Spontaneous *In Vitro* Transformation of Primary Human Osteoblast-like Cells

URSULA MAGDOLEN[1], MANFRED SCHMITT[2], BARBARA HILDEBRANDT[3], PETER DIEHL[4], JOHANNES SCHAUWECKER[1], BELMA SALDAMLI[1], RAINER BURGKART[1], JUTTA TÜBEL[1], RAINER GRADINGER[1] and BRIGITTE ROYER-POKORA[3]

Departments of [1]Orthopedic Surgery, and [2]Obstetrics and Gynecology, Klinikum rechts der Isar, Technische Universitaet Muenchen, D-81675 Muenchen, Germany;
[3]Institute of Human Genetics and Anthropology, Heinrich Heine University of Duesseldorf, D-40225 Düsseldorf, Germany;
[4]Department of Orthopedic Surgery, University of Rostock, D-868057 Rostock, Germany

Abstract. *Background: Two new tumor-like cell lines were established which developed spontaneously in vitro from normal human primary osteoblast-like cells originating from non-oncogenic bone surgery. Materials and Methods: The tumor cell properties studied included morphology, proliferation characteristics in normal and low-serum media, and anchorage-independent growth in soft agarose. Results and Conclusion: Karyotyping of the cells showed numerous rearrangements and abnormalities. These results pointed to the tumorigenic potential of the cells and demonstrate the importance of biosafety in tissue engineering and therapeutic cell applications when prolonged culture conditions are required.*

In tissue engineering, autologous primary human cells are employed to cure certain diseases and to reconstitute sites of major defects for example of bone or cartilage by reconstructive surgery (1). In particular, regarding tissue engineering conducted in bioreactors or medical devices, extracorporeal propagation of cells is needed to facilitate cultivation on biomaterials such as scaffolds of hydroxyapatite or collagen sponges. Such approaches, however, have to be performed with precaution since it has been reported that primary human cells in culture may transform spontaneously into tumor-like cells. Allen-Hoffmann *et al*. (2) showed the spontaneous transformation of human keratinocytes, Soule *et al*. (3) the spontaneous immortalization of human breast epithelial cells, Takahashi *et al*. (4) reported the same phenomenon for human endothelial cells, Zhang *et al*. (5) for fibroblasts, Rubio *et al*. (6) for human adult mesenchymal stem cells and Maitra *et al*. (7) for embryonic stem cells. In this report, for the first time the spontaneous transformation and immortalization of primary osteoblast-like cells enriched from human cancellous bone is described of which two were further characterized for their malignant properties. Thus, transformed osteoblast-like cells may provide a model for the study of the origin and evolution of human bone carcinomas.

Correspondence to: Professor Manfred Schmitt, Clinical Research Unit, Department of Obstetrics and Gynecology, Technische Universitaet Muenchen, Ismaninger Strasse 22, D-81675 Munich, Germany. Tel: +49 8941402449, Fax: +49 8941407410, e-mail: manfred.schmitt@LRZ.tum.de

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Materials and Methods

Cell culture. Primary human osteoblast-like cells were isolated from cancellous bone originating from patients undergoing non-oncological orthopedic surgery. Only bone which would otherwise have been discarded was used and its collection and use for scientific purposes was allowed by the patient by written informed consent and approved by the local Ethics Committee. Minced bone fragments were washed several times in phosphate-buffered saline (PBS) and then cultured in 75 cm^2 tissue culture flasks (Falcon; Becton Dickinson, Heidelberg, Germany) in Dulbecco's modified Eagle's medium (DMEM; Biochrom KG, Berlin, Germany) supplemented with heat-inactivated 10% fetal calf serum (FCS; Biochrom), 2 mM glutamine, 1% penicillin and streptomycin, and MEM-vitamins (Biochrom) at 37˚C in a humidified atmosphere containing 5% CO_2. For the serum starvation experiments, 0.5% FCS was used. For the proliferation assays the number of adherent cells was quantified by determination of lysosomal hexosaminidase activity using a colorimetric assay, according to the procedure described by Landegren (8).

