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Diverse Expression of G-Proteins in Human Sarcoma Cell Lines With Different Osteogenic Potential: Evidence for the Involvement of G_{i2} in Cell Proliferation

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Abstract Previously, it has been shown that the GTP-binding protein G_{i2} is implicated in cellular growth [1,2] and differentiation [2,3]. In the present paper we demonstrate that this is also the case for human sarcoma cells.

Six human osteosarcoma and three soft tissue sarcoma clonal cell lines were analyzed for levels of G-protein mRNA and polypeptide expression and effector enzyme (i.e., adenylate cyclase and phospholipase C) activation, which were all compared with individual growth rates. Unexpectedly, it appeared that the various strains exhibited large inter-individual variations in G-protein expression and signaling system activation. However, cell doubling time in the exponential phase of growth was inversely correlated ($r = 0.71$, $P < 0.05$) to immunodetected levels of intrinsic $G_{i2}\alpha$. Furthermore, cells stably transfected with a retroviral (pZipNeo(SV)X) construct containing the activating or inactivating $G_{i2}\alpha$-R179E or $G_{i2}\alpha$-G204A point mutations consistently reduced or enhanced individual cell strain doubling time, respectively.

It appeared that other parameters investigated, including cellular alkaline phosphatase and monoclonal antibody epitope binding, both being markers of the proliferating osteoblast, did not correlate with cell doubling times. © 1996 Wiley-Liss, Inc.

Key words: osteosarcomas, adenylate cyclase, phospholipase C, G-proteins, growth rate

The lifespan of the normal osteoblast entails a phenotypic change from the proliferating cell, which expresses the genes for histone 4 and collagen I through a maturation phase of alkaline phosphatase expression to the final stage of mineralization and mitotic arrest characterized by transcription of the genes for osteocalcin and osteopontin [4].

In contrast, the osteosarcoma cell is continuously dividing, albeit having retained several of the osteoblastic characteristics [5,6], is continuously dividing. This may be attributed to autonomy of some part(s) of the intracellular cascade of events triggered by growth factors (mitogens), which stimulate normal cells to reenter the cell cycle [4,5]. Each mitogen binds to specific high-affinity cell surface receptors, thereby initiating a sequence of events that culminates in DNA synthesis and mitosis. The links between occupation of the cell-surface receptors and nuclear events are poorly understood.

The transduction pathways implicated in the stimulation of cell growth are protein-tyrosine phosphorylation [7,8] and G-protein mediated activation of adenylate cyclase (AC) [9,10] and phospholipase C (PL-C) [8,10,11]. And somehow, GTP-binding proteins, such as $G_{q/11}$ (pertussis-toxin insensitive G-proteins involved in PIP_2 breakdown) and $G_s\alpha$, are involved in the mechanism of action of growth factors [8]. Recently, it has been demonstrated that the G-protein $G_{i2}\alpha$ is coupled to the insulin-like growth factor II (IGF-II) receptor [12]. Furthermore, this G-protein has been shown to facilitate IGF-II elicited DNA synthesis [13], fibroblast growth rates [1,2], and preadipocyte differentiation [2]. Since growth factors and PTH interfere with each others biological effect [14,15], it is highly probable that $G_s\alpha$, $G_{q/11}\alpha$, and $G_{i2}\alpha$ may affect cellular growth of the osteoblast and osteosarcoma cell.

Consequently, we have analyzed several different human sarcoma cell lines in terms of G-

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protein mediated cell signalling, i.e., production of the second messengers cAMP and inositol 1,4,5 trisphosphate (IP_3), and attempted to assess whether the three G-proteins may be involved in cell growth rates under equal growth conditions.

MATERIALS AND METHODS

Cell Lines

The human osteosarcoma cell lines TPXM and KPDXM, as well as the soft tissue sarcoma cell lines, were established as monolayer cultures via spheroids as previously described [16]. The osteosarcoma cell lines OHS [17] and the unclassified sarcoma cell line MHM [18] were established from biopsies by expanding primary tumor cell colonies from soft agar. The osteosarcoma line 788T was kindly supplied by Dr. E.J. Embleton (Nottingham, England), and the rat osteosarcoma line UMR-106 [6] was obtained from Dr. K. Gautvik (Oslo, Norway). The carcinoma cell line HT1080, derived from a human fibrosarcoma [19], and the SKES-1 small cell sarcoma line from ATTC (American Tissue Type Collection) were purchased from Flow Laboratories (Irvine, Scotland).

Cell Culture

Sarcoma cell lines were grown in either DMEM, RPMI, or Ham's F-10 medium (GIBCO) supplemented with up to 6.5% horse and 3–10% fetal calf serum (GIBCO) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Penicillin (50 IU/ml), streptomycin (50 μg/ml) (GIBCO), and fungizone (2.5 μg/ml) (Flow Laboratories) were added to the culture medium. This was changed every 2–3 days and always 24 h before harvest.

RNA Extraction

Total cell RNA was extracted from osteosarcoma cell lines by homogenization and centrifugation through CsCl as described elsewhere [20].

Northern Blot Analysis

RNA was separated on 1.4% agarose gels in 18% formaldehyde and 3-[N-morpholino] propane sulphonic acid (MOPS) buffer and capillary blotted onto Hybond-N nylon filters (Amersham) or BioTrans nylon filters (ICN) in Tris-Acetate-EDTA buffer. Plasmids containing cDNA clones for α-subunits of G-proteins in pGEM-2 were kindly supplied by Dr. Randall R. Reed (USA). ^{32}P-labelled cDNA probes were made from gel purified cDNA fragments containing unique sequences by the method of random priming. Prehybridization was done in 5× Denhardt's solution, 5× standard saline citrate solution (SSC), 50 mM sodium phosphate, pH 6.5, 0.1% SDS, 250 μg/ml herring sperm DNA, and 50% (v/v) formamide at 42°C for 2 h. Hybridization was performed in the same buffer, including approximately 2×10^6 dpm labelled DNA per ml at 42°C overnight. Filters were washed 4 times in 2× SSC, 0.1% SDS at room temperature, and twice for 15 min each at 50°C in 0.1× SSC, 0.1% SDS, and then exposed for various times to Hyperfilm MP (Amersham) or XAR-5 film (Kodak) at −70°C.

