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Proto-oncogene Expression in a Human Chondrosarcoma Cell Line: HCS-2/8

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HCS-2/8 is a stable human chondrosarcoma cell line with many chondrocytic characteristics and has the capacity to form chondrosarcomas in nude mice. The cells display both biochemically and morphologically definable changes in sparse, subconfluent, confluent and over-confluent phases of *in vitro* culture. Such features of HCS-2/8 cells may reflect the processes of both proliferation and differentiation of chondrocytes *in vivo*. We examined the correlations of these changes of HCS-2/8 cells with their transcript levels of 21 proto-oncogenes by Northern analysis. We found no detectable transcripts of 9 proto-oncogenes (c-*sis*, c-*met*, c-*src*, c-*lyn*, c-*fgr*, c-*ros*, c-*pim*, B*lym* and N-*myc*), but detected transcripts of 12 other proto-oncogenes (*int*-2, *erb*B, c-*abl*, c-*raf*-1, c-*fyn*, K-*ras*, H-*ras*, c-*mos*, c-*myc*, c-*myb*, c-*fos*, and c-*jun*). In the over-confluent phase, the levels of c-*fos* and c-*raf*-1 were increased several dozen times and about 5 times, respectively, while the level of c-*abl* was about 1/5th of that in the sparse, subconfluent and confluent phases of culture. The level of *int*-2 increased about 10-fold in the confluent and overconfluent phases of *in vitro* culture. The transcript levels of c-*mos* and K-*ras* were high in the sparse phase, low in the subconfluent and confluent phases and high in the over-confluent phase. The levels of the other 6 proto-oncogenes in HCS-2/8 cells were constant in all phases of *in vitro* culture.

Key words: Chondrosarcoma — Oncogene — Differentiation — Chondrocyte — Established cell line

The group of proteins encoded by so-called proto-oncogenes are essential elements in cellular signal-transducing processes to transfer extracellular stimuli to the nucleus. On the basis of their different roles, these oncoproteins have been tentatively classified into five groups: 1, growth factors, i.e., c-sis (PDGF)[4] and int-2 (bFGF); 2, receptor and non-receptor tyrosine kinases, e.g., erb-B (EGF-R), met (HGF-R) and the src family; 3, membrane-associated G proteins, e.g., ras family proteins; 4, cytoplasmic protein-serine kinases, i.e., raf and ros and 5, transcription factors, i.e., fos, jun, and rel.[1] The general expression patterns of proto-oncogenes vary depending on the origin of the cells and their stage of cellular differentiation. On the other hand, malignancies are often associated with detectable changes at either the level of expression of oncoproteins or their specific structural alteration caused by mutation, deletion, or rearrangement of one or more proto-oncogenes.[2] The involvements of two or more proto-oncogenes with different roles in cellular signal transduction systems have been demonstrated in the majority of, if not all malignant lesions examined, reflecting the multistep process of carcinogenesis.[1] Therefore, information on general expression patterns of proto-oncogenes in normal cells and their malignant counterparts is essential for understanding the mechanisms involved in cellular differentiation and in development of malignant cells from their normal counterparts *in vivo*.

Malignant tumors arising from the skeletal system are rare, constituting less than 0.5% of all malignant tumors, and 20% of them are chondrosarcomas.[3,4] The difficulty in studying this rare type of tumor has been partially overcome by our establishment of a stable human chondrosarcoma cell line, HCS-2/8.[5-7] HCS-2/8 cells possess chondrocytic features including synthesis of cartilage-specific proteoglycan and type II collagen and responses to various hormones, vitamins and growth factors.[5-7] Furthermore, HCS-2/8 cells are able to form chondrosarcomas in nude mice, indicating their malignant potency. HCS-2/8 cells *in vitro* display some characteristic changes in morphology as well as in their rate of synthesis of chondrocyte-specific proteoglycan as their growth slows down. Therefore, HCS-2/8 cells can partially mimic the process of human chondrocytic differentiation and the genesis of chondrosarcoma.[5-7] Further studies using the HCS-2/8 cell line should greatly increase our understanding of the molecular and cellular mechanisms controlling the differentiation of chondrocytes and/or the genesis of chondrosarcomas in humans.