Histological staining. The osteoblastic phenotype of the cultured cells was confirmed by histological enzymatic staining for alkaline phosphatase (ALP) and for calcification. For staining of ALP, the cells were grown for 2 weeks in a 48-well microplate (Falcon; Becton

Dickinson) in medium supplemented with 50 μg/ml ascorbic acid (Sigma-Aldrich, Steinheim, Germany) and 4 ng/ml dexamethasone (Sigma-Aldrich). The cell culture medium was removed, then the cell layer was washed with TBS (TRIS-buffered saline), fixed with 98% ethanol and subsequently incubated with the ALP-substrate nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (Roche, Mannheim, Germany). The stained cells were photographed using an Axiovert® 25 microscope (Zeiss, Jena, Germany) equipped with a Zeiss Axiocam® camera. For the determination of calcification, the cells were grown for 4 weeks as described above, fixed with 10% formaldehyde in PBS and subsequently stained with 0.5% Alizarin red, pH 4.2 (Sigma-Aldrich).

Soft agarose assay for determination of anchorage-independent cell growth. After detachment of the cultivated cells with 0.25% trypsin/0.02% EDTA (Biochrom), the cell suspension was passed through a 40-μm cell strainer (Falcon; Becton Dickinson) to remove aggregated cells. One-hundred-and-fifty μl of 0.6% agarose (A 6013; Sigma) in DMEM was poured into the wells of a 48-well microtiter plate. This agarose base was overlayed with 150 μl of 0.3% agarose in DMEM containing 10,000 cells. The cells were grown for 3 to 4 weeks at 37˚C in a humidified atmosphere containing 5% CO_2, fixed with ethanol, and subsequently stained with 0.05% crystal violet and then photographed.

Karyotyping. The cell cultures were processed for chromosome analysis as follows. Colcemid blocking was conducted for 4-6 h to arrest the cell division at metaphase. Hypotonic treatment of the cells was carried out in 0.075 M KCl and fixation by using 3:1 methanol/acetic acid. The chromosome preparations of the cultured cells were G-banded and 15 metaphases analyzed. Due to the great karyotypic heterogeneity, composite karyotyping was conducted according to the International System for Human Cytogenetic Nomenclature (9).

Results and Discussion

Morphology and growth. The first indication of phenotype changes of the osteoblast-like cells within the primary culture established from human cancellous bone was the spontaneous appearance of colonies of small, rapidly growing cells with altered cell morphology. This happened in passage 12 of the cells obtained from patients A and B, but for other patients changes in phenotype were observed at earlier passages. Cell line A (CL-A) originated from female patient A, (age 13), and cell line B (CL-B) from male patient B (age 49). The spontaneously occuring change of phenotype resembled that of the osteosarcoma cell line Saos-2 (American Type Culture Collection HTB 85), and in comparison to normal osteoblast-like cells, they were smaller and uniform in appearance with a short spindle-like morphology, forming compact and often cobblestone-like colonies (Figure 1A-C). The clonogenic growth on plastic at low cell density in DMEM/10% FCS (Figure 1B), as well as the formation of cell foci at high density (data not shown), were characteristic of transformed cells (10).

Figure 1A shows passage 12 of the CL-A cells, growing next to normal osteoblast-like cells within the same cell culture well. They stained for alkaline phosphatase and calcified (Figure 1C), in a similar way to the osteosarcoma cell line Saos-2. Proliferation of the two cell lines was accelerated and the cells divided significantly faster than did normal osteoblast-like cells (Figure 2A). The capacity to grow at low serum concentration (Figure 2B) illustrated another general feature of tumor cells, promoted by oncogene activation or autocrine secretion of growth factors (11). Under these conditions, the normal osteoblast-like cells did not proliferate.