Preparation of Subcellular Particulate Fractions

The medium was removed and the cells scraped in ice-cold 150 mM NaCl, 10 mM Tris-HCl, pH 7.5, and pelleted (700*g*, 10 min, 4°C). The cell pellet was washed once with the same buffer, resuspended in 20 volumes of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and eventually homogenized on ice using an Ultra-Turrax (Janke Kunkel, Germany) for 10 s. The homogenate was subsequently filtered through nylon mesh and centrifuged at 27,000*g* for 30 min at 4°C. Finally, the pellet was resuspended in 10 volumes of Tris-EDTA buffer containing 0.1% bovine serum albumin, using the rotating knife for 5 s.

Western Blotting and Immunostaining

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described [21]. Cell membrane samples were prepared for electrophoresis by dilution in sample buffer and heating to 100°C for 5 min. Proteins were electrophoretically transferred from gel to PVDF Immobilon-P filters (Millipore) with constant current (100 mA) for about 12 h in a Bio-Rad Transblot™ apparatus. After transfer, the filters were incubated for 2 h with phosphate-buffered saline (PBS), including 5% fat free dry milk powder. Subsequent incubation lasted for 6–24 h at room temperature in PBS containing 1% dry milk powder and anti G-protein antisera. After being washed with PBS containing 0.1% Tween 20, the filters were incubated with ^{125}I-protein A (150,000 cpm/ml, Amersham) in PBS containing 1% dry milk powder for 1 h at room temperature. The filters were then washed exten-

sively with PBS containing 0.1% Tween 20 and dried prior to autoradiography.

G-Protein α-Subunit Antibodies

Rabbit antisera against synthetic decapeptides corresponding to the predicted C-terminal amino acid sequences of different G protein α-subunits were a generous gift from Dr. Allen M. Spiegel (USA). Antisera were used at final dilutions of 1/200 to 1/400.

Adenylate Cyclase (AC) Assay

AC activity was measured in 20 μl aliquots of crude subcellular fractions containing 20–50 μg of protein per assay tube. The total incubation volume was 50 μl and consisted of 1 mM ATP (including 0.8×10^6 cpm of [α-^{32}P]-ATP (Amersham, England), 10 μM GTP, 2.8 mM $MgCl_2$, 1.4 mM EDTA, 1 mM cAMP (containing approx. 5.8×10^3 cpm of [8-^{3}H]-cAMP (Amersham)), 20 mM creatine phosphate, 0.2 mg/ml creatine kinase, 0.02 mg/ml myokinase, and 25 mM Tris-HCl, pH 7.4, in the absence or presence of PTH (1 μM), PGE_2 (10 μg/ml), Gpp(NH)p (20 μM), or forskolin (200 μM). Incubations were carried out at 35°C for 20 min. Reactions were stopped with 0.1 ml of a solution comprising 10 mM cAMP, 40 mM ATP, and 1% SDS. The [^{32}P]-cAMP formed and the [^{3}H]-cAMP added to monitor recovery (65–80%) were isolated using combined Dowex and aluminum oxide chromatography. Coefficient of variation (CV) for this effector enzyme analysis was 4.3%.

Phospholipase C Assay

Aliquots (20 μl) of diluted crude membrane suspensions (20–50 μg protein) in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, were mixed with 10 μl incubation mixture (100 mM Tris-HCl, pH 6.5, 400 μM GTP, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$) and 10 μl of PTH (10 μM) or Gpp(NH)p (200 μM) in 2.4 ml microfuge tubes on ice. Three microliters (42,000 cpm) of a [^{3}H]-PIP_2 (New England Nuclear, USA) stock solution in 5% sodium cholate were added to each tube, and incubation carried out at 35°C for 5 min. The reaction was stopped by adding in succession 150 μl $CHCl_3$:CH_3OH:HCl (1:2:0.02), 50 μl $CHCl_3$, and 50 μl 2 M KCl. After vortexing and phase separation at 5,000*g* in a microfuge, 100 μl aliquots of the aqueous layers were counted in a liquid scintillation counter. In the controls, approximately 2% of the radioactivity was retained in the aqueous phase. Coefficient of variation (CV) for this effector enzyme analysis was 8.3%.

Extraction of Genomic DNA

The monolayer cultures were washed 3 times with PBS. One ml of 20% Na-sarkosyl and 1 ml of 7.5 M NH_4-acetate were added, and the culture flask swirled until complete cell lysis. Fifty microliters of proteinase K (50 mg/ml) were added, and the lysate heated at 60°C for 1 h. This last step was repeated once. Thirty-five milliliters of absolute ethanol were subsequently added and the solution left at ambient temperature for 30 min. DNA threads were removed with a pasteur pipette, washed with 70% ethanol, and centrifuged for 5 min at 1,600*g* (room temperature). Finally, the pellet was dissolved in 1.5 ml of TE-buffer, pH 8.0, overnight.

PCR-Amplification of Genomic DNA

Approximately 1 μg of DNA was amplified in a 100 μl PCR mixture containing deoxynucleotide triphosphates (200 μM) and upstream and downstream oligonucleotide primers (0.5 μM each) annealing to the $G_s\alpha$ gene, 0.01% (w/v) gelatin, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 or 2.5 mM $MgCl_2$, and 2.5 units Taq polymerase (Perkin Elmer Cetus, USA). The reactions contained 2.5 mM $MgCl_2$ for exon 9 and 1.5 mM for exon 8. Amplification consisted of denaturing at 94°C for 5 min followed by 30 cycles of annealing at 58°C for 45 s, primer extension at 72°C for 1 min, and denaturing at 94°C for 1 min. One final cycle with a 3 min primer extension followed. The PCR products were analysed on 4–20% nondenaturing acrylamide gradient gels. For exon 8, the primers were 5′-TCGGTTGGCTTT-GGTGAGATCCA-3′ and 5′-CGCCCGCCGCGC-CCCGCGCCCGTCCCGCCGCCCCCGCCCCA-GAAACCATGATCTCTGTTATA-3′. For exon 9, the primers were 5′-AACTGCAGCCAGTCCC-TCTGGAATAACCAG-3′ and 5′-CGCCCGCCG-CGCCCCGCGCCCGTCCCGCCGCCCCCGCC-CCAGCGACCCTGATCCCTAACAAC-3′. Amplification of similar regions on the $G_{i2}\alpha$ gene (exons 5 and 6) was conducted using a similar protocol with an annealing temperature of 55°C and a Mg^{2+}-concentration of 1.5 mM. For exon 5, the primers were 5′-CATCCCCAGCTACC-TGAACG-3′ and 5′-GCTCACTTGAAGTGTAG-GTCC-3′ (with the GC-clamp attached to the 5′-end). For exon 6, the primers were 5′-GGAT-CGTGGAGACACACTTC-3′ and 5′-TCTCAC-

CATCTCCTCGTCCT-3′ (with the GC-clamp attached to the 5′-end).

