学位論文

RUNX2 expression in developing human bones and various bone tumors

（ヒト胎児骨および骨腫瘍における RUNX2 の発現）

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Abstract

Heterozygous germline mutation of *RUNX2* (*runt-related protein 2*) causes cleidocranial dysplasia. To clarify the involvement of RUNX2 in human osteogenesis, fetal bones and various bone tumors were immunohistochemically examined. During both membranous and endochondral ossification in fetus (n=8), RUNX2 was expressed not only in osteoblastic cells but also in surrounding mesenchymal cells, and at an early stage of chondrocytes. Such an expression pattern was recapitulated in bone tumors: RUNX2 was unequivocally expressed in osteosarcoma (n=20) and fibrous dysplasia (n=10), regardless of the site of occurrence, cell morphology or amount of neoplastic osteoid. RUNX2 expression was limited to less differentiated cells in chondrogenic tumors (n=20). We further analyzed whether RUNX2 expression was regulated by bone morphogenetic protein-2 (BMP-2), which is critical for osteoblastic differentiation. By real-time PCR, *RUNX2* mRNA level was correlated with *BMP-2* mRNA level, and both levels were significantly higher in 3 osteosarcoma cell lines than in 3 chondrosarcoma cell lines. By treatment with recombinant BMP-2, *RUNX2* mRNA level was significantly altered in these cell lines. RUNX2 expression is constitutive in developing and neoplastic human osteogenesis, and is most likely to be regulated by BMP-2.

Key words: RUNX2, fetal bone, osteosarcoma, osteoblastic differentiation, BMP-2

Introduction

The skeletal tissue is composed of different mesenchymal cells, including osteoblasts, chondrocytes, muscle cells and adipocytes. These cell lineages are considered to differentiate from common mesenchymal progenitor cells, under the control of different transcription factors that are specific to differentiation into each lineage. RUNX2 (runt-related protein 2), also called Cbfa1 (core binding factor alpha1), is a transcriptional factor that belongs to the RUNX family, and is a critical factor in osteoblastic differentiation in rodents[1)]. Mice with a homozygous mutation in *Runx2* showed a complete lack of ossification, including both membranous and endochondral ossification[2)]. In humans, heterozygous germline mutation of *RUNX2* causes cleidocranial dysplasia, which is characterized by hypoplasia/aplasia of clavicles and patent fontanelles[3),4)]. Considering the fact that both clavicles and cranial bones develop through membranous ossification, RUNX2 is at least necessary for membranous ossification in humans. However, it is unclear whether RUNX2 is also responsible for endochondral ossification in humans. RUNX2 expression in various bone tumors with or without osteoblastic differentiation is not yet systematically analyzed.

RUNX2 is regulated by several molecules. Previous studies showed that bone morphogenetic protein-2 (BMP-2), which is responsible for both osteoblastic and chondroblastic differentiation[5),6)], regulated RUNX2 expression or function in mouse pluripotent mesenchymal cells[7),8)]. To our knowledge, there are no data on the

relationship between BMP-2 and RUNX2 in human osteogenic or chondrogenic cells.

In the present study, we examined human fetal bones and various bone tumors for RUNX2 expression. Regulation of RUNX2 by BMP-2 was also analyzed using human osteosarcoma and chondrosarcoma cell lines.

Materials and methods

Fetal bones

Eight fetal tissues were obtained from spontaneous abortion or stillbirth. Numbers in terms of estimated gestational week were as follows: 6 weeks (n=1), 9 weeks (n=1), 10 weeks (n=1), 11 weeks (n=1), 12 weeks (n=2), 22 weeks (n=1) and 36 weeks (n=1). Among the 8 fetal tissues, seven contained limb and vertebral bones, and two (10-week and 11-week gestation) contained craniofacial bones (Table 1). Use of fetal bones was approved by the Ethics Committee of Yamagata University School of Medicine. All tissues were fixed in 10% formalin, embedded in paraffin, and sectioned without decalcification.

Tumor specimens

Tumor specimens are summarized in Table 2. Bone tumors included osteosarcoma (n=20), fibrous dysplasia (n=10), chondrosarcoma (n=10) and enchondroma (n=10), all of which were obtained from biopsy. Cell morphology of osteosarcoma was either

osteoblastic or fibroblastic, or both. Histological grade of chondrosarcoma was grade 1 (n=4) or grade 2 (n=6). Sarcomatoid renal cell carcinoma (n=5) and leiomyosarcoma of uterus (n=7) were also examined, because they should be differentiated from fibroblastic-type osteosarcoma when they metastasize to the bones. All of the specimens were fixed in 10% formalin, embedded in paraffin, and sectioned without decalcification.

