Virchows Arch (1998) 432:515–519

ORIGINAL ARTICLE

Alessandro Franchi · Anna Calzolari
Giancarlo Zampi

Immunohistochemical detection of *c-fos* and *c-jun* expression in osseous and cartilaginous tumours of the skeleton

Received: 30 July 1997 / Accepted: 15 December 1997

Abstract The products of c-*fos* and c-*jun* proto-oncogenes form the heterodimeric complex AP-1 (activator protein 1), which play an important part in the control of bone cell proliferation and differentiation and in the development of bone tumours. We examined the expression of c-*fos* and c-*jun* in a series of 52 primary skeletal neoplasms, using an immunohistochemical method on formalin-fixed, paraffin-embedded sections. The expression of c-*fos* and c-*jun* was restricted to bone-forming lesions, while cartilaginous tumours were devoid of immunoreactivity. In benign osteoblastic lesions moderate c-*fos* and c-*jun* expression was found in 2 cases (18.1%). The highest levels of c-*fos* and c-*jun* expression were detected in high-grade central osteosarcomas (7 of 15 cases with moderate/diffuse expression) while 1 telangiectatic osteosarcoma, 2 low-grade central osteosarcomas, 1 low-grade periosteal osteosarcoma and 7 low-grade parosteal osteosarcomas were either negative or had low expression. The high-grade component of a dedifferentiated parosteal osteosarcoma showed diffuse immunoreactivity for both oncoproteins. Comparison of c-*fos* and c-*jun* expression by histological grade showed that high-grade osteosarcomas had a significantly higher expression of both oncoproteins than did low-grade osteosarcomas (P = 0.01, Fisher's exact test). Thus, c-*fos* and c-*jun* overexpression may be implicated in the development of high-grade osteosarcomas, but they appear to have little or no relevance for the development of low-grade osteosarcomas and cartilaginous skeletal neoplasms.

Key words *c-fos* · *c-jun* · Bone neoplasms · Immunohistochemistry

A. Franchi (✉)[1] · A. Calzolari · G. Zampi
Institute of Anatomic of Pathology, University of Florence, Florence, Italy

Mailing address:
[1] Istituto di Anatomia Patologica, Università degli Studi di Firenze, Viale G.B. Morgagni 85, I-50134 Firenze, Italy
Fax: +39-55-437 9868

Introduction

Both c-*fos* and c-*jun* belong to a multigene family whose products form the highly stable heterodimeric complex AP-1 (activator protein 1), which regulates transcription of target genes by binding DNA at specific sites [2, 6]. These sites are *cis*-acting transcriptional regulatory sequences found in a large number of genes and are important in the regulation of a variety of processes affecting cell growth and differentiation [6].

Bone is a physiological target for the action of c-*fos* and c-*jun*. The expression of c-*fos* proto-oncogene has been demonstrated in developing bone and teeth, and elevated levels of c-*fos* have been found in osteoblasts, osteocytes, osteoclasts, periosteal cells, articular and growth plate chondrocytes by mRNA in situ hybridization studies and immunohistochemistry [1, 3, 4, 10, 13, 15, 19]. The role of c-*fos* proto-oncogene expression in skeletal development and remodelling processes has been investigated using in vivo approaches employing mice with both loss of function and gain of function of the gene. Mice lacking c-*fos* are affected by a severe form of osteopetrosis owing to lack of osteoclast activity [9, 25]. Overexpression of c-*fos* in transgenic mice results in increased formation of woven bone, increased resorption, and the development of osteosarcomas [7, 17, 26].

Subsequently, an association between c-*fos* overexpression and human osteosarcomas has been postulated on the basis of the results of immunohistochemical studies showing significantly higher oncoprotein expression in osteosarcoma than in normal tissues or nonosteosarcoma lesions [27]. In order to clarify the possible role of c-*fos* and c-*jun* expression in the development of skeletal neoplasms further, we analysed the in situ expression of the two oncoproteins in a series of primary bone neoplasms, including bone- and cartilage-forming lesions, using an immunohistochemical method. The results indicate that c-fos and c-jun oncoproteins are expressed specifically in bone-forming lesions, and at a significantly higher level in high-grade osteosarcomas.