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed in a Hoefer SE600 vertical apparatus maintained at 60°C using an LKB Multitemp II thermostatic circulator. PCR samples (10–15 μl) were electrophoresed for 15–20 h at 85 V on a 7% acrylamide vertical gel with parallel gradients containing linearly increasing denaturing conditions. The denaturing gradients used to reveal mutations on the $G_s\alpha$ gene were 35% to 65% for exon 8 and 35% to 90% for exon 9. The multiplied $G_{i2}\alpha$ gene stretches were analysed on 35% to 90% gels (both exons 5 and 6) (100% denaturing condition is 7M urea and 40% v/v formamide). Gels were stained with silver (Bio-Rad kit).

Site Directed $G_{i2}\alpha$ Mutations and Construction of Expression Vectors

The insert of plasmid DJG18, encoding a rat α_{i2} cDNA (provided by R. Reed, Johns Hopkins University) was subcloned into M13mp19 and site-directed mutagenesis was performed by a modification of the method of Nakamaye and Eckstein [22]. The mutagenic oligonucleotides had the following sequences: R179E α_{i2}; 5′-GCCTGTGGTCTTCACTTCGGTCCGCAG-CACATC-3′ and G204A α_{i2}; 5′-CCGCTCAGATC-GCTGGGCGCCCACATCAAACAT-3′. Mutations were confirmed by dideoxy sequencing. Wild-type and mutant α_{i2} cDNAs were ligated into the EcoRI site of a shuttle vector pSP72NotI and then into the NotI site of a modified retroviral vector, pZipNeoSV(X) [23]. In the latter constructs, the transcription of α_{i2} cDNAs and the G418-resistance gene is directed by the Moloney murine leukemia virus long terminal repeat.

Transfection and Clone Selection

Cell lines were transfected with 1 μg of linearized DNA per 10-cm plate by calcium phosphate precipitation. Twenty-four hours after transfection, cells were grown in selective medium containing G418 (0.60 mg/ml) for at least 3 weeks. G418-resistant clones were selected by serial dilution. Ten clones were selected for each construct, and the studies described here were performed with the clones (three) demonstrating the highest level of α_{i2} expression.

^{3}H-Thymidine Incorporation

Cells were incubated with 0.1 μCi of ^{3}H-thymidine for 1 h six times during exponential growth in 6.5% horse and 3.5% fetal calf serum. Subsequent to each incubation period, the cells were washed (3 times) with ice-cold PBS and solubilized with 0.1% SDS. Material precipitated by 20% TCA was dissolved in 1N NaOH and neutralized with 1N HCl.

Antibody Binding and Blocking Assays

An IRMA was used to quantitate the antigen. Triton X-100 extracts were prepared from unlabeled cells grown in vitro. The TP-MoAbs were directly coupled to tosylated monodisperse beads (Dynabeads CA-031-A; Dynal, Oslo, Norway) according to the method of Nustad et al. [24]. The immunobeads were allowed to react with the different extracts at 4°C overnight and pelleted. After a washing in PBS containing 0.5% Tween 20 and 1% HSA, the particle-bound material was assayed by incubating the washed beads with radiolabelled TP-1 or TP-3 for 4 h. After washing, solid phase bound radioactivity was measured in a multiwell-type gamme counter (LKB-Wallace, Bromma, Sweden).

Alkaline Phosphatase Determinations

Measurements were performed on incubated cells or washed Immunobeads (see above) in a pH 10.5 buffer (1 M diethanolamine/0.5 M $MgCl_2$) (Merck, Darmstadt, West Germany), containing 10 mM p-nitrophenylphosphate (Sigma, St. Louis, MO). The standard for enzymatic activity was purchased from Sigma. After 10 min of incubation at 37°C, the reaction was terminated by the addition of 0.1 M KOH, the samples were cleared by centrifugation, and the absorbance at 405 nm was measured [18].

Statistical Evaluations

Between group analyses were performed by Wilcoxon rank test at the level of $P = 2\alpha = 0.05$. Covariations between variables were assessed by correlation matrix analyses (Statpro, Penton Software Inc., NY) accounting and correcting for mass significance.

RESULTS

Growth Characteristics of Osseous Sarcoma Cell Lines

The doubling time (T_d) of each cell line was assessed in the exponential phase of growth. For

the various cell lines, T_d ranged from 24.7 to 54.6 h (Table I). Parallel measurements of the proliferation parameter cellular alkaline phosphatase activities in the same cells, ranged from almost nil (0.01) to 4.12 U/mg cell protein (Table I). Monoclonal antibody TP-1 and TP-3 reactive epitopes, which have been shown to serve as a marker of the proliferating osteoblast/osteosarcoma cell, were only expressed in demonstrable quantities on the SaOS-2, KPDXM, and OHS osteosarcoma cells (Table I).

Hormone-Elicited Signalling Systems In Osseous Sarcoma Cell Lines

Since cAMP and DAG as second messengers might play a role in cell proliferation, basal and hormone-elicited adenylate cyclase (AC) and phospholipase C (PL-C) activities were determined in crude membranes from the cell lines in question. Basal AC of the various sarcoma cell lines ranged between 28.4 and 90.6 pmol cAMP/mg protein/min with the exception of the clones 788T (osteosarcoma) and HT1080 (soft tissue sarcoma), which displayed activities of 453 and 145 pmol cAMP/mg protein/min, respectively. Basal PL-C activities ranged between 3800 and 8300 cpm of IP_3 + IP_2/mg protein/min for all the cell lines (tested) (Fig. 1). Basal effector enzyme activities showed normal distribution (Q-Q plot) when AC activities of the cell lines 788T and HT1080 were exempted from the analysis. The reason for such high basal AC activities may reside in activating $G_s\alpha$ mutations, and is discussed later.