Cells and culture

Three human osteosarcoma (OS) cell lines, designated as NOS-1, NOS-2 and HuO9, and three human chondrosarcoma (CS) cell lines, designated as CRL-7891, HTB-94 and OUMS-27, were used. The NOS-1 and NOS-2 were established by one of the authors (T. M.), and the HuO9 was obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan). CRL-7891 (designation: Hs 819.T) and HTB-94 (designation: SW1353) were purchased from American Type Culture Collection (Manassas, VA). OUMS-27 was kindly donated by Dr. M. Namba (Institute of Molecular and Cellular Biology, Okayama University Medical School). When the cell lines are heterotransplanted into nude mice, NOS-1 and HuO9 form bone and osteoid, NOS-2 forms osteoid and cartilage[9)-11)], and HTB-94 and OUMS-27 form cartilage[12),13)]. The OS lines were cultured in α-MEM (Cosmo Bio, Tokyo, Japan) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria), and the CS lines were cultured in

RPMI-1640 (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal bovine serum. All cell lines were incubated in a humidified 5% CO_2 atmosphere at 37 °C.

Immunohisto/cytochemistry for RUNX2

Immunohistochemistry and immunocytochemistry were performed on paraffin sections and chamber slides, respectively. Four-micrometer-thick paraffin sections of archival specimens were deparaffinized. The confluent cells on chamber slides were washed with phosphate-buffered saline, and then fixed with 80% ethanol at 4°C for 30 minutes. For antigen retrieval, the paraffin sections and chamber slides were pretreated with autoclave heating (paraffin sections) at 121°C for 15 minutes and microwave heating (chamber slides) at 95°C for 20 minutes in sodium citrate buffer (10 mM sodium-citrate monohydrate, pH 6.0). Endogenous peroxidase was inactivated with 0.3% hydrogen peroxide for 30 minutes, and the slides were exposed to 3% skim milk in phosphate-buffered saline for 20 minutes to block nonspecific binding of the antibody. Then, the slides were incubated with rabbit anti-RUNX2 polyclonal antibody (M70; dilution 1/50; Santa Cruz Biotechnology, CA, USA) for 2 hours at room temperature. They were visualized using UltraTech HRP Streptavidin-Biotin Detection System (Beckman-Coulter, Marseille, France).

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from each cell line using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse-transcribed using mRNA Selective PCR Kit Ver.1.1 for first-strand cDNA synthesis (TaKaRa, Otsu, Japan). All real-time PCR reactions contained the first-strand cDNA, Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and each primer mix. Real-time PCR primers are summarized in Table 3. The SYBR Green-based quantitative real-time PCR analysis was carried out using the Applied Biosystem 7500 Real-Time PCR System (Applied Biosystems). The cycling program of RUNX2, BMP-2 and GAPDH was as follows: 95 °C × 10 minutes for one cycle, 40 cycles at 92 °C × 20 seconds, 57 °C × 30 seconds and 72 °C × 40 seconds, followed by a plate read at 72 °C for each cycle. All samples were normalized by the expression level of GAPDH. Triplicate reactions were carried out for each sample.

Treatment with recombinant human BMP-2

1×10^5 cells of each cell line were inoculated in 35-mm tissue culture dishes. When they reached at 80% confluent state, they were incubated for 72 hours with medium containing 500 ng/ml recombinant BMP-2 (R&D Systems, Inc., Minneapolis, USA). As a control, culture without recombinant BMP-2 was used. After 72 hours, total RNA was obtained from each dish, and *RUNX2* mRNA level was evaluated by quantitative real-time PCR.

Statistical analysis

The data values are shown as the average of experiments (means ± SD). Significance of differences was analyzed using Student's t-test. A value of $p < 0.05$ was regarded as statistically significant.

Results

RUNX2 expression in human fetal bones

In the formation of craniofacial bones, bone matrix was accompanied by osteoblasts and osteocytes, but not accompanied by cartilage. Distinct nuclear expression of RUNX2 was observed in osteoblasts, osteocytes, and mesenchymal cells of primitive appearance that aggregated around the bone matrix (Fig. 1a,b).

At the earlier stage (6～11 weeks) of osteogenesis of limb or vertebral bones, cartilage was first formed, followed by osteoblastic lining, then bone collar was formed between cartilage and osteoblastic layer. Distinct nuclear expression of RUNX2 was observed not only in osteoblastic cells but also in mesenchymal cells that surrounded the osteoblastic cells. Some nuclei of chondrocytes were also positive for RUNX2 (Fig. 1c). At the later stage of osteogenesis, the cartilage was invaded by blood vessels, then endochondral ossification occurred. RUNX2 was still expressed by osteoblasts, but not expressed by hypertrophic chondrocytes (Fig. 1d).

