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Distribution, gene expression, and functional role of EphA4 during ossification

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ABSTRACT

EphA4 receptor tyrosine kinase has been shown to be critically involved in neural tissue development. Here, we found EphA4 was also distributed among hypertrophic chondrocytes and osteoblasts in the growth plate of developing mouse long bones. *In vitro* evaluation revealed that *ephA4* expression was elevated upon hypertrophic differentiation of chondrocytes and that markedly stronger expression was observed in osteoblastic SaOS-2 than chondrocytic HCS-2/8 cells. Of note, RNAi-mediated silencing of *ephA4* in SaOS-2 cells resulted in the repression of osteocalcin gene expression and alkaline phosphatase activity. Interestingly, confocal laser-scanning microscopic analysis revealed the presence of EphA4 molecules in the nucleus as well as on the surface of SaOS-2 cells. These findings are the first indication of a critical role of EphA4 in ossification, especially at the final stage in which osteoblasts and hypertrophic chondrocytes play major roles.

In mammals, body length is critically determined by the skeletal growth that is conducted by endochondral ossification. Endochondral ossification occurs mainly by the action of growth plate chondrocytes developed in a precisely organized architecture with four-dimensional polarity of cytodifferentiation, in collaboration with vascular endothelial cells and osteoblasts. This complex biological process is under the control of a vast number of systemic hormones and local growth factors or cytokines [1]. However, the ligand–receptor interactions and subsequent signal transduction pathways for all of the growth factors and hormones involved are not fully understood yet. Therefore, we decided to seek the receptor that receives the signal from those signaling molecules and examined a human chondrocytic cell line, HCS-2/8, for the presence of tyrosine kinase-type receptors. As a result, we found that the genes for macrophage colony-stimulating factor receptor (M-CSFR/FMS), ErbB4, and HEK8 were expressed in them (Fig. 1A) [2,3]. Among these three genes, only HEK8 has been left uncharacterized in our hands. However, recent research has been uncovering the biological significance of this molecule, which is currently re-designated as EphA4.

The Eph receptor family constitutes one of the largest groups of transmembrane receptor tyrosine kinases [4,5]. In the vertebrate genome EphA4 is a member of the Eph family that comprises 14 members in mammals, and this family is divided into 2 subclasses, EphA (EphA1–EphA8) and EphB (EphB1–EphB6). They are activated by a second family of cell surface-anchored ligands, the ephrins, that are attached to the plasma membrane via either a glycosylphosphatidylinositol (GPI) anchor (type A) or a transmembrane amino acid sequence (type B). The Eph receptors are also divided into type A or type B according to their ligand-binding specificities. In general, type A receptors bind to type A ephrin ligands, and type B ephrin ligands stimulate type B receptors. These Eph receptors and their ligands have been implicated in playing important roles in a variety of biological activities including axon guidance and migration of neural crest cells in the nervous system, establishment of segmental boundaries, and formation of angiogenic capillary plexuses [6–11]. Among the Eph receptor family members, EphA4 shows a defined spatiotemporal pattern of expression within the developing forebrain, hindbrain, and mesoderm [12,13]. EphA4 mutant mice display a gross motor abnormality in their hindlimbs, and anatomical analysis and anterograde tracing of cortical neurons have demonstrated a severe disruption of the corticospinal tract (CST) in these animals [14]. Thus, EphA4 is expressed in various tissues at the time of morphosis, and it is thought that this receptor participates in the accurate distribution of cell. It is repulsion arising by interaction of EphA4 with ephrin that enables these roles. Ephrin/Eph

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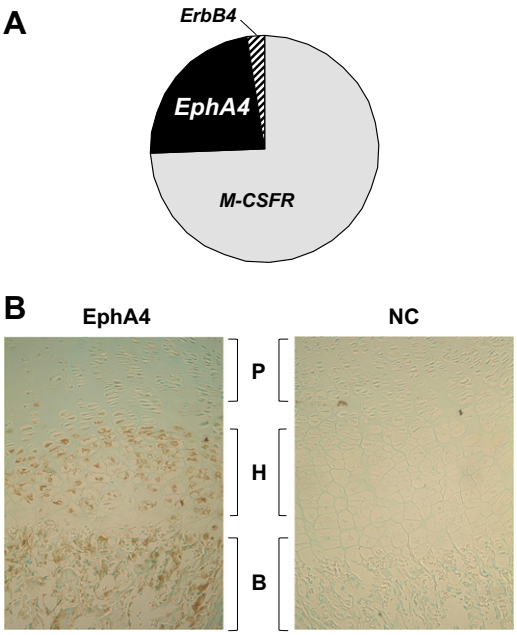