Anchorage-independent growth of cell lines CL-A and CL-B in soft agarose. As the cultures showed an absence of contact inhibition and the formation of foci and clonogenic colonies, which are all typical tumor cell growth characteristics, another cell feature was examined, namely anchorage-independent growth in the semisolid medium agarose. The loss of anchorage-dependent growth is a key criterion for the neoplastic phenotype and reflects invasive or metastatic potential (7). After up to 4 weeks of incubation in 0.3% agarose, colonies had developed from cell lines CL-A and CL-B as well as from the osteoblast-like control tumor cell line Saos-2 (Figure 3). The normal osteoblast-like cells obtained from patient A prior to transformation (Figure 3A) did not proliferate when they were embedded in agarose, a further indication of the acquired tumor cell properties of CL-A and CL-B in culture.

Chromosome analysis. All the spontaneously transformed cells described so far in the literature have shown major rearrangements of their chromosomes (2-7) and the same was true for cell lines CL-A and CL-B (Table I). CL-A was hypotetraploid, CL-B near tetraploid, both with numerous complex aberrations of chromosomal structures (Figure 4).

Many of the alterations were found in both cell lines CL-A and CL-B, a phenomenon which was also reported by Rubio and coworkers (6) for spontaneously transformed human adult mesenchymal stem cells, a topic which was discussed by Kassem *et al.* (12). Earlier, cytogenetic characterization of various osteosarcoma specimens revealed tumor-specific chromosomal abnormalities (13, 14). Interestingly, several of the most frequent abnormalities were observed in CL-A and/or CL-B as well, such as involvement of chromosomal bands 1q11, 1q12, 1p11, 2p23, 3q10, 12p11, 14p11, 17p11 and 19p13. Some of these genomic segments are related to oncogenes or tumor suppressor genes (14). Members of the human rat sarcoma (*RAS*) oncogene family have been mapped to chromosomal breakpoints 11p15, 12p13 and the genes of tumor suppressors *p53* and retinoblastoma 1 are located at 17p13

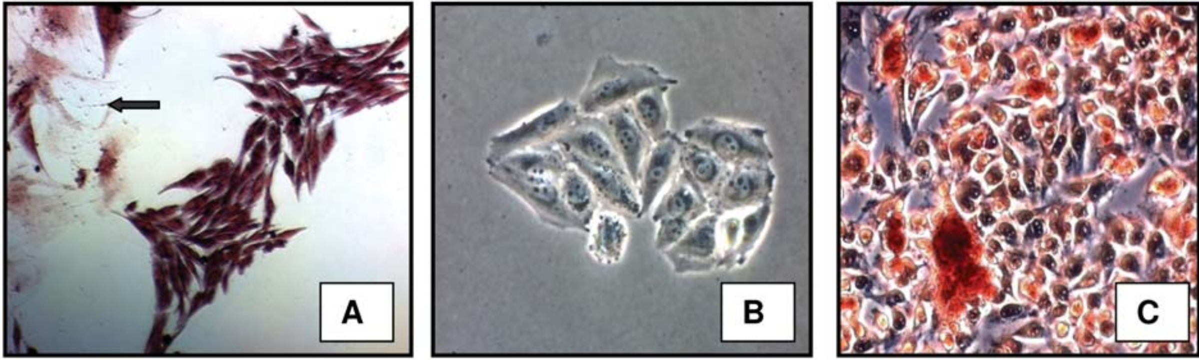

Figure 1. *A: CL-A cells shortly after transformation, stained for alkaline phosphatase. The arrow indicates normal osteoblast-like cells of the culture next to transformed cells on the right (magnification: ×100). B: Clonogenic growth of CL-A cells (unstained, magnification: ×200). C: CL-B cells stained for calcification with Alizarin red (magnification: ×100).*