RUNX2 expression in tumor specimens

RUNX2 expression in tumor specimens is summarized in Table 4. In each tumor type (i.e., osteosarcoma, fibrous dysplasia, chondrosarcoma and enchondroma), all cases were positive for RUNX2. In osteosarcomas, almost all tumor cells showed nuclear positivity for RUNX2, regardless of the site of occurrence (limb/pelvic bones or jawbones), the amount of osteoid (rich or poor) or the cell morphology (osteoblastic or fibroblastic). In fibrous dysplasia, not only osteoblastic cells rimming the woven bones but also fibroblastic cells were positive for RUNX2 in their nuclei, regardless of the amount of woven bones or site of occurrence. On the other hand, chondrosarcoma and enchondroma showed heterogeneity in RUNX2 expression: nuclear RUNX2 staining was found in tumor cells that were small in size and lacked lacunar formation, whereas it was rarely found in tumor cells that were hypertrophic or associated with lacunae. Neither sarcomatoid renal cell carcinomas nor uterine leiomyosarcomas showed RUNX2 expression (Fig. 2).

RUNX2 and BMP-2 expression in cell lines

Three OS lines, NOS-1, HuO9 and NOS-2, showed distinct nuclear immunostaining for RUNX2. Among 3 CS lines, CRL-7891 and OUMS-27 were scarcely immunostained with RUNX2, and HTB-94 showed weak immunostaining (Fig. 3a).

Such *in situ* expressions were almost concordant with the *RUNX2* mRNA level, which was significantly higher in OS lines than in CS lines ($p<0.0001$) (Fig. 3b). *BMP-2* mRNA level was also significantly higher in OS lines than in CS lines ($p<0.0001$) (Fig. 3c).

Effect of BMP-2 on RUNX2 expression

After treatment with recombinant BMP-2, *RUNX2* mRNA level significantly decreased in 3 OS lines, whereas it increased in 3 CS lines ($p<0.0001$) (Fig. 4).

Discussion

The present analysis using human fetus showed that RUNX2 was expressed during both membranous and endochondral ossification at all gestational stages examined. In either mode of ossification, RUNX2 was expressed not only in osteoblastic cells but also in mesenchymal cells that surrounded the osteoblastic cells. Such an expression pattern was concordant with the *in situ* expression pattern of *RUNX2* mRNA observed in membranous ossification[14]. Mesenchymal cells that aggregate around the osteoblasts are considered to be precursors of osteoblasts, including so-called preosteoblasts[15], although they have not been specified morphologically. The present finding is compatible with an experimental finding that *Runx2* knockout mice did not even show aggregation of mesenchymal cells[16),17]. As for RUNX2 expression in fetal

cartilage, there was a tendency that RUNX2 immunostaining was diminished as the chondrocytes became more hypertrophic. Any products associated with chondrocyte maturation might possibly act as repressors of RUNX2.

RUNX2 expression in various bone tumors were fairly analogous to that observed in fetal skeleton. In fibrous dysplasia, RUNX2 was strongly expressed not only in osteoblasts rimming the woven bones but also in fibroblastic cells, which is concordant with a previous finding on RUNX2 expression in fibrous dysplasia of jawbones[18]. For a long time, it has been presumed that fibroblastic cells of fibrous dysplasia share phenotypic features with osteoprogenitor cells. A previous study showed that fibroblastic cells were immunoreactive for osteonectin[19]. We consider that the RUNX2 immunohistochemistry gives stronger support for this presumption, since RUNX2 is a more fundamental molecule in osteogenic differentiation[20]. Regarding the RUNX2 expression in osteosarcoma, previous studies showed RUNX2 overexpression by immunohistochemistry or quantification of mRNA[21),22]. However, they did not refer the site of occurrence or the amount of neoplastic osteoid. In the present study, we showed that RUNX2 was constitutively expressed in osteosarcomas, regardless of the affected site (limb/pelvic bones or jawbones). In every case, almost all tumor cells were distinctly positive for RUNX2, even if they showed fibroblastic morphology and there was little osteoid. On the contrary, sarcomatoid renal cell carcinomas or uterine leiomyosarcomas, bone metastasis of which is sometimes difficult

to distinguish from osteosarcomas[23], were completely negative for RUNX2. RUNX2 is likely to be helpful to differentiate osteosarcoma from these metastatic tumors. Further study in a larger series is necessary to examine whether RUNX2 could be a marker of practical use.

RUNX2 is transcriptionally regulated by various molecules, including fibroblast growth factors, TGF-β and BMPs[17]. BMP-2 is the most potent osteogenic agents, which strongly promotes the differentiation of pluripotent mesenchymal cells into osteoblastic cells[5], and is also involved in chondrogenic differentiation[6]. Previous studies using pluripotent mesenchymal cell lines showed that *RUNX2* mRNA was induced by BMP-2[7], or that RUNX2 activity was reduced by blocking BMP-2[8]. As far as we are aware, there are no data on the relationship between BMP-2 and RUNX2 expressions in human osteogenic or chondrogenic cells. In the present study, we examined 3 osteosarcoma (OS) cell lines and 3 chondrosarcoma (CS) cell lines in terms of the relationship between BMP-2 and RUNX2. Semi-quantification of intrinsic mRNA showed that *RUNX2* mRNA level was correlated with *BMP-2* mRNA level, suggesting that intrinsic BMP-2 positively regulates RUNX2 expression in human osteogenic and chondrogenic cells. To verify this speculation, we treated the 6 cell lines with recombinant BMP-2 and analyzed the *RUNX2* mRNA level. In both OS and CS lines, significant alteration in *RUNX2* mRNA level was found after treatment with BMP-2. The pattern of alteration, however, was different between OS lines and CS