Table 1 Expression of c-*fos* and c-*jun* in a series of 52 primary bone tumours

Tumor Entity	c-fos/c-jun			
	– *n* (%)	+ *n* (%)	++ *n* (%)	+++ *n* (%)
Osteoid osteoma	3 (50)	2 (33.3)	1 (16.7)	0
Osteoblastoma	3 (60)	1 (20)	1 (20)	0
Conventional OS*	5 (33.3)	3 (20)	3 (20)	4 (26.7)
Telangiectatic OS	1 (100)	0	0	0
Intraosseous well differentiated OS	2 (100)	0	0	0
Parosteal OS	4 (66.6)	2 (33.3)	0	0
Periosteal OS	1 (100)	0	0	0
Dedifferentiated Parosteal OS (high-grade component)[a]	0	0	0	1 (100)
Dedifferentiated parosteal OS (low-grade component)[a]	1 (100)	0	0	0
Chondroma	5 (100)	0	0	0
Chondrosarcoma	10 (100)	0	0	0

[a] Same lesion

Materials and methods

Fifty-two primary bone tumours were selected from the files of the Institute of Anatomical Pathology of the University of Florence Medical School. For diagnostic and grading purposes we followed the criteria proposed by Unni [21]. The series included benign and malignant osseous and cartilaginous neoplasms. In the first group there were 6 osteoid osteomas, 5 osteoblastomas, 15 intramedullary high-grade (grades III and IV) osteosarcomas, 1 telangiectatic osteosarcoma, 2 intraosseous well-differentiated (grade I) osteosarcomas and 8 peripheral osteosarcomas (6 parosteal low-grade, 1 periosteal low-grade, and 1 dedifferentiated parosteal osteosarcoma). The group of cartilaginous lesions included 5 enchondromas and 10 chondrosarcomas (8 conventional chondrosarcomas, 1 dedifferentiated chondrosarcoma, 1 chondrosarcoma arising in osteochondroma); of these, 3 were grade I, 4 were grade II, and 2 were grade III chondrosarcomas.

For light microscopic and immunohistochemical examination tissue samples were fixed in 10% buffered formalin and embedded in paraffin. When needed specimens were decalcified in a solution of formic acid. Serial 5-µm sections were prepared for staining with haematoxylin and eosin and for the immunohistochemical studies, which were performed by the avidin-biotin complex (ABC) technique (Dakopatts, High Wycombe, Bucks., UK). The primary monoclonal mouse antibodies against c-*fos* and c-*jun* (Oncogene Science, Cambridge, Mass.) were applied at 1:40 dilution. In parallel, a number of sections were subjected to microwave irradiation and the staining obtained was similar to the untreated sections. Negative controls were performed by substituting the primary antibody with nonimmune mouse serum.

The results of immunohistochemical staining were evaluated semiquantitavely on the basis of a four-point scale: – negative staining; + low expression, less than 10% of positive cells; ++ moderate expression, 10–50% of positive cells; +++ diffuse expression, more than 50% of positive cells. Statistical analysis was performed using Fisher's two-tailed exact test.

Results

Table 1 summarizes the results of the immunohistochemical study. Overall, the immunohistochemical distribution of c-*fos* and c-*jun* was similar in our series of bone-forming lesions. Positivity was mainly localized in the nucleus, although in 6 lesions (33.3% of positive tumours; 1 osteoid osteoma, 1 osteoblastoma, and 4 osteosarcomas) both nuclear and cytoplasmic staining were observed. The immunoreactions were not affected by the decalcification procedure: the number of decalcified specimens in the group of lesions showing absent/low positivity was comparable to that in the group with moderate/diffuse positivity (67.8% vs 60%, $P = 0.7$).

Osteoid osteomas were characterized by a central nidus made of plump osteoblasts producing woven bone with numerous osteoclasts and a richly vascular stroma. Staining for both c-*fos* and c-*jun* was observed in osteoblasts and osteoclasts in 3 of 6 lesions (50%), and positivity was scored as low in 2 cases and moderate in 1 (Fig. 1).