TABLE I. Growth Characteristics and Cellular Expression of Alkaline Phosphatase or Epitopes for the Monoclonal Antibodies TP-1 and TP-3 in Human Sarcoma Cell Lines

Cell lines	t_d (h)[a]	ALP (U/mg protein)[b]	TP1-1, TP-3 expression[c]	
Human osteosarcomas				
KPDXM	54.6	2.12	2+	2+
OHS	29.2	3.92	3+	3+
SaOS-2	40.8	3.08	3+	3+
TPXM	53.0	1.01	—	—
SKES-1	47.3	nd	—	—
788T	45.3	4.12	—	—
Rat osteosarcoma				
UMR 106	nd	0.82	—	—
Human soft tissue sarcoma				
HEXM	24.7	nd	nd	nd
MHM	37.3	0.02	—	—
HT1080	27.4	0.01	—	—

[a]Cell growth (t_d = doubling time) was measured as ^{3}H-thymidine incorporation into TCA-precipitable material during the exponential growth phase in the presence of 10% serum (see Materials and Methods).
[b]Alkaline phosphatase activity (units/mg protein) was measured by incubating whole cells in a mixture containing p-nitrophenylphosphate.
[c]TP expression was assessed by incubating Triton X-100 cell extracts with TP-MoAbs coupled to tosylated monodispersed beads and expressed as relative intensities of cell surface fluorescence staining.

Relative PTH-elicited AC activity was normally distributed and ranged between 1.2 and 4.9, when the value 17.1, belonging to the rat osteosarcoma cell line UMR-106, was excluded. Otherwise, relative PGE_2-, Gpp(NH)p-, and forskolin-activated AC activities were normally distributed with ranges of 1.0–4.4, 1.1–2.0, and 2.9–10.0, respectively (Fig. 2A). Relative PTH- and Gpp(NH)p-stimulated PL-C activities ranged between 1.2–1.8 and 1.4–2.6, respectively, and were both normally distributed (Fig. 2B). There appeared to be a significant relationship ($r = 0.71$, $P < 0.05$, $n = 9$) between PTH-AC and PTH-PL-C activities of the human sarcoma cell lines tested.

Steady-State Levels of G-Protein mRNA and Polypeptide Levels Under Conventional Growth Conditions

The relative contents of G-protein mRNA, based on densitometric analyses of Northern blot autoradiograms, are listed in Table II. It appeared that mRNA for $G_s\alpha$, $G_{i2}\alpha$, and $G_{36}\beta$, when excluding data on the rat UMR-106 osteosarcoma cell line, ranged from 1.0 to 20, 1.0 to 10, and 1.0 to 16, respectively. Similar wide distributions were observed for corresponding polypeptide levels ($G_s\alpha$, 1.0–26; G_{i2}, 1.0–13; $G_{q/11}$, 1.0–3.2; $G_{36}\beta$, 1.0–4.8) as well. However, there were no positive correlations ($P > 0.05$) between steady state G-protein mRNA and corresponding membrane polypeptide levels (data not shown).

Assessment of Signal System Elements as Predictors of Cell Growth

In an attempt to analyse the possible impact of various elements of the signaling system cascade analyzed, a matrix correlation analysis was performed (Table III). It appeared that the doubling time, t_d, was significantly ($P = 0.03$) and inversely ($r = -0.71$, $n = 9$) associated with the

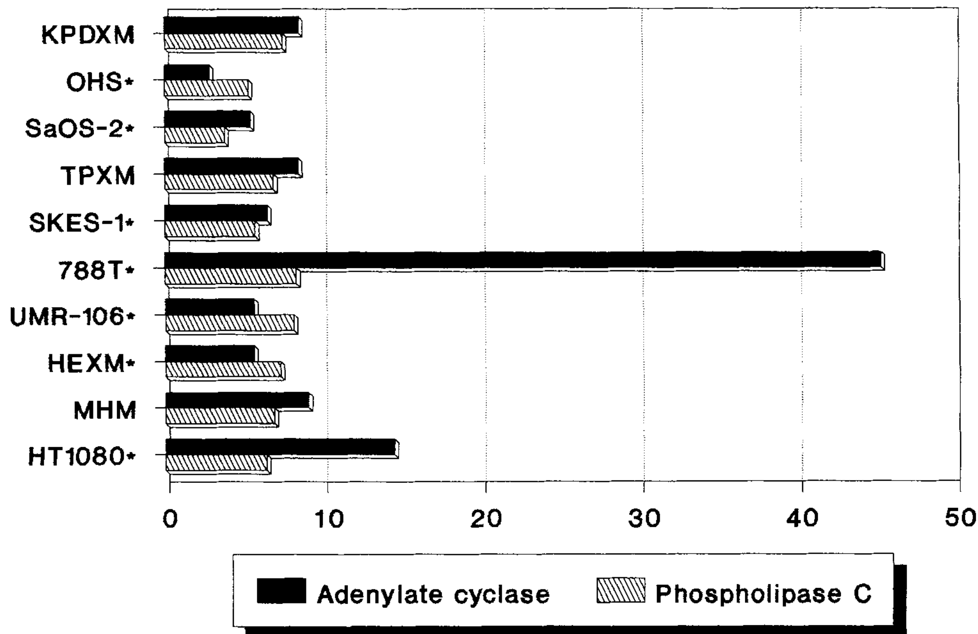

Fig. 1. Basal adenylate cyclase (AC) and phospholipase C (PL-C) activities in human sarcoma cell membrane fractions. Activities are expressed as 10^1 pmol cAMP/mg protein/min (AC) or 10^3 cpm IP_3 + IP_2/mg protein/min (PL-C) and depicted as means of quadruplicate determinations. The effector enzyme analyses were repeated twice with similar results. The average coefficients of variation (CVs) for the enzyme assays were 4.4% and 10.8%, respectively. *Indicates (Wilcoxon rank test) significant differences ($P < 0.05$) from the KPDXM osteosarcoma cell line in terms of both AC and PL-C activities.

steady-state polypeptide level of $G_{i2}\alpha$ only. This correlation, as well as the relationships t_d vs $G_s\alpha$ and $G_{q/11}\alpha$ protein levels, is depicted in Figure 3.