Fig. 1. (A) Receptor tyrosine kinase genes expressed in human chondrocytic HCS-2/8 cells. Among the cDNA clones isolated, 9 out of 39 were those of EphA4. The rest included 29 macrophage colony stimulating factor receptor (M-CSFR) ones and 1 ErbB4 cDNA, as described previously [2]. (B) Distribution of EphA4 in mouse growth plate. Tibial sections from postnatal day 1 mice were probed with an anti-EphA4 antibody (left). Negative control without primary antibody reaction is also shown (right). P, proliferative layer; H, hypertrophic layer; B, cancellous bone.

signaling is thought to control actin dynamics by regulating the balance between Rho family kinases [15,16]. However, no information suggesting a functional role for EphA4 in the ossification process has been presented yet.

Based on these recent findings and the fact that *ephA4* is expressed in chondrocytic cells, we suspected a role for EphA4 in the process of endochondral ossification. Therefore, we precisely analyzed the distribution and gene expression of EphA4 during endochondral ossification *in vivo* and *in vitro*. Furthermore, we examined the functional contribution and possible mechanism of action of EphA4 at particular stages of endochondral ossification as well.

Materials and methods

Materials: Dulbecco's modified Eagle's medium (D-MEM), alpha modification of Eagle's medium (α-MEM), and fetal bovine serum (FBS) were purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan), ICN Biomedicals, Inc. (Aurora, OH), and Cancera International (Rexcalale, Ontario, Canada), respectively. Plastic tissue culture dishes and multiwell plates were obtained from Becton Dickinson (Franklin Lakes, NJ) [15]. A rabbit polyclonal antibody against a peptide mapping near the C-terminus of EphA4 of human origin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used for immunofluorescence microscopy, immunocytochemistry, and immunoblot analysis. Anti-human nucleolin, anti-human actin and rhodamine phalloidin were obtained from Medical & Biological Laboratories (Nagoya, Japan), Sigma (St. Louis, MO) and Molecular Probes (Eugene, OR), respectively. The secondary antibodies used were Alexa Fluor 488- or 568-labeled goat anti-rabbit IgG (Molecular Probes and American Qualex [San Clemente, CA]) and horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Dako; Trappes, France).

Immunohistochemistry: Paraffin sections were prepared and deparaffinized, and endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide in methanol as described previously [17]. After washed in 0.1 M Tris-buffered saline (TBS; pH 7.4), the sections were incubated with 10% normal serum to eliminate nonspecific binding. Thereafter, they were incubated overnight with a 1:200 dilution of the anti-EphA4 antibody at 4 °C. After a wash in TBS with 0.1% Tween 20, the sections were incubated with a peroxidase-conjugated anti-rabbit IgG as a secondary antibody (P1-1000, Dako) for 60 min at room temperature and washed in TBS. Finally, color development was performed by using 3,3-diaminobenzidine tetrachloride (Dojindo; Tokyo, Japan). The sections were also counterstained with methyl green and mounted. Controls samples were processed with the omission of the primary antibody. The Animal Committee of Okayama University Dental School approved all of the procedures used in the present study.

Cell culture: Mouse growth cartilage (MGC) cells were isolated from cartilage in the ribs of an E18.5 mouse by digestion with 0.1% collagenase A (Roche; Mannheim, Germany) and cultured in α-MEM containing 10% FBS. The cells were collected at various times after the cultures had become confluent, and chondrogenic differentiation was monitored. HeLa (a human cervical cancer cell line), SaOS-2 (a human osteoblastic cell line), MDA-MB-231 (MDA-231, a human breast cancer cell line) and HCS-2/8 (a human chondrocytic cell line) [18–20] were cultured in DMEM containing 10% FBS. The cells were cultured at 37 °C in humidified air with 5% CO_2.