lines: *RUNX2* mRNA level significantly decreased in OS lines, whereas it increased in CS lines. This indicates that RUNX2 expression is positively regulated by BMP-2 in some conditions, but negatively regulated in other conditions, in human osteogenic or chondrogenic cells. It is known that BMP signaling is transduced by the Smad family, such as Smad1, Smad4 and Smad5. They form a complex, move into the nucleus and activate target genes[24]. It is interesting that the activated genes include *Smad6* or *noggin*, the products of which inhibit BMP signaling and thereby preclude cells from exhibiting too much BMP signaling[25),26)]. In the present study, such a negative feedback loop might have come into play in the 3 OS lines because they already had a significant amount of intrinsic BMP-2. Systemic studies on the BMP-2 signaling pathway are needed to clarify how RUNX2 expression is regulated by BMP-2.

In summary, RUNX2 is expressed from the early to late stage of osteoblastic differentiation during both membranous and endochondral osteogenesis. RUNX2 is also constitutively expressed in osteogenic tumors, regardless of the site of occurrence, cell morphology or amount of osteoid. At the mRNA level, RUNX2 expression is most likely to be regulated by BMP-2 in both osteogenic and chondrogenic cells.

References

1. Banerjee C, McCabe LR, Choi JY, Hiebert SW, Stein JL, Stein GS et al. : Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1 is a major component of a bone-specific complex. J Cell Biochem 1997 ; 66 : 1-8

2. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K et al. : Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. Cell 1997 ; 89 : 755-764

3. Mundlos S, Otto F, Mundlos C, Mulliken JB, Aylsworth AS, Albright S et al. : Mutations involving the transcription factor CBFA1 cause cleidocranial dysplasia. Cell 1997 ; 89 : 773-779

4. Mundlos S : Cleidocranial dysplasia: clinical and molecular genetics. J Med Genet 1999 ; 36 : 177–182

5. Cheng H, Jiang W, Phillips FM, Haydon RC, Peng Y, Zhou L et al. : Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). J Bone Joint Surg Am 2003 ; 85-A : 1544-1552

6. Goldring MB, Tsuchimochi K, Ijiri K : The control of chondrogenesis. J Cell Biochem 2006 ; 97 : 33-44

7. Lee KS, Kim HJ, Li QL, Chi XZ, Ueta C, Komori T et al. : Runx2 is a common target of transforming growth factor beta1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the

pluripotent mesenchymal precursor cell line C2C12. Mol Cell Biol 2000 ; 20 : 8783-8792

8. Phimphilai M, Zhao Z, Boules H, Roca H, Franceschi RT : BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. J Bone Miner Res 2006 ; 21 : 637-646

9. Hotta T, Motoyama T, Watanabe H : Three human osteosarcoma cell lines exhibiting different phenotypic expressions. Acta Pathol Jpn 1992 ; 42 : 595-603

10. Kawai A : A newly established human osteosarcoma cell line with osteoblastic properties. Clin Orthop Relat Res 1990 ; 259 : 256-267

11. Ogose A, Motoyama T, Hotta T, Watanabe H, Takahashi HE : Bone formation in vitro and in nude mice by human osteosarcoma cells. Virchows Arch 1995 ; 426 : 117-125

12. Kunisada T, Miyazaki M, Mihara K, Gao C, Kawai A, Inoue H et al. : A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. Int J Cancer 1998 ; 77 : 854-859

13. Klenke FM, Abdollahi A, Bertl E, Gebhard MM, Ewerbeck V, Huber PE et al. : Tyrosine kinase inhibitor SU6668 represses chondrosarcoma growth via antiangiogenesis in vivo. BMC Cancer 2007 ; 7 : 49

14. Bronckers AL, Sasaguri K, Engelse MA : Transcription and immunolocalization of Runx2/Cbfa1/Pebp2alphaA in developing rodent and human craniofacial tissues: further

evidence suggesting osteoclasts phagocytose osteocytes. Microsc Res Tech 2003 ; 61 : 540-548

15. Dunlop LL, Hall BK : Relationships between cellular condensation, preosteoblast formation and epithelial-mesenchymal interactions in initiation of osteogenesis. Int J Dev Biol 1995 ; 39 : 357-371

16. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR et al. : The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell 2002 ; 108 : 17-29

17. Komori T : Regulation of skeletal development by the Runx family of transcription factors. J Cell Biochem 2005 ; 95 : 445-453