Transfection of Osteosarcoma Cell Lines With a Retroviral Construct Containing Mutated Rat $G_{i2}\alpha$ cDNA

The pZipNeo(SX) retroviral construct containing either the activating R179E or the inactivating G205A missense mutations of $G_{i2}\alpha$ were transfected into six human osteosarcoma cell lines and selected for by using geneticin (G418), 600 μg/L. Stably transfected clones were isolated by the dilution method and tested separately for G-protein translation. All clones tested (at least three per cell line and construct) expressed at least six times the endogenous level of $G_{i2}\alpha$ (Fig. 4).

Adenylate Cyclase Activation in Transfected Human Osteosarcoma Cell Lines

The PTH- and Gpp(NH)p-elicited adenylate cyclase (AC) in transfected cell lines were assessed and computed as percent of net control stimulation (100%) for each individual cell line containing the various constructs (R179E or G204A). The average PTH- or Gpp(NH)p-stimulated activations compared with controls are depicted in Figure 5. It appeared that expression of the missense mutation R179E in average reduced PTH- and Gpp(NH)-elicited AC activities by some 24% and 20%, respectively, while G204A enhanced them by some 52% and 30%.

Impact of Activating and Inactivating $G_{i2}\alpha$ Mutants on Human Osteosarcoma Cell Growth Rates

Doubling time, t_d, of each of the six individual cell lines were consistently lowered in the presence of the activating missense mutation R179E, while consistently enhanced in the presence of the inactivating point mutation G204A (Fig. 6).

DISCUSSION

Normal bone growth and bone remodelling depends on the orderly interplay of various regulatory factors that affect the osteoblast [25]. The most widely studied of these are the classic humoral hormones parathyroid hormone (PTH) and 1,25-dihydroxyvitamin D_3. More recently, however, considerable work has focused attention on the importance of various para- or autocrine peptide growth factors, such as transform-

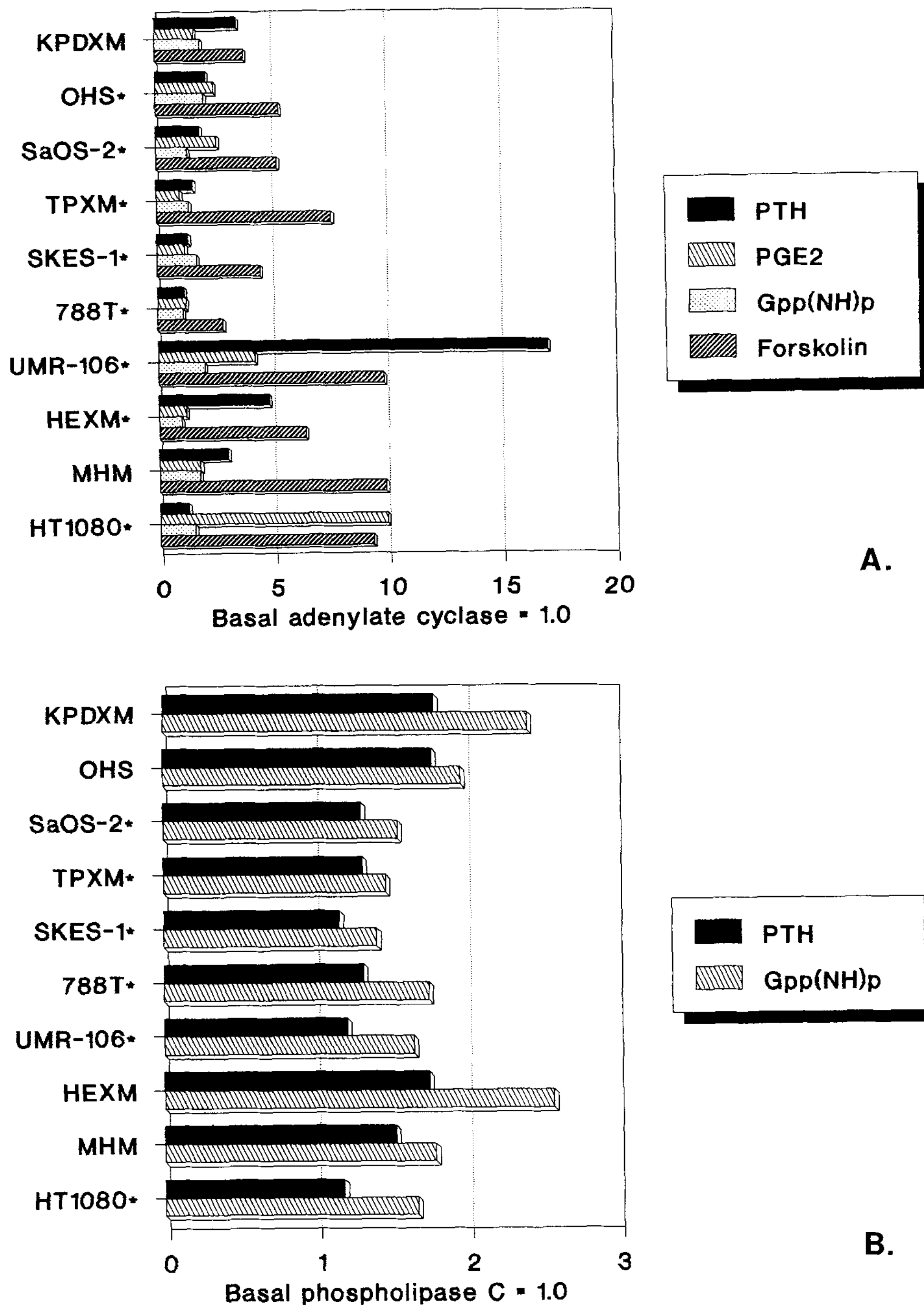

Fig. 2. Hormone-, Gpp(NH)p- and forskolin-enhanced adenylate cyclase (AC) and phospholipase C (PL-C) activities in human sarcoma cell membrane fractions. Effector enzyme stimulation was accomplished in the presence of 1-84 hPTH (10^{-6}M), PGE_2 (10 μg/ml), Gpp(NH)p ($4 \cdot 10^{-5}$M), forskolin ($4 \cdot 10^{-4}$M) (AC); or 1-84 hPTH (10^{-6}M), Gpp(NH)p ($4 \cdot 10^{-5}$M) (PL-C). Activities are given as stimulations relative to basal (=1.0) and depicted as means of quadruplicate determinations. This set of data is representative of three individual experiments. The average coefficients of variation (CVs) for the enzyme assays were 3.0% and 6.4%, respectively. *Indicates (Wilcoxon rank test) significant differences ($P < 0.05$) from the KPDXM osteosarcoma cell line in terms of both PTH- and Gpp(NH)p-susceptibility.