The histological appearance of osteoblastomas was similar to the nidus of osteoid osteomas. Both c-fos and c-jun oncoproteins were detected in 2 of 5 lesions (40%), with a cellular distribution similar to that observed in osteoid osteomas. Low expression was observed in 1 lesion and moderate expression, in 1.

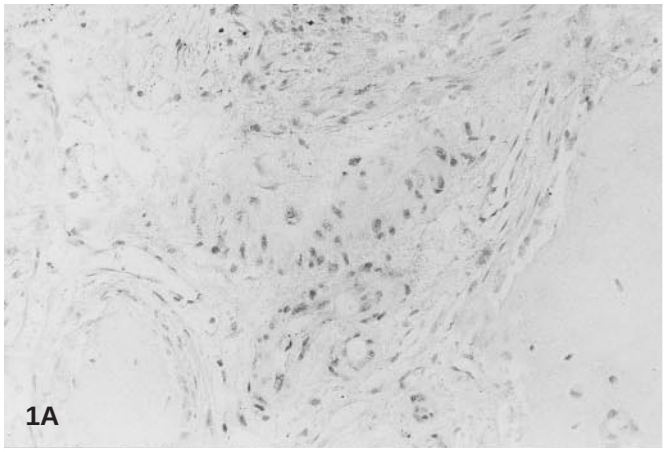
Conventional intramedullary osteosarcomas were characterized by a proliferation of highly atypical cells producing variable quantities of woven bone-like matrix and cartilaginous matrix. The expression of c-fos and c-jun oncoproteins was demonstrated in 10 cases (66.6%), varying from low (3 cases) to moderate (3 cases) or diffuse (4 cases) (Fig. 2). The telangiectatic osteosarcoma, in which the sarcomatous elements formed septa delimiting cystic spaces filled with blood, was negative for the expression of both oncoproteins.

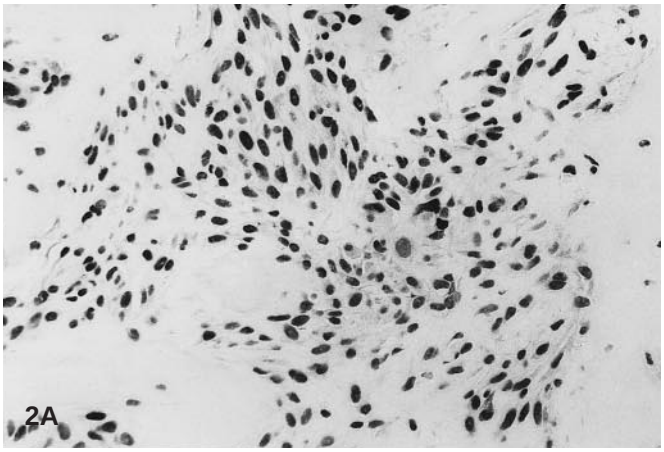
Fig. 1 Focal nuclear immunoreactivity for **A** c-*fos* and **B** c-*jun* in the nidus of an osteoid osteoma. ×180 ▶

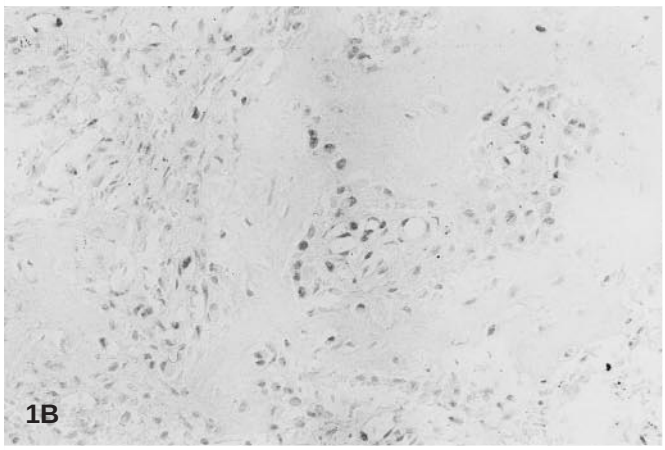
Fig. 2 High-grade central osteosarcoma showing diffuse and intense nuclear positivity for **A** c-*fos* and **B** c-*jun.* ×180