18. Toyosawa S, Yuki M, Kishino M, Ogawa Y, Ueda T, Murakami S et al. : Ossifying fibroma vs fibrous dysplasia of the jaw: molecular and immunological characterization. Mod Pathol 2007 ; 20 : 389-396

19. Sakamoto A, Oda Y, Iwamoto Y, Tsuneyoshi M : A comparative study of fibrous dysplasia and osteofibrous dysplasia with regard to expressions of c-fos and c-jun products and bone matrix proteins: a clinicopathologic review and immunohistochemical study of c-fos, c-jun, type I collagen, osteonectin, osteopontin, and osteocalcin. Hum Pathol 1999 ; 30 : 1418-1426

20. Cohen MM Jr : The new bone biology: pathologic, molecular, and clinical correlates. Am J Med Genet A 2006 ; 140 : 2646-2706

21. Andela VB, Siddiqui F, Groman A, Rosier RN : An immunohistochemical analysis to evaluate an inverse correlation between Runx2/Cbfa1 and NF kappa B in human osteosarcoma. J Clin Pathol 2005 ; 58 : 328-330

22. Sadikovic B, Thorner P, Chilton-Macneill S, Martin JW, Cervigne NK, Squire J et al. : Expression analysis of genes associated with human osteosarcoma tumors shows correlation of RUNX2 over expression with poor response to chemotherapy. BMC Cancer 2010 ; 10 : 202

23. Unni KK, Inwards CY, Bridge JA, Kindblom LG, Wold LE : Conditions that simulate primary neoplasms of bone. In : Silverberg SG, eds. AFIP Atlas of Tumor Pathology Series 4: Tumors of the Bones and Joints. Washington, DC ; ARP/AFIP, 2005 : 321-324

24. Miyazono K : Positive and negative regulation of TGF-beta signaling. J Cell Sci 2000 ; 113 : 1101-1109

25. Gazzerro E, Gangji V, Canalis E : Bone morphogenetic proteins induce the expression of Noggin, which limits their activity in cultured rat osteoblasts. J Clin Invest 1998 ; 102 : 2106-2114

26. Takase M, Imamura T, Sampath TK, Takeda K, Ichijo H, Miyazono K et al. : Induction of Smad6 mRNA by bone morphogenetic proteins. Biochem Biophys Res Commun 1998 ; 244 : 26-29

Table 1. Fetal bones

Week	Membranous ossification	Endochondral ossification
6		limb, vertebral bone
9		limb, vertebral bone
10	craniofacial bone	
11	craniofacial bone	limb, vertebral bone
12		limb
12		limb
22		vertebral bone
36		vertebral bone

Table 2. Tumor specimens

Tumors	No. of cases
Osteosarcoma	20
limb or pelvic bones	16
jawbones	4
Fibrous dysplasia*	10
Chondrosarcoma*	10
Enchondroma**	10
Sarcomatoid renal cell carcinoma	5
Uterine leiomyosarcoma	7

* Limb or pelvic bones, except for jawbones.

** Limb bones.

Table 3. Primers for real-time PCR

Primer	Sequence (5' to 3' orientation)
RUNX2 (F)	GACACCACCAGGCCAATC
RUNX2 (R)	AGAACAAGGGGGCCGTTA
BMP-2 (F)	GGGCATCCTCTCCACAAA
BMP-2 (R)	GTCATTCCACCCCACGTC
GAPDH (F)	CAGCGACACCCACTCCTC
GAPDH (R)	TGAGGTCCACCACCCTGT

F, forward; R, reverse

Table 4. RUNX2 expression of tumor specimens

Tumors	Positive	Negative
Osteosarcoma	20	0
Fibrous dysplasia	10	0
Chondrosarcoma	10	0
Enchondroma	10	0
Sarcomatoid renal cell carcinoma	0	5
Uterine leiomyosarcoma	0	7