TABLE II. Relative Amounts of G-Protein mRNA and Polypeptide Levels in Human Osseous Sarcoma Cell Lines*

	mRNA			Polypeptide			
Cell lines	$G_s\alpha$	$G_{i2}\alpha$	$G_{36}\beta$	$G_s\alpha$	$G_{i2}\alpha$	$G_{q/11}$	$G_{36}\beta$
Human osteosarcomas							
KPDXM	1.5	1.4	5.5	1.0	5.2	3.1	3.4
OHS	3.5	4.8	15.6	1.4	8.0	2.7	1.9
SaOS-2	3.7	2.6	6.9	6.1	1.7	1.5	1.0
TPXM	19.8	1.7	4.8	25.5	1.0	1.7	2.4
SKES-1	3.8	5.7	10.1	4.8	4.3	1.2	3.0
788T	13.5	1.0	1.0	20.1	3.1	2.3	1.6
Rat osteosarcoma							
UMR 106	21.0	31.8	1.2	8.5	3.7	1.0	5.0
Human soft tissue sarcoma							
HEXM	1.0	9.8	3.1	5.3	13.4	3.2	4.8
MHM	1.6	4.3	2.8	14.4	10.4	1.7	5.1
HT1080	1.9	1.7	1.8	2.3	6.1	1.5	2.9

*Total cell mRNA was extracted, separated, blotted onto filters, and hybridized with ^{32}P-labelled cDNA probes as described in Materials and Methods. Cell membrane protein was separated by PAGE, blotted, and incubated with crude antisera against G-protein C-terminal decapeptides followed by ^{125}I-protein A. Autoradiograms were scanned in an automatic XRS OmniMedia Scanner. The lowest level of G-protein mRNA or peptide species, irrespective of cell line, was arbitrarily set equal to 1. Differences in relative steady-state contents of G-protein mRNA or peptide less than 0.35 (35%) are not considered statistically significant (Wilcoxon rank test, n = 6). The experiment was repeated once with similar results.

TABLE III. Binary Correlations Between Doubling Time (t_d, h) and G-Protein Levels as Well as Basal, Hormone-Elicited, or Gpp(NH)p-Enhanced Adenylate Cyclase (AC) and Phospholipase C (PL-C) Activities in Human Osseous Sarcoma Cell Lines*

Variables	r	*P*	n
t_d versus—			
$G_{i2}\alpha$	−0.71	0.03	9
$G_s\alpha$	−0.37	0.26	9
Basal AC	0.18	0.64	9
PTH-AC	−0.28	0.47	9
Gpp(NH)p-AC	0.15	0.73	8
t_d versus—			
$G_{q/11}$	−0.17	0.66	9
Basal Pl-C	0.20	0.60	9
PTH-PL-C	−0.20	0.60	9
Gpp(NH)p-PL-C	−0.30	0.47	8
t_d versus—			
$G_{36}\beta$	−0.25	0.51	9

*Correlations were obtained through a PC-based matrix analysis using Statpro software (Penton Software Inc., NY).

ing growth factor β (TGF_β), epidermal growth factor (EGF), fibroblast growth factor (FGF), tumor necrosis factor (TNF), the insulin like growth factors (IGF-I and IGF-II), and other lymphokines and cytokines [25]. In the presence of these factors, the normal osteoblast undergoes a phenotypic development from a state of proliferation to a phase where mineralization prevails [4]. The osteosarcoma cell is continuously proliferating with an apparent abberation in its phenotypic development, which never completely attains mineralizing characteristics [4,6]. Apart from their prevailing activation of tyrosine kinases, the growth factors are also involved in the stimulation of second messenger synthesis, that is, the production of both cAMP and 1,2-diacyl glycerols (DAG) [7,8,26–28]. Hence, both protein kinases A and C may be actively involved in cellular growth. The IGF-II receptor has been shown to couple to the GTP-binding protein G_{i2}, which, through a hitherto unknown effector element, mediates at least some effects of the IGF-II molecule [12,13,29]. Consequently, this paper deals with the impact of G-protein mediated signalling on growth of the human osseous sarcoma cell lines.

The sarcoma cell lines tested have been subjected to an exhaustive analysis of conditions normally determining aberrant growth, such as the presence of mutations in the antioncogene encoding the p53 protein [30], but not to contingent over-expression of the aberrant transcription factors jun, fos, or myc, autonomously functioning truncated tyrosine kinase receptors etc [31]. It is therefore not feasible to explain why there is such a large variation in individual cell line doubling time. Furthermore, their exact phenotype as regards transition between the