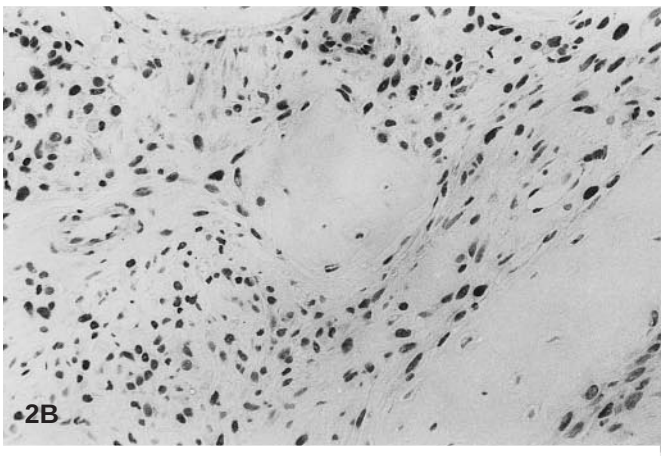
Fig. 3 Low-grade parosteal osteosarcoma showing focal nuclear immunoreactivity for **A** c-*fos* and **B** c-*jun.* ×330

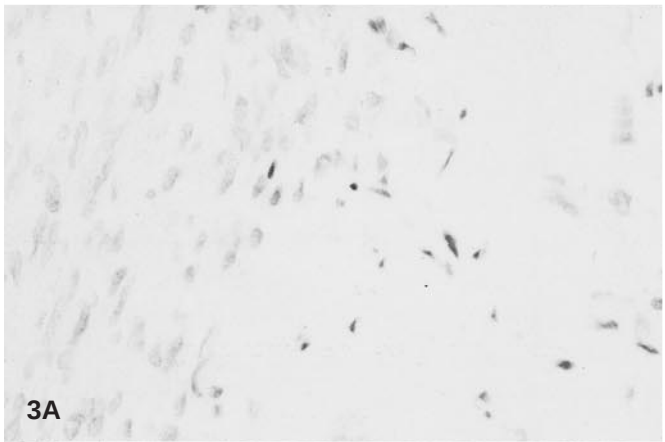
Fig. 4 Absence of **A** c-*fos* and **B** c-*jun.* expression in a grade I chondrosarcoma. ×180


1A

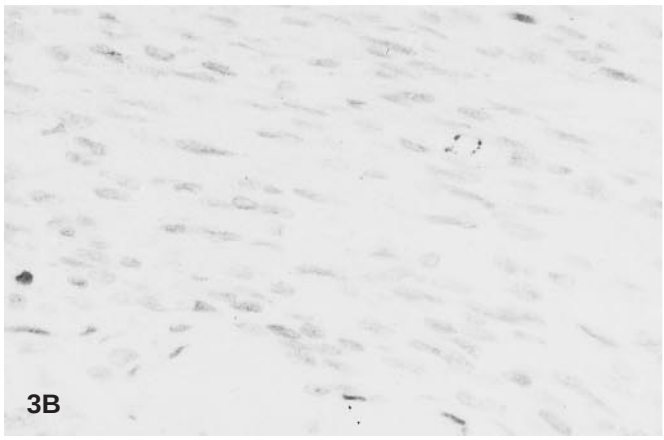

2A


1B


2B


3A


4A


3B


4B

Intraosseous well-differentiated osteosarcomas were formed by fibroblast-like cells with little or no cellular atypia producing osteoid matrix, with a pattern resembling that of fibrous dysplasia (fibrous dysplasia-like low-grade osteosarcomas). Both tumours were negative for the expression of c-*fos* and c-*jun*.