Fig. 1

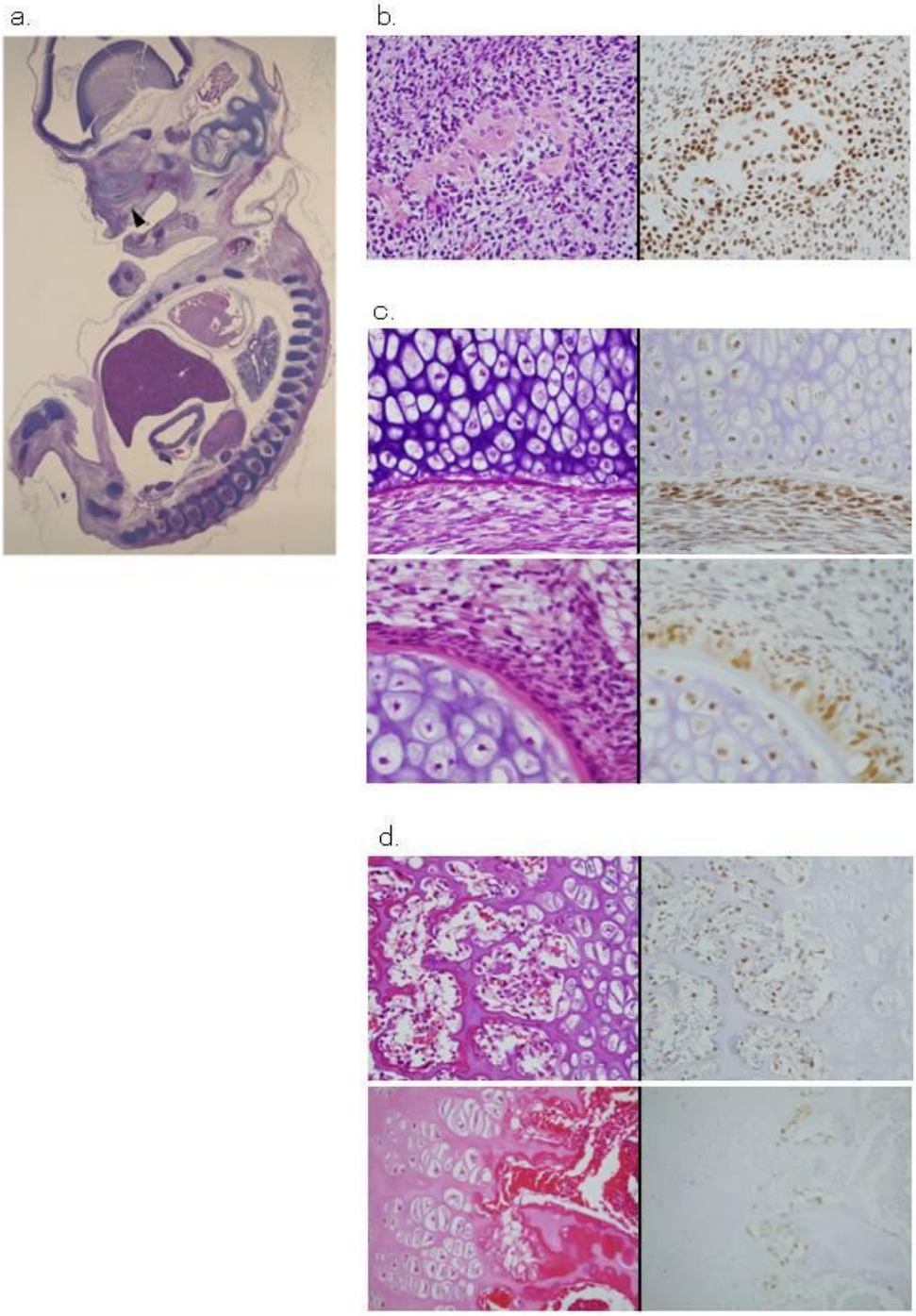


Fig. 2

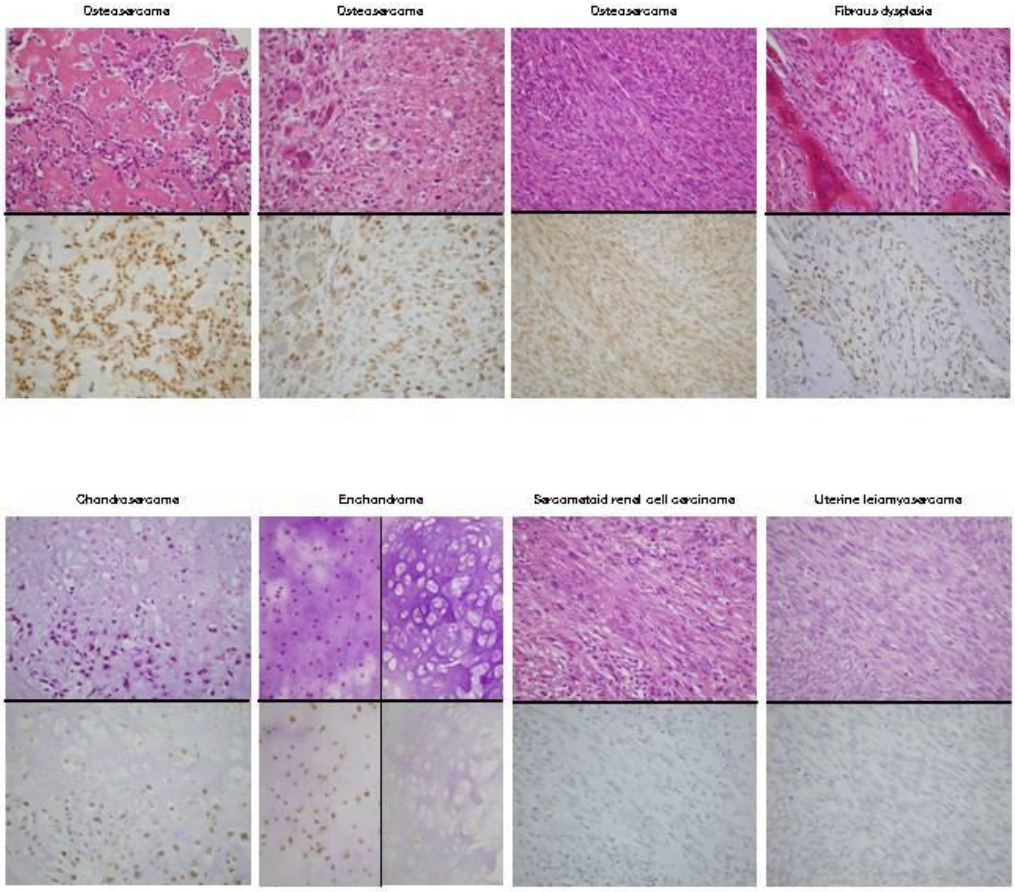
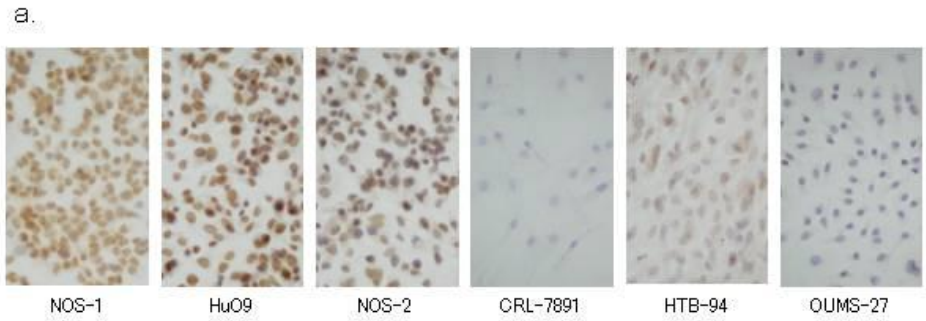
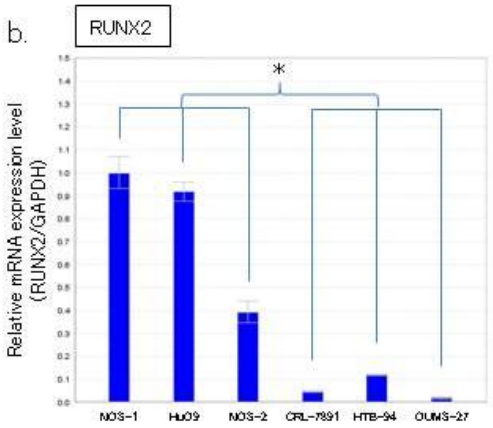

Osteosarcoma
Osteosarcoma
Osteosarcoma
Fibrous dysplasia
Chondrosarcoma
Enchondroma
Sarcomatoid renal cell carcinoma
Uterine leiomyosarcoma

Fig. 3


a.
NOS-1
HuO9
NOS-2
CRL-7891
HTB-94
OUMS-27


b.
RUNX2
*
Relative mRNA expression level
(RUNX2/GAPDH)
NOS-1
HuO9
NOS-2
CRL-7891
HTB-94
OUMS-27

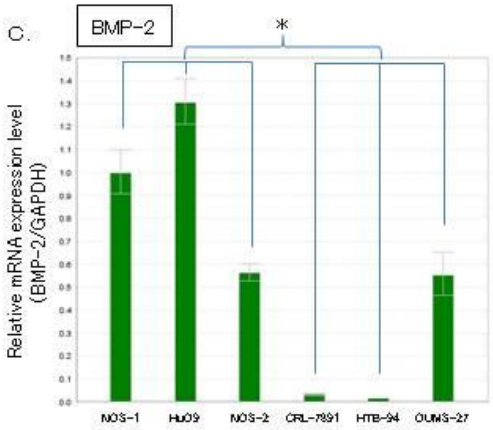

c.
BMP-2
*
Relative mRNA expression level
(BMP-2/GAPDH)
NOS-1
HuO9
NOS-2
CRL-7891
HTB-94
OUMS-27