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[4] The abbreviations used are: PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; EGF-R, epidermal growth factor receptor; HGF-R, hepatocyte growth factor receptor; HCS-2/8, human chondrosarcoma cell line clone No. 2/8; MEM, minimum essential medium; FBS, fetal bovine serum; SDS, sodium dodecyl sulfate.

In this study, we examined the expression patterns of 21 proto-oncogenes in HCS-2/8 cells by measuring the steady-state levels of their transcripts in four morphological and biochemical phases of *in vitro* culture. We observed correlations of these phases with changes in the transcript levels of c-*fos*, c-*raf*-1, c-*mos*, K-*ras*, c-*abl*, and *int*-2.

MATERIALS AND METHODS

Cell culture The human chondrosarcoma cell line HCS-2/8 cells (passage no. 23-31) was maintained in MEM containing 20% FBS (Gibco) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were photographed at the indicated times (Fig. 1) and then harvested.

Probes and labeling Proto-oncogenes containing plasmid clones were all generously provided by the Japanese Gene Bank, Tokyo, and oligoprobes were purchased from Oncogene, Co. (Table I).

Plasmid DNA was prepared from a 20 ml overnight culture under appropriate antibiotic selection by a modified alkaline lysis and polyethylene glycol precipitation method and restricted with suitable restriction enzymes (Stratagene). DNA inserts were separated from the vector DNA by agarose gel electrophoresis with 1× TAE buffer (40 m*M* Tris-acetate, pH 8.0, and 1 m*M* EDTA) and purified using a Geneclean kit (B101). The DNA probes were labeled with [α-^{32}P]dCTP using a Prime-it kit (Stratagene). The 40 mer oligonucleotide probes were purchased from Oncogene Co. and labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase.

Table I. Proto-oncogene Probes Used

Proto-oncogene	Insert [Restriction enzyme] (kb)	Reference
c-*sis*	0.6 [*Pst* I]	(8)
int-2	0.6 [*Sac* I]	(9)
c-*src*	40 mer	(10)
c-*fgr*	0.22 [*Sac* I]	(11)
c-*fyn*	1.8 [*Eco*R I/*Hin*d III]	(12)
c-*lyn*	1.2 [*Eco*R I/*Hin*d III]	(13, 14)
c-*abl*	2.4 [*Eco*R I]	(15)
c-*ros*	3.2 [*Bam*H I/*Hin*d III]	(16)
*erb*B [EGF-R]	40 mer	(17)
c-*met*	1.6 [*Sal* I/*Eco*R I]	(18)
c-H-*ras*	2.9 [*Sac* I]	(19)
c-K-*ras*	3.8 [*Hin*d III]	(19)
c-*raf*-1	2.9 [*Eco*R I]	(20, 21)
c-*pim*	0.9 [*Bam*H I]	(22)
c-*mos*	2.8 [*Eco*R I]	(23)
c-*myc*	1.5 [*Sac* I]	(24)
c-N-*myc*	2.7 [*Eco*R I]	(25)
c-*myb*	2.0 [*Eco*R I]	(26)
c-*fos*	2.1 [*Eco*R I]	(27)
c-*jun*	40 mer	(28, 29)
B*lym*	0.95 [*Eco*R I]	(30)
β-Actin	1.9 [*Hin*d III]	