The group of peripheral osteosarcomas included 7 parosteal osteosarcomas and 1 periosteal low-grade osteosarcoma. Parosteal osteosarcomas were characterized by a proliferation of uniform bland spindle cells producing woven bone with a trabecular appearance; focal production of cartilaginous matrix was also observed. The histological profile of one lesion corresponded to the so-called dedifferentiated parosteal osteosarcoma, with foci of pleomorphic high-grade sarcoma present within the tumour. In this group of neoplasms, immunoreactivity for c-*fos* and c-*jun* was demonstrated only in 2 parosteal low-grade osteosarcomas (25%), both showing low expression of the oncoproteins (Fig. 3). The high-grade component of the dedifferentiated parosteal osteosarcoma showed diffuse immunoreaction for both c-*fos* and c-*jun*.

The series of osteosarcomas included 17 high-grade (including all central high-grade lesions and the high-grade component of the dedifferentiated parosteal osteosarcoma) and 10 low-grade tumours. Overall, 12 (70.5%) high-grade osteosarcomas were positive for c-fos and c-jun, while in the group of low-grade lesions only 2 cases (20%) expressed the oncoproteins. Moderate or diffuse expression was observed in 8 (47.5%) of the high-grade lesions, while none of the low-grade osteosarcomas showed positivity to a similar degree. This difference was statistically significant ($P = 0.01$, Fisher's exact test). The levels of c-*fos* and c-*jun* were comparable in low-grade osteosarcomas and benign osteoblastic lesions ($P = 0.2$, Fisher's exact test).

Finally, no immunoreactivity for c-*fos* or c-*jun* was found in the group of chondromas and chondrosarcomas (Fig. 4).

Discussion

The results of the present study confirm that the c-*fos* gene is frequently overexpressed in human osteosarcomas [27]. In addition, our results contribute to these observations by finding that in osteosarcomas the expression of the oncogene c-*jun* parallels that of the oncogene c-*fos*, indicating that the two oncogenes may co-operate during the development of these neoplasms. Overall, we observed a lower percentage of osteosarcomas with diffuse expression of c-*fos* than in the study of Wu et al. [27]. This is probably because we performed our investigation on formalin-fixed, paraffin-embedded material, while Wu et al. used fresh tumour sections. In addition, tissue processing may also influence the distribution of the immunostaining for c-*fos* and c-*jun*. Indeed, nearly exclusive nuclear staining has been obtained in studies on fresh tissue sections [23], while both cytoplasmic and nuclear staining has been observed in fixed material [8, 12]. However, the study of bone-forming lesions is extremely difficult in fresh tissue sections owing to the presence of calcified matrix, which is particularly abundant in benign neoplasms and in some variants, of osteosarcoma. Nevertheless, it has been shown that immunohistochemical studies of c-*fos* and c-*jun* expression can be performed on formalin-fixed, paraffin-embedded decalcified sections of bone tissue without significant loss of information [8].

However, benign and malignant cartilaginous tumours do not express immunohistochemically detectable levels of c-*fos* and c-*jun*, suggesting that these oncogenes are not primarily involved in the development of these neoplasms. This is in apparent contrast to the observations that ectopic expression of c-*fos* in chimeric mice is associated with frequent development of cartilaginous tumours [24]. However, it should be noted that these experimentally induced tumours do not reproduce the same histological profile of human chondrosarcomas exactly, as they also contain foci of bone-forming neoplastic cells and undifferentiated mesenchymal spindle cells [24].

Elevated levels of c-*fos* and c-*jun* have been described in several tumour types, [12, 20, 23] and it has been suggested that elevated c-*fos* and c-*jun* expression is an important event in tumorigenesis, because it may determine an increased proliferation rate [12]. With specific reference to osteosarcomas, it is of interest that the expression of both c-*fos* and c-*jun* is significantly higher in high-grade osteosarcomas (characterized by aggressive growth with tendency to systemic spread) than in low-grade osteosarcomas (locally aggressive lesions with infrequent metastases) [21], suggesting that these oncogenes may be involved in determining the clinical behaviour of these neoplasms.