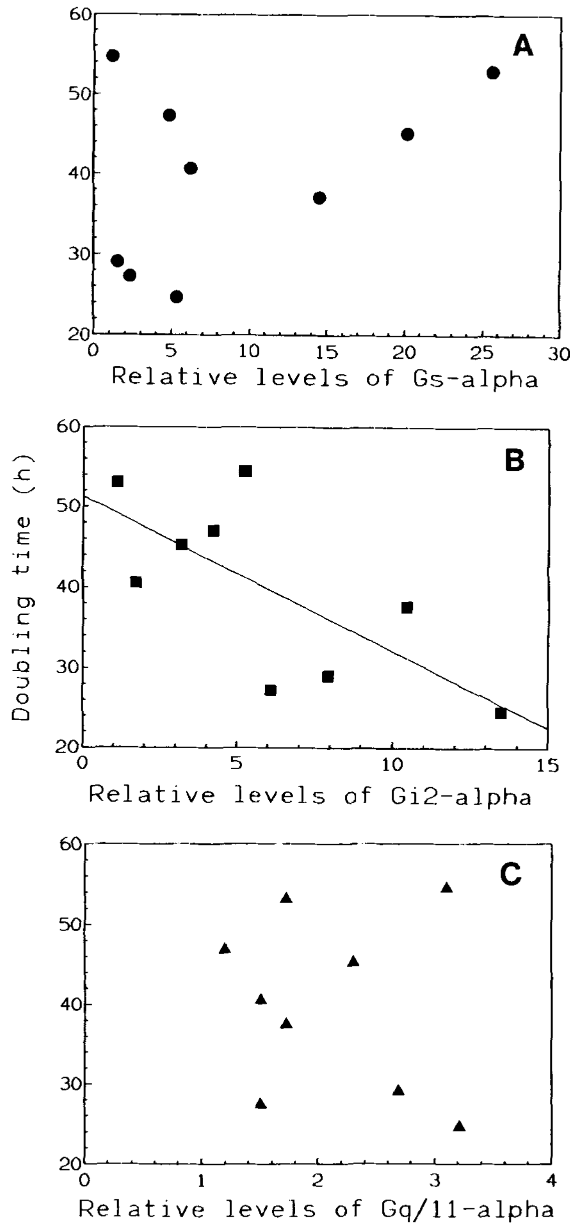

Fig. 3. Relationship between immunodetected G-protein α-subunit levels and doubling time (t_d) of human sarcoma cell lines. $G_s\alpha$ (**A**), $G_{i2}\alpha$ (**B**), and $G_{q/11}$ (**C**) levels and t_d (h) of individual cell lines wre assessed as described (see Tables I and III). Linear regresion analyses revealed that $G_{i2}\alpha$ and t_d correlated inversely (r = −0.71) at the level of $P = 2\alpha < 0.05$.

proliferating and mineralizing cell has not been assessed.

Alkaline phosphatase (ALP), which in some osteosarcoma patients was strongly linked to progression of the disease, proved not to be related to individual cell clone growth rate as assessed by population doubling time. According to Pockwinse et al. [32], the ALP gene is not an irrefutable marker for proliferation. It is therefore not surprising that cellular ALP activities did not correlate well with cell growth rates. Neither were the presence of epitopes for the monoclonal antibodies predicitive of growth rate. It has previously shown that the epitope for these antibodies, which clearly are markers for osteosarcomas and proliferating osteoblasts, are lost in xenografted lines, and when permanent monolayer cultures are established [18].

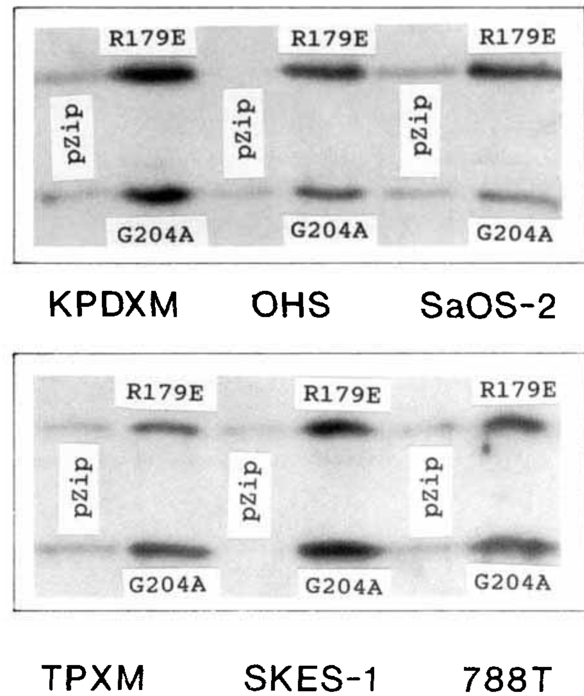

Fig. 4. Expression of the $G_{i2}\alpha$ subunit in osteosarcoma cells transfected with the vector pZipNeoSX(V) or the mutants R179E and G204A (H21a). Crude membrane proteins (20 μg/lane) were resolved on SDS popyacrylamide, trans-blotted, and the α-subunit visualized with antisera/^{125}I-protein A as described in Materials and Methods.

As mentioned earlier, signal flow through the adenylate cyclase and phospholipase C pathways have been implicated in the effect of both peptide hormones and growth factors on cell proliferation. However, neither basal- nor hormone-elicited adenylate cyclase, or the steady-state levels of $G_s\alpha$ seemed to consistently play a role in osseous sarcoma cell growth. This contention was further substantiated by the fact that the osteosarcoma cell line 788T, which displayed the highest basal adenylate cyclase activity, was among the slowest-growing cell lines (t_d = 45.3 h). This cell line most probably contained a monoallelic missense mutation in exon 9 of the $G_s\alpha$ gene, which rendered the activating