Northern analysis Total RNA was prepared from HCS-2/8 cells by the guanidium thiocyanate/acid phenol method.[31] Total RNA (10 μg) was denatured in 0.02 *M* 3-[N-morpholine]propanesulfonic acid, pH 7.0], 5 m*M* sodium acetate, 1 m*M* EDTA, 2.2 *M* formaldehyde, and 50% formamide at 65°C for 10 min and the products was separated by electrophoresis in 1% agarose gel in the same buffer but without formamide.[32] It was located under UV-light after staining with acridine orange[33] and then transferred and fixed to Hybond N$^+$ (Amersham). Hybridization was carried out essentially according to Henderson's protocol.[34] The temperatures for hybridization and washing with oligonucleotide probes were calculated according to Henderson's formulae. For the double-stranded DNA probe, the temperature for hybridization was 60–65°C, and that for the final post-hybridization washing was 55–60°C in 0.1×SSPE [1× SSPE: 3.6 *M* NaCl, 0.2 *M* $NaHPO_4$ (pH 7.4), 0.02 *M* EDTA (pH 7.4)]/ 0.5% SDS, depending upon the G+C content of the probe. The sizes of specific RNAs hybridized to individual probes were estimated with reference to acridine orange-stained bands of 18S (2.4 kb) and 28S (6.3 kb) rRNA and the band hybridizing with the β-actin (2.2 kb) probe. Autoradiography was performed at −70°C for 1 to 5 days using an intensifying screen and Kodak XAR5 film.

Before the next round of hybridization, the previous probes were removed by immersing the filters in 0.01× SSPE, pH 7.0/0.01% SDS at 95°C for 30 min.

Determination of rate of proteoglycan synthesis Proteoglycan synthesis was monitored by determining the incorporation of [^{35}S]sulfate into materials precipitated with cetylpyridinium chloride, as previously described.[35–37]

RESULTS

When plated at low density (1 million cells/100 mm dish), HCS-2/8 cells proliferated with a cellular doubling time of 3.5 days. After about three cell cycles (10 days), the doubling time decreased to 7 days, and after another cell cycle, the cells reached confluence. During the rapidly growing phase (sparse phase, from day 1–10) (Fig. 1A) and subconfluent phase (from day 10 to day 15) (Fig. 1B), the cells had a slightly elongated polygonal shape and very little or no extracellular matrix. As they approached confluence, the cells became polygonal and surrounded by an extracellular matrix (day 16–18) (Fig. 1C). Then, the cells proliferated very slowly with a doubling time of about 14 days, and formed both multilayers of cells and so-called 'cartilage nodules' surrounded by further extracellular matrix (Fig. 1D).

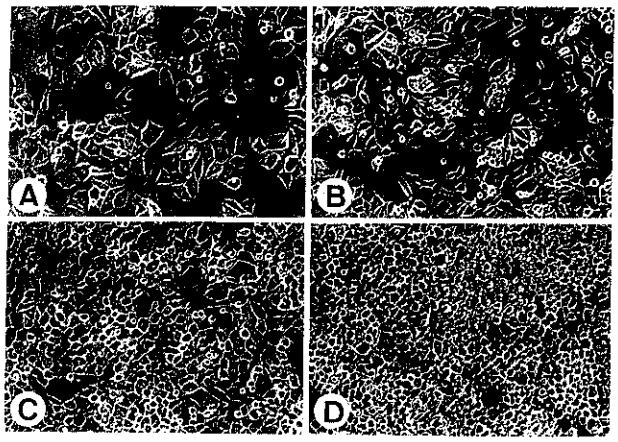

Fig. 1. Morphology of HCS-2/8 cells in different phases of *in vitro* culture. The cells were plated at a density of 1 million cells/100 mm dish and cultured in MEM medium containing 20% fetal bovine serum. Phase-contrast photomicrographs of the cells in sparse (A), subconfluent (B), confluent (C) and over-confluent (D) phases of culture were taken on day 5 (A), day 10 (B), day 16 (C) and day 24 (D). ×35.

Proteoglycan synthesis has been regarded as one of the major phenotypic properties of chondrocytes. The profile of proteoglycan synthesis in HCS-2/8 cells during *in vitro* culture is shown in Fig. 2. Proteoglycan synthesis increased and reached a maximum during the confluent phase of culture. In the over-confluent phase, proteoglycan continued to accumulate, although its rate of synthesis decreased slightly as indicated by a decrease in the rate of [^{35}S]sulfate incorporation. This profile of proteoglycan synthesis has been suggested to reflect the maturation process of HCS-2/8 cells along the chondrocytic differentiation pathway.[7]