Immunofluorescence staining: SaOS-2 cells were cultured on 4- or 8-well glass coverslips, fixed in 4% paraformaldehyde (w/v) in phosphate buffer (PB) for 15 min, and if necessary, permeabilized with 0.2% Triton X-100 for 5–15 min. For the primary reaction, anti-EphA4 (1:50) or anti-nucleolin (1:200) antibody was used. Alexa Fluor-labeled secondary antibodies were thereafter applied to the cells at a dilution of 1:800. In several experiments, the cells were further stained with rhodamine phalloidin in TBS [21] in order to visualize actin fibers.

RNA extraction and real-time PCR analysis: Total RNA was isolated from the cells by using the commercially available reagent ISOGEN (Nippongene, Tokyo, Japan). Polyadenylated RNAs were selectively reverse-transcribed from 500 ng of each total RNA by using avian myeloblastosis virus (AMV) reverse transcriptase (TAKARA). Relative cDNA levels of *ephA4*, *osteocalcin*, *ccn2,* and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) were evaluated by real-time PCR with a LightCycler system (Roche), in combination with SYBR Green Realtime PCR Master Mix (TOYOBO, Tokyo, Japan). The nucleotide sequences of the primers used in the quantification of *ephA4, osteocalcin, ccn2* and *gapdh* were as follow: *ephA4 (mouse)* sense, 5′-GAGGAGCAGCAGAATGGTGAATGCC-3′; *ephA4 (mouse)* anti-sense, 5′-AATTCTCGAACTGCCTGGTTGG-3′; *ephA4 (human)* sense, 5′-AAGATTCCTATCCGGTGGAC-3′; *ephA4 (human)* anti-sense, 5′-ACATCACTTCCCACATAACG-3′; *osteocalcin (human)* sense, 5′-CCCAGGGGCTACCTATCA-3′; *osteocalcin (human)* anti-sense, 5′-CTCCTGAAAGCCGATGTGGTC-3′; *ccn2 (mouse)* sense, 5′-CCACCCGAGTTACCAGTGAC-3′; *ccn2 (mouse)* anti-sense, 5′-GTGCAGCCAGAAAGCTCA-3′; *gapdh (human, mouse)* sense, 5′-TCACCATCTTCCAGGAGCGA-3′; *gapdh (human, mouse)* anti-sense, 5′-CACAAT GCCGAAGTGGTCGT-3′. The absence of nonspecific PCR products was checked by melting curve and agarose electrophoresis analyses. The relative copy numbers were computed based on data obtained with a serial dilution of a representative sample for each target gene [21].

Western blotting analysis: Western blotting was performed as essentially described previously [22]. Briefly, total cellular protein was prepared by lysing cells in a RIPA buffer [22] at various time points. Twenty-microgram protein samples were separated by

SDS–PAGE, and transferred to a polyvinylidene difluoride (PVDF) membrane. After having been blocked with 1% BSA/TBS, each membrane was incubated for 24 h at 4 °C with the primary antibody against EphA4 or actin. The membranes were then incubated with HRP-labeled anti-rabbit IgG (DAKO). The signals were detected by using an enhanced chemiluminescence method (ECL Western Blotting Detection System; Amersham Biosciences, Piscataway, NJ).

Gene silencing by siRNAs: Two siRNAs specific for human EphA4 were predicted and synthesized by iGENE (Sapporo, Japan). One of them (siR-14) targeting *EphA4* was an oligoduplex of 5′-GGGUAA UCCUGGAAUAUGAAGUCAA-3′ (sense) and 5'-CCCAUUAGGACCUU AUACUUCAGUU-3′ (anti-sense). The other, siR-17, was composed of 5′-ACAGUAAAGCCAAACAAGAAGCGGA-3′ (sense) and 5′-UGU-CAUUUCGGUUUGUUCUUCGCCU-3′ (anti-sense). The control was an oligoduplex of a scrambled nucleotide sequence. For gene knock-down studies, cells were seeded in 6-well plates and then cultured for 24 h. Cells were transfected with the siRNA (50 nM) with the aid of 10 μl of siPORT™ *NeoFX*™ (Ambion Austin, TX), and further cultured for 24 h. RNA was isolated and was forwarded for quantitative analysis, as described [21].