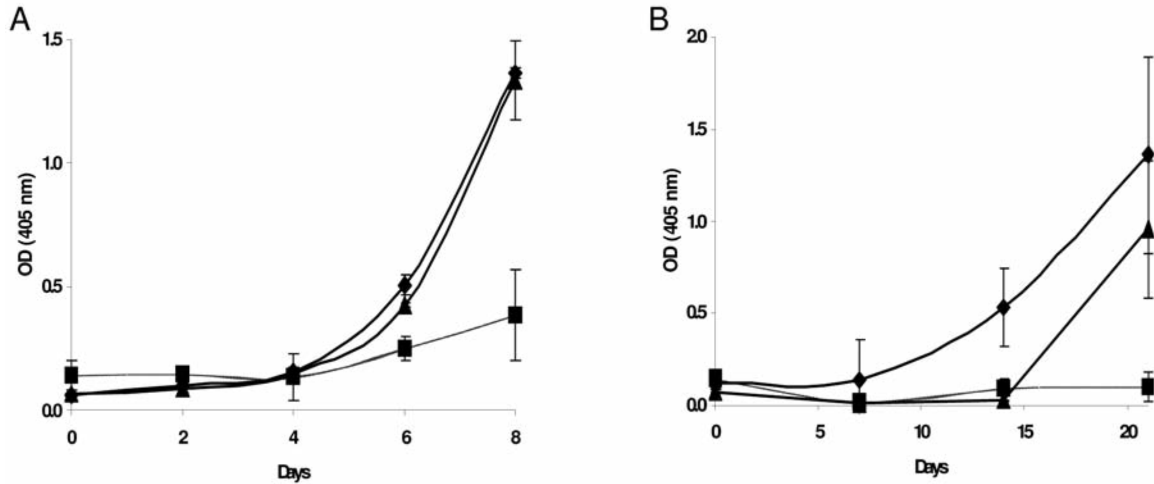

Figure 2. *Growth of normal osteoblast-like cells and of cell lines CL-A and CL-B in cell culture medium supplemented with 10 % FCS (A) or 0.5 % FCS (B). Five-hundred cells were seeded in the wells of a 96-well culture plate. After 4 h of adhesion (day 0) and at indicated intervals, lysosomal hexosaminidase activity was determined by a colorimetric assay according to Landegren (8), where absorbance at 405 nm is proportional to the number of cells. Experiments were performed three times in triplicate; mean values plus standard deviation are shown. ■ Normal osteoblast-like cells; ▲ CL-A; ♦ CL-B.*

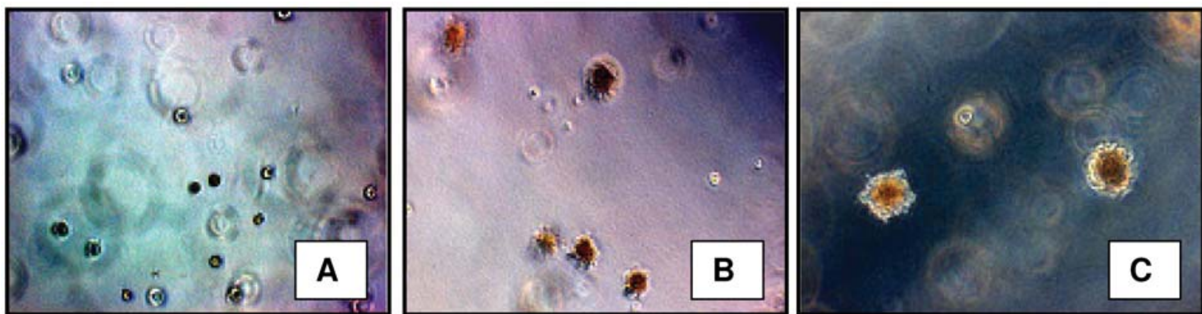

Figure 3. *Anchorage-independent growth of normal osteoblast-like cells of patient A and transformed cells CL-A in soft agarose. A total of 10,000 normal osteoblast-like cells, CL-A, and Saos-2 cells were grown for 2 weeks in 0.3% soft agarose, fixed with ethanol, and stained with 0.05% crystal violet. A: Normal osteoblast-like cells of patient A before spontaneous transformation. B: Osteoblast-like cells (CL-A) of patient A after spontaneous transformation. C: Saos-2 control cells.*