We examined the correlations of these defined morphological and biochemical changes of HCS-2/8 cells in four phases (Figs. 1 and 2) with the steady-state levels of expression of 21 proto-oncogenes (Table I) by conventional Northern analysis.[32] As HCS-2/8 cells grow slowly, isolation of large quantities of RNA was not possible. Therefore, 10 μg samples of RNA at each culture phase were analyzed. Thus, results were only obtainable for RNAs that were present at moderate to high levels, and the absence of detectable c-*sis*, c-*met*, c-*src*, c-*fgr*, c-*lyn*, c-*ros*, c-*pim*, B*lym* and N-*myc* (data not shown) means only that their levels were not detectable by this relatively insensitive assay. Further analyses by more sensitive methods including multiple S1 assay of poly A$^+$ RNA and the quantitative polymerase chain reaction of reversely transcribed RNA seem necessary for determination of their actual levels.

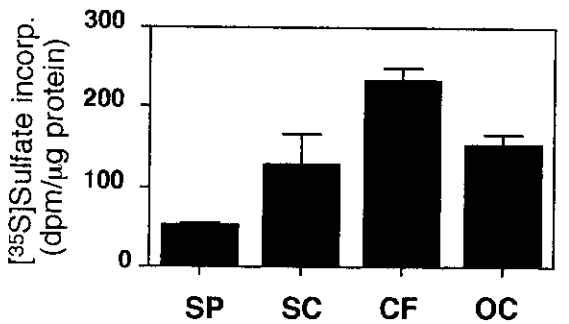

Fig. 2. Profile of proteoglycan synthesis in HCS-2/8 cells during *in vitro* culture. The cells in sparse (SP), subconfluent (SC), confluent (CF) and over-confluent (OC) phases of culture were labeled for 3 h in a mixture of Gey's solution and Hanks' solution (9:1, v/v) containing 1 μCi of [^{35}S]sulfate. Values are means±SD for 3 dishes.

Table II. Relative Transcript Levels of Proto-oncogenes in HCS-2/8 Cells

	Sparse	Subconfluent	Confluent	Over-confluent
int-2	+	+	++++	++++
c-*fyn*	+	+	+	+
c-*abl*	+++	+++	++	+
*erb*B[EGF-R]	+	+	+	+
c-H-*ras*	+	+	+	+
c-K-*ras*	+++	+	+	+++
c-*raf*-1	+	++	++	+++
c-*mos*	+++	+	+	++
c-*myc*	+	+	+	+
c-*myb*	+	+	+	+
c-*fos*	+	++	++	+++++
c-*jun*	+	+	+	+
β-Actin	+	+	+	+

Relative transcript levels of each proto-oncogene among the four phases in *in vitro* culture were scored as + the lowest level among four phases; ++ about two to three times +: +++ about 5 times +; ++++ about ten times +: +++++ several dozen times +.

On the other hand, transcripts of 12 proto-oncogenes in HCS-2/8 cells were readily detectable (Table II and Fig. 3). Equivalent sample loading of HCS-2/8 cellular RNA was carefully controlled as indicated by the uniform staining patterns of 28S and 18S rRNAs in gels stained with acridine orange and by rehybridization to a β-actin probe (panels 7 and 12, Fig. 3). Hence, it was possible to correlate the relative transcript levels of these proto-oncogenes with the morphological and biochemical changes in HCS-2/8 cells (Figs. 1 and 2).

Of the 12 proto-oncogenes detected, the transcript levels of c-*myc*, H-*ras*, c-*myb*, c-*fyn*, *erb*B, and c-*jun* were