The elevated levels of c-fos and c-jun oncoproteins in high-grade osteosarcomas may be the result of the alteration of several pathways that ultimately control cell proliferation. First, c-*fos* and c-*jun* are under the regulation of other oncogenes, such as the retinoblastoma tumour suppressor gene (*RB*), whose product can down regulate c-*fos* transcription and AP-1 activity [16]. The *RB* gene is frequently altered in human osteosarcomas [22], and loss of *RB* activity could be involved in determining increased levels of c-*fos* and c-*jun* in these tumours. In addition, the expression of c-*fos* appears to be regulated by transforming growth factor beta (TGFβ), one of the major growth factors for bone tissue. Recent studies have shown that TGFβ induces an increase of c-*fos* mRNA levels in cultured normal and transformed human osteoblast-like cells [18], and this may be responsible for an increase in proliferative activity, since the uptake of antisense c-*fos* oligonucleotide abolishes the mitogenic effect of TGFβ on osteoblast-like cells [11]. Our analysis supports the existence of a strong direct relationship between the expression of c-*fos* and TGFβ in human osteosarcomas, since high-grade osteosarcomas show significantly higher levels of c-*fos* and of TGFβ1 than low-grade lesions [5]. The observation that high-grade osteosarcomas have a signifi-

cantly higher proliferative activity than low-grade osteosarcomas [14] suggests that in these variants of osteosarcoma the elevated expression of TGFβ1 and c-*fos* may sustain a higher proliferative activity and may contribute substantially to establishment of an aggressive phenotype. Conversely, in low-grade osteosarcomas lower levels of TGFβ1 and c-*fos* may result in lower proliferative activity [14] and ultimately in less aggressive growth. Taken together, these data indicate that the control pathways of the expression of c-*fos* and c-*jun* could play an important part in determining the clinical behaviour of osteosarcomas. Further studies with larger series are needed to determine whether the evaluation of c-*fos* and c-*jun* expression may be useful in predicting of clinical outcome in these neoplasms.

Acknowledgement This work was supported in part by a grant from Associazione Italiana per la Ricerca sul Cancro (AIRC).