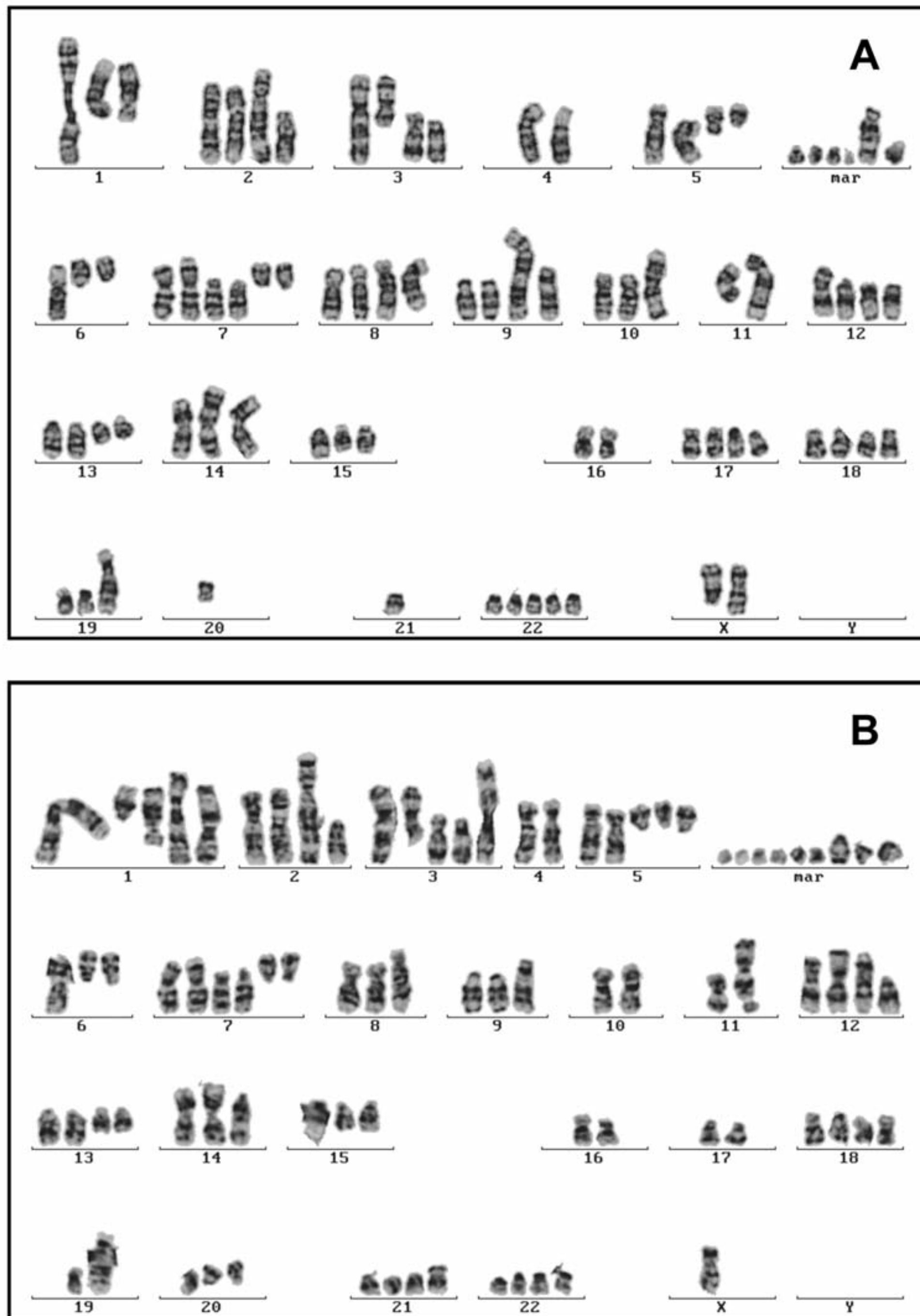

Figure 4. *G-banded karyotype of cell lines CL-A and CL-B.*

and 13q14. Some of these loci were also affected in CL-A and CL-B, as were the abnormalities found in osteosarcoma tissue specimens (13, 14).