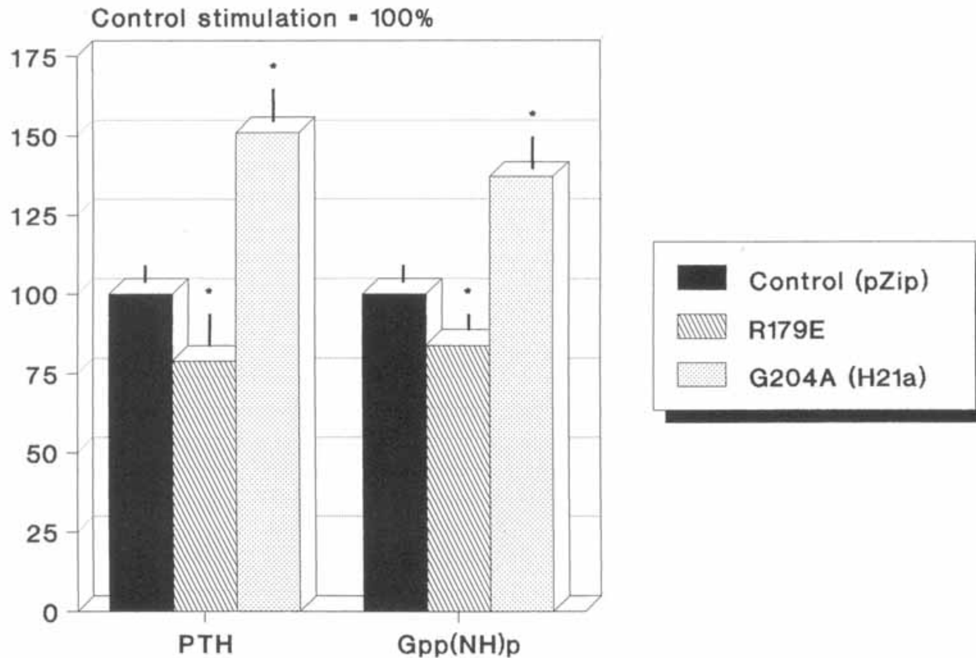

Fig. 5. Average alteration in relative PTH-elicited adenylate cyclase (AC) in human osteosarcoma cell lines after stable transfection with either vector alone or constructs with mutated rat $G_{i2}\alpha$ cDNA. The R179E and G204A (H21a) mutants were inserted into a modified pZipNeoSX (V) retroviral vector (see Materials and Methods). Clones, stably transfected by the calcium-phosphate precipitation technique, were selected for by using geneticin (G418), and three individual clones containing each construct were routinely used expressing at least 4 times as much $G_{i2}\alpha$ protein as the controls (vector transfected strains). The AC activation evoked by each agent is arbitrarily set to 100%. The data are depicted as means ± SD of six osteosarcoma cells analysed. *Indicates significant differences ($P < 0.05$, Wilcoxon rank test) between $G_{i2}\alpha$-transfected cells and controls.

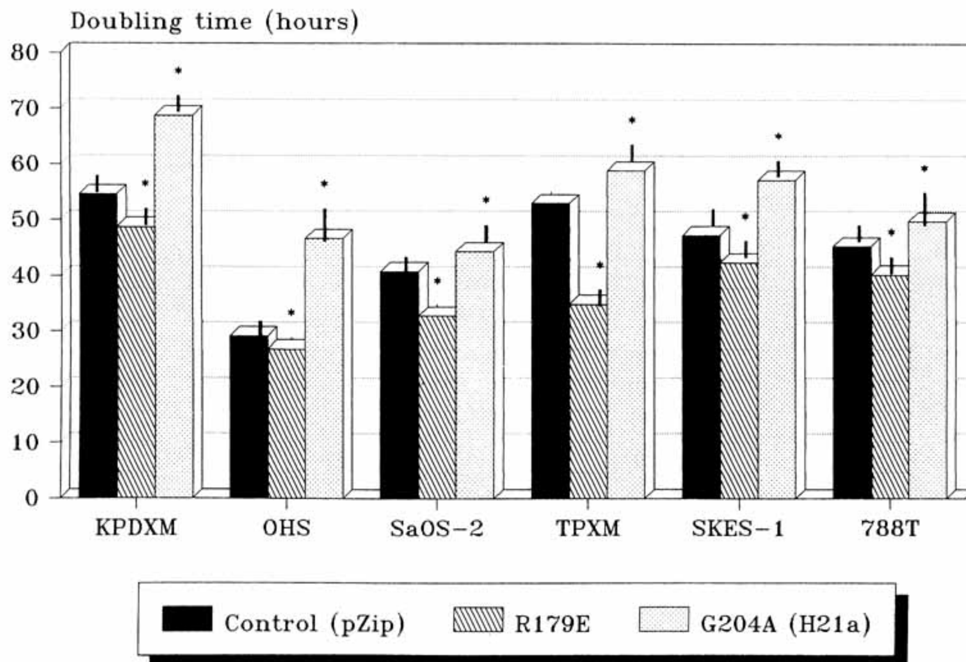

Fig. 6. Doubling time (t_d, h) of osteosarcoma cell lines before and after stable transfection with the activating and inactivating R179E and G204A $G_{i2}\alpha$-mutants. Cell clones used are described in legend to Figure 4 and in Materials and Methods. The data are depicted as mean ± SD of four replicate experiments. *Indicates significant differences ($P < 0.05$, Wilcoxon rank test) between $G_{i2}\alpha$-transfected cells and controls.

G_s protein consitutively active. The missense mutation Q227R has previously been found in the $G_s\alpha$ gene of human growth hormone secreting adenomas [33], and may thus be involved in cAMP mediated aberrant growth. This was not the case for the osseous sarcomas tested. Neither were basal- or hormone-elicited phospholipase C activities nor the steady state levels of the G-protein $G_{q/11}\alpha$ good predictors of individual sarcoma strain growth. Basal phospholipase C activities were normally distributed, rendering the probability of one or more cell lines containing activating missense mutations extremely small. Naturally occurring activating mutations in the $G_{q/11}\alpha$ gene has to date not been reported, despite the fact that cell lines transfected with such mutations displayed altered growth characteristics [11].

The last candidate among the G-proteins tested is $G_{i2}\alpha$. This G-protein has been shown to be implicated in cell proliferation of both NIH 3T3 cells and 3T3-L1 fibroblasts, and naturally activating mutations R179E, corresponding to the cholera toxin activated ADP-ribosylation site R201 of $G_s\alpha$, have been reported [34]. The findings that the IGF-II receptor has been shown to couple to this protein [12], and that mitose activating kinases (MAPK) are constitutively activated in *gip2* transformed cells [35,36], have increased the biological importance of this G-protein beyond its role as the adenylate cyclase inhibiting G-protein [37]. The effector through which IGF-II elicits its biological effect is not known; however, intracellular injections of antibodies against the C-terminal decapeptide of $G_{i2}\alpha$ effectively blocked IGF-II stimulated DNA synthesis in Balb/c 3T3 fibroblasts [13]. Doubling time for individual osseous sarcoma cell strains correlated negatively with steady state levels of $G_{i2}\alpha$. Furthermore, osteosarcoma cell lines transfected with the activating missense mutation R179E or the inactivating point mutation G204A displayed consistently decreased or enhanced population doubling times, respectively. It appeared that those cell lines which possessed the largest endogenous $G_{i2}\alpha$ levels were the least affected by the activating mutation R179E and vice versa, reinforcing the contention that $G_{i2}\alpha$ expression does hold an impact on osteosarcoma cell growth. The effect of $G_{i2}\alpha$ on cell proliferation was apparently not related to alterations in the hormone-elicited adenylate cyclase subsequent to the transfection, nor alterations in basal adenylate cyclase activity, which was virtually unaltered.

In summary, we have shown that the expression of $G_{i2}\alpha$ in osteosarcoma cells somehow, and at least partly, affects cell proliferation rate. Whether this G-protein, which is partly responsible for differentiation of preadipocytes into mature adipocytes [1], also plays a role in the phenotypic change of the osteoblast, is currently being investigated in our laboratory.

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