Alkaline phosphatase assay: For the measurement of alkaline phosphatase activity (ALPase) in SaOS-2 cells, the cells were rinsed with phosphate-buffered saline (PBS) and then homogenized in 0.5 M Tris–HCl (pH 9.0) containing 0.9% NaCl and 1% Triton X-100 on ice; and thereafter the homogenate was centrifuged at 12,000*g* for 15 min. ALPase activity in the resultant supernatant was determined by the method of Bessey et al. [23] with a few modifications described previously [24].

Results

Distribution of EphA4 in mouse growth plate

In a previous study, we surveyed receptor tyrosine kinase genes that were expressed in chondrocytic HCS-2/8 cells and found EphA4 as one such gene (Fig. 1A). Therefore, we initiated the present study by analyzing the production and distribution of EphA4 molecules in the growth plate cartilage *in vivo* (Fig. 1B). Immunohistochemical analysis revealed that EphA4 was present specifically in the hypertrophic layers of the growth plate cartilage, whereas no prominent signals were detected in the resting and proliferative layers. Furthermore, strong immunoreactivity was observed in osteoblasts as well as osteoclasts in the cancellous bone adjacent to the growth plate. These findings suggest the regulated expression of EphA4 along with chondrocytic differentiation and a specific role for EphA4 at a late stage of endochondral ossification executed by hypertrophic chondrocytes and osteoblasts.

Gene expression profile of EphA4 during endochondral ossification in vitro

In order to confirm that *ephA4* was expressed at a late stage of chondrocyte differentiation, we isolated MGC cells from normal mice and maintained them under differentiation-inducing conditions in long-term cultures (Fig. 2A). Immediately after the cells had reached confluence, *ephA4* expression was quite low. Such low-level expression of *ephA4* was also observed at 25 days after the cells reached the initial confluent stage. However, by 31 days, *ephA4* expression had drastically increased, which occurred after the peak expression of *ccn2*, an early hypertropic marker gene expression. These results *in vitro* are consistent with the findings of *in vivo* analysis (Fig. 1B). Next, to evaluate the gene expression and production of EphA4 in osteoblasts, we used a human osteoblastic cell line, SaOS-2. Comparative real-time RT-PCR and Western blotting analyses with chondrocytic HCS-2/8 cells revealed