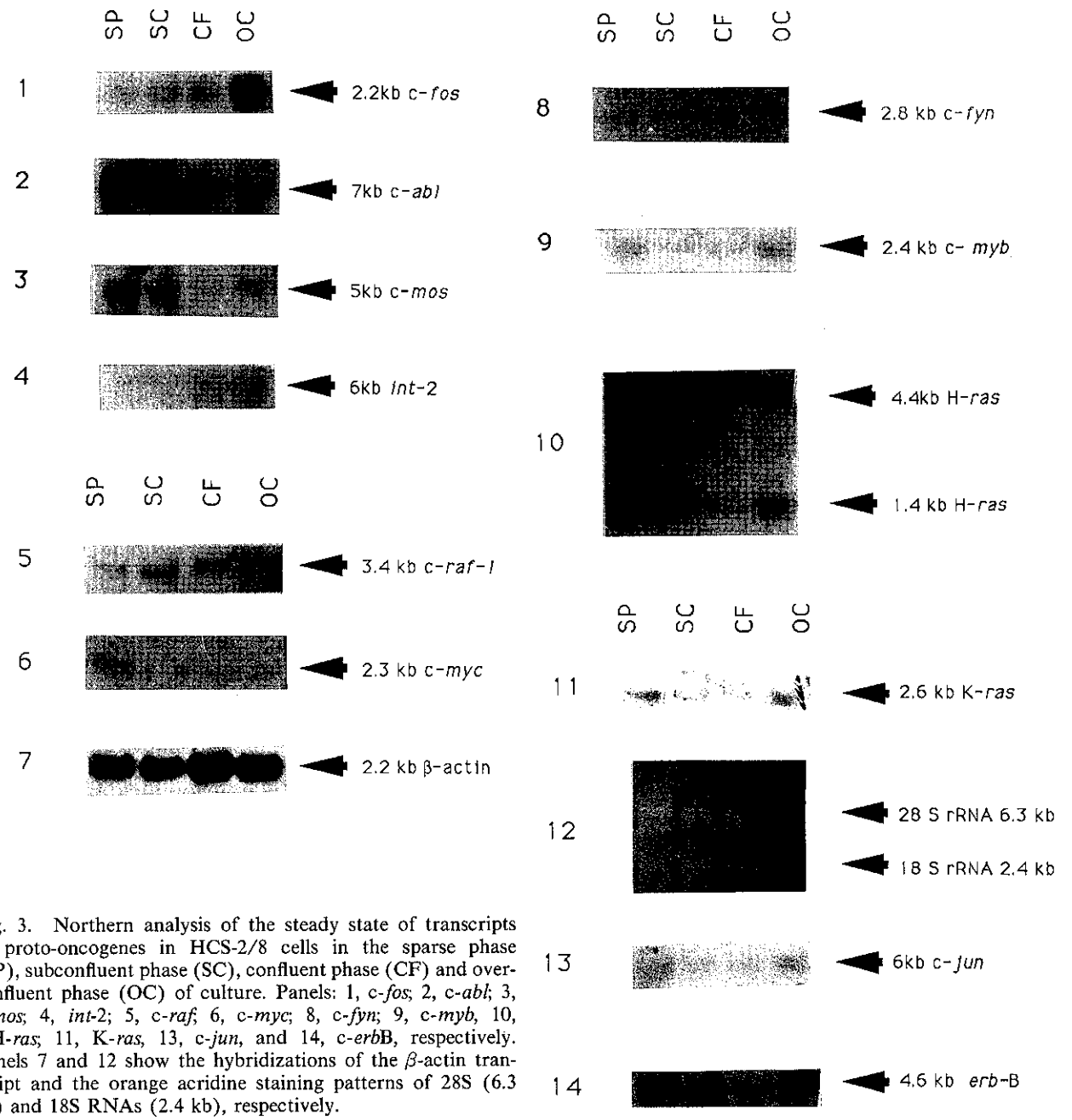

Fig. 3. Northern analysis of the steady state of transcripts of proto-oncogenes in HCS-2/8 cells in the sparse phase (SP), subconfluent phase (SC), confluent phase (CF) and over-confluent phase (OC) of culture. Panels: 1, c-*fos*; 2, c-*abl*; 3, c-*mos*; 4, *int*-2; 5, c-*raf*; 6, c-*myc*; 8, c-*fyn*; 9, c-*myb*, 10, c-H-*ras*; 11, K-*ras*, 13, c-*jun*, and 14, c-*erb*B, respectively. Panels 7 and 12 show the hybridizations of the β-actin transcript and the orange acridine staining patterns of 28S (6.3 kb) and 18S RNAs (2.4 kb), respectively.

maintained at the same level throughout the various phases of culture (Fig. 3). But in the over-confluent phase, the levels of c-*fos* and c-*raf*-1 transcripts were increased several dozen-fold and approximately 5-fold, respectively, while that of c-*abl* decreased 5-fold in comparison to their levels in the earlier phases of culture. The level of *int*-2 transcripts was increased 10-fold in both the confluent and over-confluent phases of *in vitro* culture. The transcript levels of c-*mos* and K-*ras* were higher in the sparse phase, lower in both the subconfluent and confluent phases and higher in the over-confluent phase.

