and 50% formamide at 55°C, while being shaken at 90 strokes/min to remove the excess riboprobe; and then they were incubated at 37°C for 30 min with 20 µg/ml of RNase A in NTE buffer [0.5 mM NaCl, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA]. After having been rinsed in NTE buffer at 37°C for 5 min, the slides were washed 3 times for 20 min each time with 0.5 × SSC at 37°C and incubated at room temperature for 30 min in TBS buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl and 150 mM NaCl] containing 1% normal goat serum. Next, the sections were incubated with 1/500-diluted alkaline phosphatase (AP)-conjugated sheep anti-digoxigenin antibody at room temperature for 1 hr. To visualize digoxigenin-labeled riboprobe-tissue mRNA complexes, we incubated the sections in a freshly prepared solution of nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) in an alkaline buffer [100 mM Tris-HCl (pH9.5), 100 mM NaCl and 50 mM MgCl₂] containing 1 mM levamisole in a dark room at room temperature. Finally, the slides were washed in distilled water 3 times and mounted in aqueous mounting material.