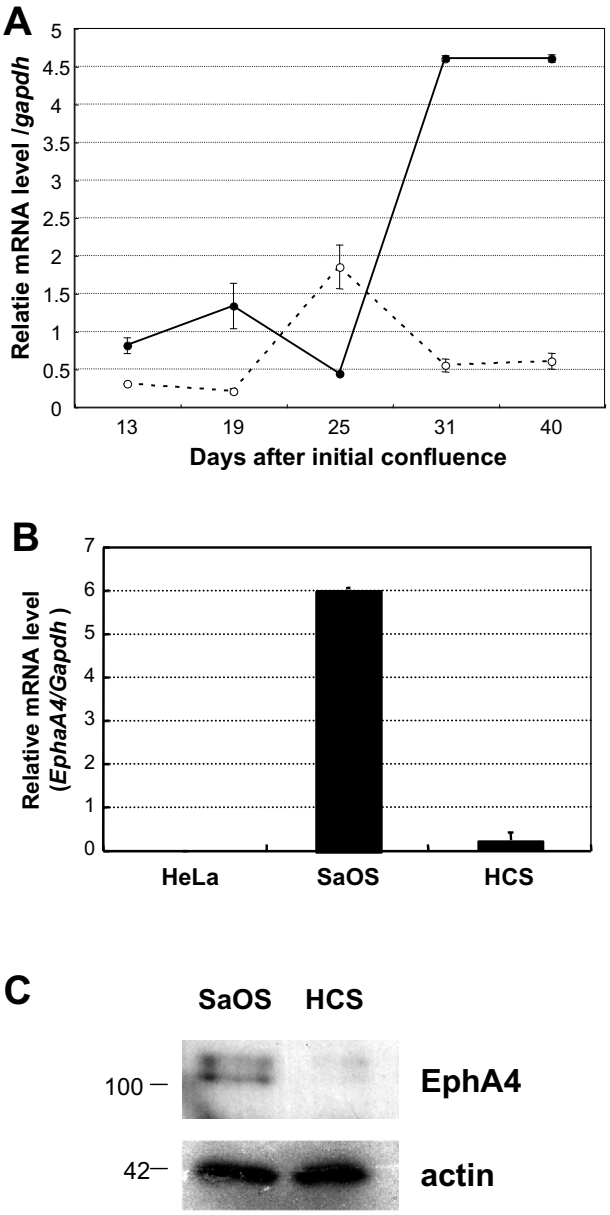

Fig. 2. (A) Gene expression of *ephA4* during *in vitro* differentiation of MGC cells. RNAs were sampled and quantitatively analyzed by real-time RT-PCR at the indicated time points after the cells had reached initial confluence. Relative gene expression levels of *ephA4* (solid circles) and *ccn2* (open circles) were computed by normalizing against *gapdh* gene expression levels. (B) Gene expression of EphA4 in osteogenic cell lines. RNAs from HeLa (control), chondrocytic HCS-2/8, and osteoblastic SaOS-2 cells were quantitatively evaluated for the expression of EphA4. (C) Production of EphA4 protein by HCS-2/8 and SaOS-2 cells as evaluated by Western blotting. The same membrane was reprobed with anti-actin antibody to serve as a control.

remarkably higher mRNA expression and protein production in the SaOS-2 cells (Fig. 2B and C). Collectively, the osteogenic cell-specific expression of EphA4 shown above suggests the involvement of EphA4 molecules in the ossification process that follows the endochondral growth of long bones.

Effects of RNAi-mediated knockdown of EphA4 on osteoblastic phenotype

Since elevated expression of *ephA4* was observed in osteoblastic SaOS-2 cells, the functional requirement of EphA4 in maintaining the osteoblastic phenotype was evaluated by using a