DISCUSSION

The general expression pattern of proto-oncogenes has been described in a number of cell lineages as well as

their malignant counterparts, but little has been reported on either human chondrocyte or chondrosarcoma systems, due to the lack of appropriate *in vitro* and *in vivo* systems for their study. However, in this work use of a stable human chondrosarcoma cell line, HCS-2/8 cells, allowed us examine the steady-state levels of transcripts of moderately abundant proto-oncogenes (Fig. 3 and Table II). Since both acquisition and maintenance of a differentiated state are associated with qualitative and quantitative changes in the expression pattern of genes, we also examined the correlations of the transcript levels of these proto-oncogenes with definable morphological and biochemical changes of the cells in culture (Figs. 1 and 2).

The proto-oncogene subjected to the most intensive study in the chondrocytic differentiation system is c-*fos*. Closs *et al.*[38] demonstrated c-*fos* expression in the chondrocytes in the hypertrophic zone of mouse mandibular condyles by *in situ* hybridization and suggested that c-*fos* plays a crucial role in the perturbation of determined pathways of skeletoblast differentiation and in the regulation of endochondral bone formation. On the other hand, Sandberg *et al.*[39] showed that c-*fos* expression was particularly prominent in cells of the perichondrium/periosteum and in the chondrocyte layers nearest to the synovial cavity. Studies on osteogenic tumors from c-*fos* transgenic mice showed that elevated c-*fos* expression is associated with a variety of expression patterns of the genes characteristic of differentiated bone cells.[40] Moreover, mice lacking c-*fos* as a result of gene targeting were reported to have bone and cartilage defects[41,42]; that is, the zone of proliferating cartilage was drastically reduced with a corresponding increase in the relative size of the hypertrophic chondrocyte zone. While it is certain that c-*fos* is involved in endochondral ossification, its role in cartilage is still obscure. In the over-confluent phase of culture, HCS-2/8 cells had a several dozen-fold higher level of c-*fos* transcripts than in all earlier phases. This was confirmed by repeated experiments, although we did not examine the time course in detail. Moreover, a preliminary experiment showed that the cells in the center of nodules formed by HCS-2/8 cells stain positively with an antibody against the vitamin-D_3 receptor (unpublished), which has been shown to be abundant in hypertrophic chondrocytes.[43] Hence, HCS-2/8 cells in the over-confluent phase, especially cells in nodules, are likely to be phenotypically similar to hypertrophic chondrocytes *in vivo*. Our findings also suggest that c-*fos* plays a role in chondrocyte differentiation rather than proliferation, especially at a late stage of chondrocyte maturation.

It is now fairly clear how Fos and Jun form a transcription factor, AP-1 to control the transcription of a set of genes containing AP-1-binding sites.[44] However, the transcript level of c-*jun* in HCS-2/8 cells does not change significantly (panel 13, Fig. 3). Therefore, it would be interesting to assay AP-1 activity in HCS-2/8 cells in each phase of *in vitro* culture.

The expression of c-*myc* has been reported not to alter the process of chondrocyte differentiation.[45,46] However, recently, Quarto *et al.*[47] found that constitutive *myc* expression impairs hypertrophy and calcification in cartilage. In HCS-2/8 cells, the transcript level of c-*myc* does not change significantly, although the doubling time of cells changes greatly (panel 6, Fig. 3), suggesting that c-*myc* expression in HCS-2/8 cells is not closely associated with cell proliferation. As a 2.4 kb c-*myb* mRNA which is likely to be produced by alternative splicing[48] was maintained at a constant level (panel 9, Fig. 3), c-*myb* is not likely to be directly involved in the control of the proliferation of HCS-2/8 cells.