References

1. Caubet J-F, Bernaudin J-F (1988) Expression of the *c-fos* proto-oncogene in bone, cartilage and tooth forming tissues during mouse development. Biol Cell 64:101–104
2. Curran T, Franza R Jr (1988) *Fos* and *Jun*: the AP-1 connection. Cell 55:395–397
3. De Togni P, Niman H, Raymond V, Sawchenko P, Verma IM (1988) Detection of *fos* protein during osteogenesis by monoclonal antibodies. Mol Cell Biol 8:2251–2256
4. Dony C, Gruss P (1987) Proto-oncogene *c-fos* expression in growth regions of fetal bone and mesodermal web tissue. Nature 328:711–714
5. Franchi A, Arganini L, Baroni G, Calzolari A, Capanna R, Campanacci D, Caldora P, Masi L, Brandi ML, Zampi G (1998) Expression of transforming growth factor β isoforms in osteosarcoma variants: association of TGF-β1 with high grade Osteosarcomas. J Pathol (Lond) (in press)
6. Franza RB Jr, Rauscher FJ III, Josephs SF, Curran T (1988) The *Fos* complex and *Fos*-related antigens recognize sequence elements that contain AP-1 binding sites. Science 239:1150–1153
7. Grigoriadis AE, Schellander K, Wang Z-Q, Wagner EF, (1993) Osteoblasts are target cells for transformation in c-*fos* transgenic mice. J Cell Biol 122:685–701
8. Hoyland J, Sharpe PT (1994) Upregulation of c-*Fos* protooncogene expression in pagetic osteoclasts. J Bone Miner Res 9: 1191–1194
9. Johnson RS, Spiegelman BM, Papaioannu V (1992) Pleiotropic effects of a null mutation in the c-*fos* proto-oncogene. Cell 71:577–586
10. Machwate M, Julienne A, Mouktar M, Marie PJ (1995) Temporal variation of c-*fos* proto-oncogene expression during osteoblast differentiation and osteogenesis in developing rat bone. J Cell Biochem 57:62–70
11. Machwate M, Jullienne A, Moukhtar M, Lomri A, Marie PJ (1995) c-*fos* protooncogene is involved in the mitogenic effect of transforming growth factor-β in osteoblastic cells. Mol Endocrinol 9:187–198
12. Magrisso IJ, Richmond RE, Carter JH, Pross CB, Gilfillen RA, Carter HW (1993) Immunohistochemical detection of RAS, JUN, FOS, and p53 oncoprotein expression in human colorectal adenomas and carcinomas. Lab Invest 69:674–681
13. Mason DJ, Hillam RA, Skerry TM (1996) Constitutive in vivo mRNA expression by osteocytes of β-actin, osteocalcin, connexin-43, IGF-I, c-*fos* and c-*jun*, but not TNF-α nor tartrate-resistant acid phosphatase. J Bone Miner Res 11:350–357
14. Oda Y, Wehrmann B, Radig K, Walter H, Röse I, Neumann W, Roessner A (1995) Expression of growth factors and their receptors in human osteosarcomas. Gen Diagn Pathol 141: 97–103
15. Ohta S, Yamamuro T, Lee K, Okumura H, Kasai R, Hiraki J, Ikeda T, Iwasaki R, kikuchi H, Konishi J, Shigeno C (1991) Fracture healing induces expression of the proto-oncogene c-*fos* in vivo. Possible involvement of the Fos protein in osteoblastic differentiation. FEBS Lett 284:42–45
16. Robbins PD, Horowitz JM, Mulligan RC (1990) Negative regulation of human c-*fos* expression by the retinoblastoma gene product. Nature 346:668–671
17. Rüther U, Komitowski D, Schubert FR, Wagner EF (1989) c-*fos* expression induces bone tumors in transgenic mice. Oncogene 4:861–865
18. Subramaniam M, Oursler MJ, Rasmussen BL, Riggs BL, Spelberg TC (1995) TGF-β regulation of nuclear proto-oncogenes and TGF-β gene expression in normal human osteoblast-like cells. J Cell Biochem 57:52–61
19. Sandberg M, Vuorio T, Hirvonen H, Alitalo K, Vuorio E (1988) Enhanced expression of TGF-β and c-*fos* mRNAs in the growth plates of developing human long bones. Development 102:461–470
20. Tiniakos DG, Scott LE, Corbett IP, Piggott NH, Horne CH (1994) Studies of c-*jun* oncogene expression in human breast using a new monoclonal antibody, NCL-DK4. J Pathol (Lond) 172:19–26
21. Unni KK (1996) Dahlin's bone tumors: general aspects and data on 11087 cases. Lippincott-Raven, Philadelphia
22. Wadayama B, Toguchida J, Shimizu T, Ishizaki K, Sasaki MS, Kotoura Y, Yamamuro T (1994) Mutation spectrum of the retinoblastoma gene in osteosarcomas. Cancer Res 54:3042–3048
23. Walker RA, Cowl J (1991) The expression of c-*fos* protein in human breast. J Pathol (Lond) 163:323–327
24. Wang Z-Q, Grigoriadis AE, Möhle-Steinlein U, Wagner EF (1991) A novel target cell for c-*fos*-induced oncogenesis: development of chondrogenic tumours in embryonic stem cell chimeras. EMBO J 10:2537–2450
25. Wang Z-Q, Ovitt C, Grigoriadis AE, Möhle-Steinlein U, Rüther U, Wagner EF (1992) Bone and haematopoietic defects in mice lacking c-*fos*. Nature 360:741–745
26. Wang Z-Q, Liang J, Schellander K, Wagner EF, Grigoriadis AE (1995) c-*fos*-induced osteosarcoma formation in transgenic mice: cooperativity with c-*jun* and the role of endogenous c-*fos*. Cancer Res 55:6244–6251
27. Wu J-X, Carpenter M, Gresens C, Keh R, Niman H, Morris JWS, Mercola D (1990) The proto-oncogene c-*fos* is over-expressed in the majority of human osteosarcomas. Oncogene 5: 989–1000