Involvement of 8q24, the location of the *Myc* oncogene, was a common feature of CL-A and CL-B, as well as of tumor cell lines U-2OS and Saos-2 (15). In CL-B, the Y-chromosome was lost. This loss conformed with the spontaneously immortalized human adult mesenchymal stem cells described by Rubio *et al*. (6) and Allen-Hoffmann *et al*. (2) for human keratinocytes and with the osteosarcoma cell line CAL 72 (16). As a probable cause for the appearance of such altered cells oxidative damage by the high oxygen

Table I. *Karyotypes of cell lines CL-A and CL-B.*

A: 81-85,X,-X,-X,del(X)(q22),i(1)(q10),del(1)(q12)x2,-1,der(2)del(2)(p23)add(2)(q32),der(2),del(2)(p11), add(3)(q29), der(3;21)(q10;q10),add(3)(p11),del(3)(p11),add(4)(p11),-4,-4,del(5)(q11)x2,add(6)(p11),del(6)(q11)x2,-6,add(7)(p22),del (7)(p11)x2,+del(7)(q11)x2,der(8)del(8)(p21)add(8)(q24)x2,der(9)del(9)(p11)add(9)(q34)1-3x,der(9)add(9)(p22)dup(9) (q12q34),del(9)(p22),add(10)(p13),-10,del(11)(q21),add(11)(q23),-11,-11,add(12)(p11),del(12)(p11)x3,del(13)(q14)x2,add (14)(q11),add(14)(p11),add(14)(p11),-14,del(15)(q22)x2,-15,-16,-16,del(17)(p11)x2,der(19)t(1;19)(q21;p13),-19,del(20) (q11),-20,-20,-20,-21,-21,+22,+5-15mar[cp15]

B: 85-94,-X,-X,-Y,-Y,i(1)(q10),del(1)(q11),del(1)(q12),+add(1)(p11),add(2)(p11),del(2)(p11)x2,add(3)(q29),der(3;?;21) (p10;q10),add(3)(p11), del(3)(p11),add(4)(p11),-4, -4,del(5)(q11)x3,add(6)(p11), del(6) (q11)x2,-6,add(7)(q22),del(7) (p11),del(7)(q11),+del(7) (q11),der(8),del(8)(p21),add(8) (q24),der(9),del(9)(p11),add(9) (q34)x3,-10,-10,del(11)(q21), der(11),add(11)(p15),add (11)(q23),-11,-11,add(12)(p12),add(12)(p13),add(12)(p11),del(12)(p11),del(13)(q14)x2,add(14) (p11)x2,add(14)(p11), del(12)(p11), del(13)(q14)x2,add(14)(p11)x2, add(14)(q32),-14,del(15)(q22)x2,-15,-16,-16,del(17) (p11)x2,-17,-17,der (19),t(1;19)(q21;p13),-19,del(20)(q11) x2-20,add(21)(p11),+22, +5-15mar[15]

add: addition, de: deletion, der: derived, dup: duplication, i: insertion, mar: marker, t: translocation.

concentration during cultivation has been considered (17), where an atmospheric concentration of 20% is present, while under physiological conditions in tissue, only about 3% of oxygen is present. In a mouse model, Busuttil *et al*. (18) showed that at 20% oxygen more mutations occurred, mainly genome rearrangements, than under 3% physiological oxygen. These genomic aberrations led to spontaneous immortalization of cells. *In vitro*, however, no immune system is present to eliminate malignant cells thus allowing altered cells to develop and overgrow normal cells. In particular, these spontaneous transformations do often occur in rodent cell cultures (17-20), but are a rather rare event in human cells *in vitro*. Nevertheless, our data show that for therapeutic use in regenerative medicine (1), prolonged cultivation of osteoblast-like cells requires very strict supervision of culture conditions, and also of the pheno- and karyotype of the cells.

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