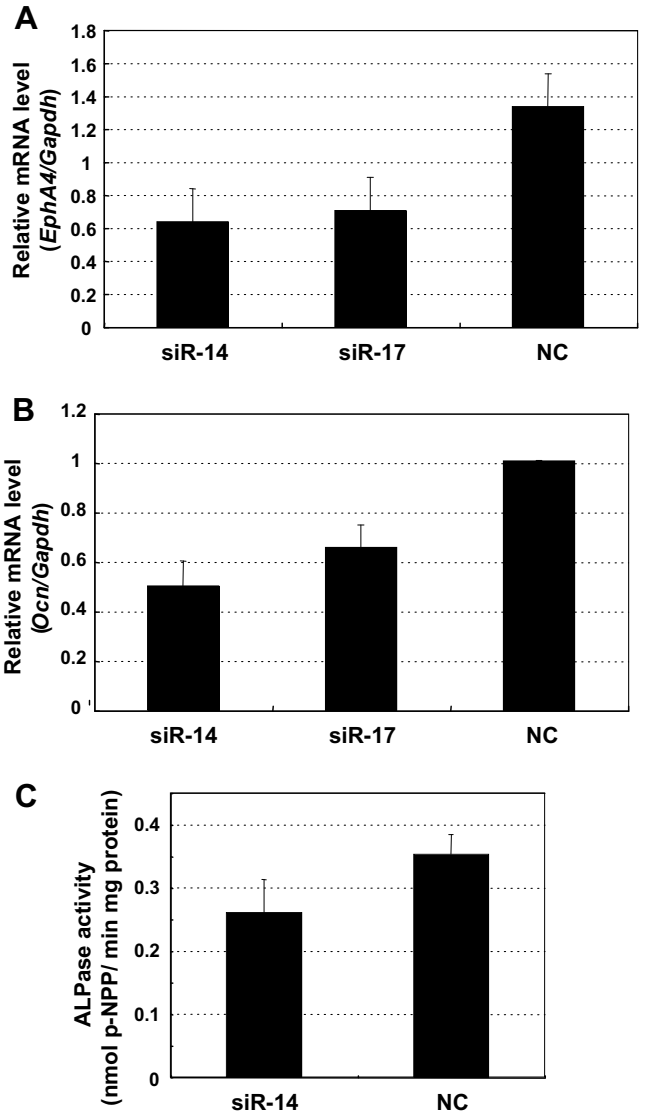

Fig. 3. Effects of EphA4 knock-down in osteoblastic cells by siRNAs. (A) EphA4 gene silencing in SaOS-2 cells by two different siRNAs, siR-14 and siR-17. SaOS-2 cells were transfected with the respective siRNA or control siRNA (scrambled nucleotide sequence), and total RNA was quantitatively analyzed by real-time RT-PCR. Relative mRNA levels were computed by standardizing the values against those of *gapdh*. (B) Effect of EphA4 gene silencing on the steady-state osteocalcin mRNA level in SaOS-2 cells. (C) Effect of EphA4 gene silencing on ALPase activity displayed by SaOS-2 cells. SaOS-2 cells were transfected with siR-14, and ALPase activity was measured. Columns and error bars indicate averages and standard deviations, respectively, of at least two independent evaluations.

set of siRNAs targeted to EphA4 (Fig. 3). The introduction of either of 2 synthetic siRNA duplexes caused a significant reduction in the EphA4 mRNA level. Particularly, siR-14 decreased the EphA4 mRNA level down to less than 50% of the control (Fig. 3A). Under these conditions, the mRNA level of an osteoblastic marker gene was evaluated. Real time RT-PCR analysis of the cDNAs reverse-transcribed from the RNA of those cells revealed that the expression of the osteocalcin gene was diminished in proportion to the silencing potential of the respective siRNAs (Fig. 3B). Subsequently, as another marker representing mature osteoblastic phenotype, alkaline phosphatase (ALPase) activity was examined under the same conditions. As a result, SaOS-2 cells with their EphA4 knocked down by siR-14 displayed less ALPase activity (Fig. 3C); whereas the effect of the less-efficient siR-17 was not significant (data not shown). These results indicate that EphA4 was functionally required in SaOS-2 cells in displaying the osteoblastic phenotype in full.

Intracellular localization of EphA4 in SaOS-2 cells

Eph A4 is known to transmit signals into cells to induce cellular repulsion in neural tissues, which is called "forward signaling." Nevertheless, the role of EphA4 observed in osteoblastic cells appears quite different from the outcome of the classical forward signaling in neural cells, indicating a different function of EphA4 through a different intracellular behavior in osteoblasts. To gain insight into this point, we precisely analyzed the intracellular localization of EphA4 in SaOS-2 cells by using laser confocal microscopy (LCM). Immunofluorescence analysis with LCM showed EphA4 molecules on the plasma membrane, in the cytoplasm, and even in the nucleus (Fig. 4A). The nuclear EphA4 was found to have accumulated in particular areas in the nucleus; however, double staining for nucleolin (a marker of nucleoli) and EphA4 indicated that the area where EphA4 accumulated was distinct from the nucleoli. Thus, it was shown that EphA4 or its subfragment was translocated into the nucleus in osteoblastic cells.

Discussion

In this study, EphA4 was re-discovered in hypertrophic chondrocytes and osteoblasts that execute the final process of endochondral bone formation *in vivo* and *in vitro*. Importantly, the functional contribution of EphA4 in maintaining the mature phenotype of osteoblastic cells was for the first time indicated herein. Since this novel function of EphA4 in osteoblasts is quite different from the reported function through the forward signaling pathway mediated by this molecule, an alternative pathway of EphA4 signaling is suggested, as represented by the nuclear translocation of EphA4 in osteoblastic cells.

Recently, the involvement of the Eph–ephrin interaction in bone tissue has been uncovered and is now attracting the interest of scientists involved in bone research. Indeed, it is now clear that EphB4 and ephrin B2 play a central role in bone remodeling as coupling factors to mediate osteoblast-osteoclast interactions [25], which is reminiscent of the classical receptor activator of NF-κB (RANK)—RANK ligand (RANKL) interaction between these cells. Nevertheless, no information has been available concerning the role of other Eph/ephrin family members in bone formation/remodeling, save for a report describing the expression of EphA4 in osteoclasts [25]. In this context, our present study has uncovered another critical aspect of the function of an Eph receptor tyrosine kinase, further emphasizing the involvement of various Eph/ephrin family members in bone formation and remodeling.