The transcript level of c-*raf*-1 gradually increased during *in vitro* culture of HCS-2/8 cells (panel 5, Fig. 3), suggesting that expression of c-*raf*-1 may be a good marker of chondrocyte differentiation. As a cytoplasmic protein-serine kinase, and a key factor in the intracellular network of signal transduction pathways,[44,49] c-*raf*-1 has been shown to enhance transcription of c-*fos*,[7] and so might be responsible for the increased transcript level of c-*fos* (panel 1, Fig. 3). However, further investigation should be performed to prove this hypothesis because expression of c-*raf*-1 was not exactly correlated with that of c-*fos*.

Among other cytoplasmic protein-serine kinases, the c-*mos* transcript was readily detectable (panel 3, Fig. 3). The transcript level of c-*mos* was high in this sparse phase, reduced in both the subconfluent and confluent phases and increased in the over-confluent phase. Although c-*mos* is important in control of the M-phase in meiosis, its role in control of the M-phase in somatic cells is still unknown.[50] In this regard, there is a recent report demonstrating c-*mos* expression in all types of human cells investigated.[51] Therefore, c-*mos* may play a physiological role(s) not only in germ cells but also in somatic cells.

The transcript level of H-*ras* remains constant throughout the four phases of *in vitro* culture, while the level of the K-*ras* transcript changes in a similar manner to that of the c-*mos* transcript (panels 10 and 11, Fig. 3). This suggests that the two *ras* oncoproteins may play different roles during the processes of proliferation and differentiation of HCS-2/8 cells. In some malignant tumors, *ras* oncogenes are activated by specific point-mutations,[52] so it will be of interest to determine if there is any specific point mutation in either the K-*ras* or H-*ras* gene in the chondrosarcoma-derived HCS-2/8 cell line.

The transcript of *int*-2 (FGF-related) was detected at a greatly increased level in both the confluent and over-confluent phases of culture (panel 4, Fig. 3). We have

detected abundant FGF-like molecules in HCS-2/8 cells by radioimmunologic assay (unpublished) and shown that basic FGF is a potent mitogen in chondrocytes.[53] Therefore, *int*-2 may be one of the autocrine FGF-related factors produced by HCS-2/8 cells and may play an important role in the growth of HCS-2/8 cells in late phases of *in vitro* culture.

In the category of non-receptor tyrosine kinases, no transcripts of c-*lyn*, c-*fgr*, and c-*src* were detected, while a 2.8 kb c-*fyn* transcript was readily detected at a constant level (panel 8, Fig. 3). However, due to lack of information concerning the involvement of non-receptor trks in chondrocytic lineages, the implication of these findings is unknown. A 7 kb species of c-*abl* transcript was found in HCS-2/8 cells, although in most human tissues there are two species of c-*abl* mRNA formed by alternative splicing of the primary transcript.[54] The level of this transcript was very high in the sparse and subconfluent phases, significantly lower in the confluent phase and further reduced in the over-confluent phase. It is not clear at the moment if there is a functional significance of the correlation between the transcript level of c-*abl* and the growth rate of HCS-2/8 cells.

In the category of receptor tyrosine kinases, *erb*B transcript was readily detected in HCS-2/8 cells (panel 14, Fig. 3). Because *erb*B encodes epidermal growth factor receptor lacking the ligand-binding domain,[17] the finding might be related to the lack of responsiveness of HCS-2/8 cells to EGF (unpublished).

Many oncoproteins are subjected to both activation and inactivation-related post-translation modifications such as phosphorylation, glycosylation and myristoylation. In this report, we observed moderately high levels of transcripts of the following proto-oncogenes with these properties: c-*fyn*, c-*abl*, c-H-*ras*, c-K-*ras*, c-*raf*, c-*mos*, c-*myc*, c-*myb*, c-*fos*, and c-*jun*. Therefore, for full understanding of the molecular and cellular mechanisms controlling the differentiation of chondrocytes and genesis of chondrosarcomas, it would be very informative to study post-translation modification in the context of consistent morphological and biochemical changes of HCS-2/8 cells during *in vitro* culture.

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