Fig. 4

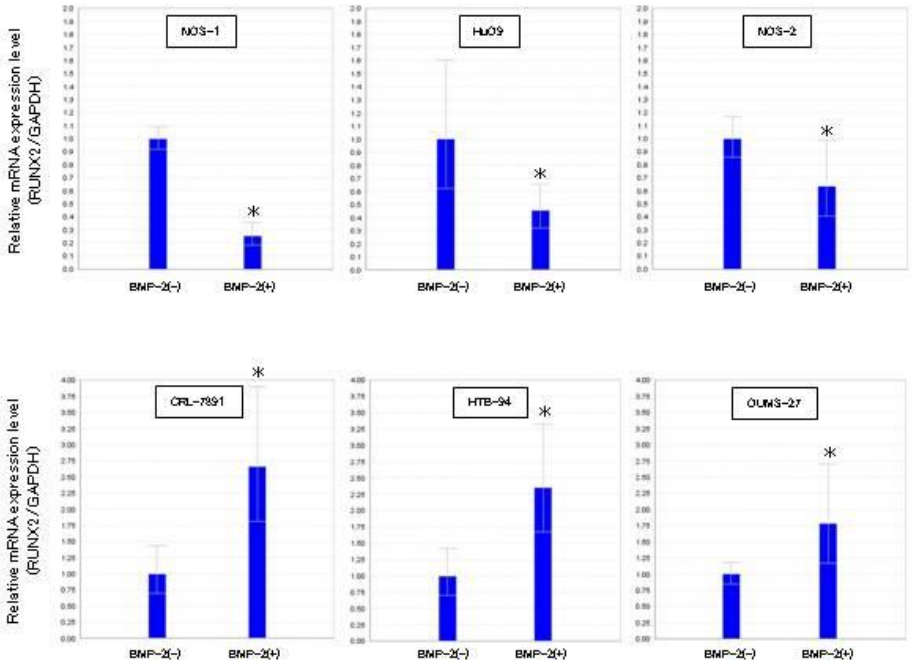

Relative mRNA expression level (RUNX2/GAPDH)
NOS-1
HuO9
NOS-2
CRL-7891
HTB-94
OUMS-27
BMP-2(-)
BMP-2(+)
*

Figure legends

Fig. 1. RUNX2 expression during osteogenesis in human fetus. **a.** Longitudinal section of a fetus at 11 gestational weeks. Ossification center appeared in the maxillar region (arrowhead), whereas primordia of vertebral column and limbs consisted of cartilage. **b.** High magnification of the ossification center appeared in the maxillar region at 11 gestational weeks. It was membranous ossification without cartilage. RUNX2 was expressed not only in osteoblasts or osteocytes but also in mesenchymal cells that proliferated around the bone matrix. **c.** High magnification of tibia at 9 gestational weeks (upper) and forearm at 11 gestational weeks (lower). Osteoblastic lining on cartilage and subsequent bone collar formation was observed. RUNX2 was expressed not only in osteoblastic cells but also in spindle-shaped mesenchymal cells that surrounded the osteoblastic cells. Some chondrocytes were also immunoreactive with RUNX2. **d.** Endochondral ossification in vertebral bone of a fetus at 22 (upper) and 36 (lower) gestational weeks. Osteoblasts that lined the bone trabeculae showed distinct RUNX2 expression. Hypertrophic chondrocytes rarely showed RUNX2 expression (a, hematoxylin and eosin. b-d, left, hematoxylin and eosin; right, immunostaining for RUNX2.).

Fig. 2. RUNX2 expression in tumor specimens. In osteosarcomas, tumor cells of

both osteoblastic and fibroblastic morphology showed distinct RUNX2 expression, even in the area where osteoid was inconspicuous. Reactive osteoclast-like cells (arrows) were negative for RUNX2. RUNX2 expression was also found in both fibroblastic cells and osteoblastic cells that rimmed the woven bones of fibrous dysplasia. In chondrosarcoma and enchondroma, small-sized cells showed RUNX2 immunostaining, whereas differentiated cells with lacunae showed scarce immunostaining. Sarcomatoid renal cell carcinoma and uterine leiomyosarcoma, bone metastasis of which should be differentiated from osteosarcoma, was completely negative for RUNX2 (upper, hematoxylin and eosin ~~stain~~; lower, immunostaining for RUNX2.).

Fig. 3. a. Immunocytochemistry for RUNX2 in osteosarcoma (OS) lines (NOS-1, HuO9, NOS-2) and chondrosarcoma (CS) lines (CRL-7891, HTB-94, OUMS-27). The three OS lines showed distinct RUNX2 immunostaining. Two of the 3 CS lines (CRL-7891 and OUMS-27) did not show significant immunostaining for RUNX2, and HTB-94 showed faint immunostaining (immunostaining for RUNX2).

b. *RUNX2* mRNA level in 6 cell lines. It was significantly higher in OS lines (NOS-1, HuO9, NOS-2) than in CS lines (CRL-7891, HTB-94, OUMS-27)

(*p<0.0001).

c. *BMP-2* mRNA level in 6 cell lines. Like the *RUNX2* mRNA level, this was significantly higher in OS lines than in CS lines (*p<0.0001).

Fig. 4. Alteration of *RUNX2* mRNA level following BMP-2 treatment of osteosarcoma cell lines (NOS-1, HuO9, NOS-2) and chondrosarcoma cell lines (CRL-7891, HTB-94, OUMS-27). All 6 cell lines were treated with recombinant BMP-2 (500 ng/ml) for 72 hours. After treatment, *RUNX2* mRNA level significantly decreased in the 3 osteosarcoma cell lines, whereas it increased in the 3 chondrosarcoma cell lines (*p<0.0001).