In contrast to the function in mesenchymal tissues, the function of EphA4 in neural tissue has been profoundly investigated; and its intracellular signaling pathway has been uncovered. It is widely known that EphA4 forward signaling is critical to the control of neuronal circuit formation. According to a recent study, this forward signal emitted by the binding of ephrin B3 to EphA4 is transmitted through α-chimerin Rac GTPase-activating protein to inactivate Rac, a Rho family member that promotes neuronal outgrowth in motor circuit formation. Indeed, knockout mice of ephrin B3, EphA4 or α-chimerin commonly display characteristic phenotypes, including a rabbit-like hopping gait and impairment of motor circuits [16]. It remains to be clarified how EphA4 functions in promoting or maintaining the osteoblast phenotype in SaOS-2 cells. Also, since Eph receptors have been indicated to interact with a number of different ligands, specific ligands involved in the EphA4 function in osteoblasts need to be identified.

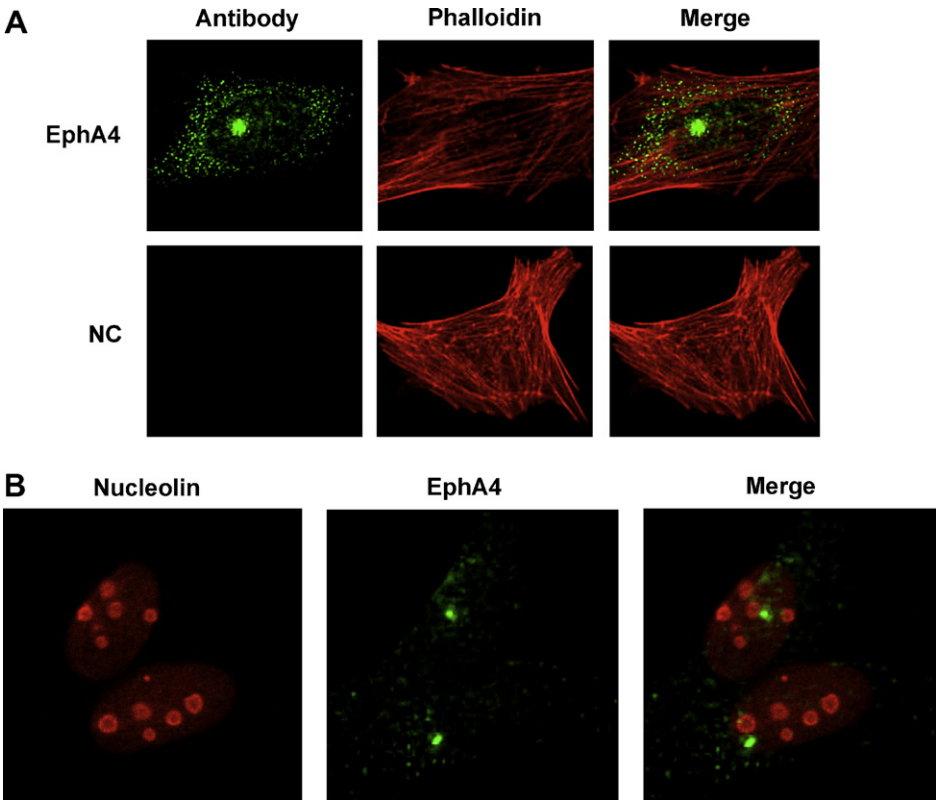

Fig. 4. Subcellular localization of EphA4 in SaOS-2 cells as evaluated by LCM. (A) Double staining for EphA4 (green) and actin (red). (B) Double staining for nucleolin (red) and EphA4 (green). Note that nuclear EphA4 has accumulated as a single large spot distinct from the nucleoli. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

Recent research has revealed the nuclear function of receptor tyrosine kinases. A typical example is ErbB4. Upon the interaction with a ligand, ErbB4 not only transmits phosphorylation signal to STAT5, but also undergoes intramembranous processing by presenilin-dependent γ-secretase. Thereafter, the cytoplasmic fragment of ErbB4 migrates into the nucleus where it regulates the transcription of target genes [26]. By immunofluorescence analysis by LCM, we found EphA4 to be distinctly present in the nuclei of osteoblastic cells. Therefore, in analogy to ErbB4, an alternative function other than the classical signal transduction observed in the neuronal cells is also suspected for EphA4. Investigation on the molecular behavior of EphA4 and its associated signal transduction pathways is currently in progress in order to clarify the hypothetical role of EphA4 in the nucleus.

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