# Living cells in 1 of 2 frozen femoral heads

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**ABSTRACT Allogeneic, frozen bone is now the most commonly grafted tissue (Norman-Taylor and Villar 1997). Tissue banks collect bone material according to protocols developed with the aim of maintaining osseoinductive properties of grafts as well as preventing transmission of viral or bacterial diseases (Standards from the American Association of Tissue Banks (AATB) or from the European Association for Musculo-skeletal Transplanting (EAMST)). Standard procedures include cryopreservation of tissue at –80 °C, which is generally considered to devitalize the bone by killing all cells present, resulting in reduced immunogenicity of the graft. The osseoinductive properties of frozen, allogeneic bone grafts have therefore mainly been attributed to the dead bone matrix, that may provide osteoblast-stimulating growth factors and other essential proteins, and/or an osteoclast substrate to direct bone remodeling (Aspenberg et al. 1996, Kingsmill et al. 1999). Recently however, it was suggested that some cells in bone biopsies may survive standard bone bank freezing procotols. It is unclear whether vital cells are present in other bone banks and whether these cells can contribute to the clinical outcome of frozen allogeneic bone grafting.**

**In this report, we show that frozen bone biopsies, obtained from the Erasmus Medical Center bone bank may contain living cells that can be cultured in vitro. These cultured cells were found to originate from the donor by genotyping.**

### *Cell cultures from frozen bone bŠpsies*

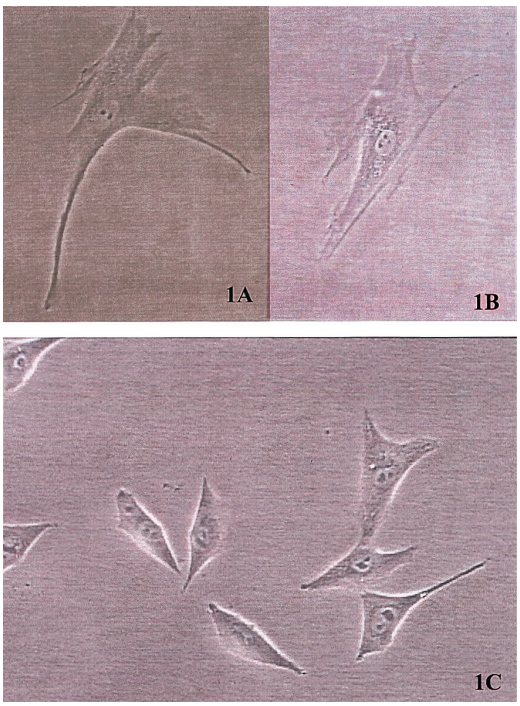
Two human femoral heads were obtained from primary hip replacement surgery and frozen at –80 °C for a minimum of 6 months. To test for the presence of viable cells in these samples, femoral heads were defrosted at room temperature and cortical and trabecular bone chips, ranging in size from 1–3 $mm^3$, were made aseptically. Part of the chips were refrozen for later genotyping. Other chips were treated with 1 mg/mL collagenase H at 37 °C for 1 or 3h or left untreated. Subsequently, bone chips were cultured in αMEM supplemented with penicillin/streptomycin and 5% or 20% FCS. Media were replaced once a week and cell outgrowth was monitored by microscopy. Following outgrowth of cells, bone chips were removed and the cells washed and passaged. This treatment ensures that genotyping is performed on vital cells and not on dead bone.

### *Cell cultures from retrieved graft material*

To determine the persistence of donor cells in the recipient following bone grafting, biopsies were taken from the graft site in two female patients returning for retrieval surgery, 3 or 18 months after receiving male donor bone grafts. Biopsies were fragmented to 1–3 $mm^3$ chips, treated with 1 mg/mL collagenase H for 1h at 37 °C, and cultured in αMEM supplemented with penicillin/streptomycin and 10% FCS. Following the outgrowth of cells, bone chips were removed and the cells washed and passaged.

### *Genotyping*

Genomic DNA was extracted from cultured cell pellets, or control bone chips, using standard protein K digestion and phenol/chloroform extraction. Allelotyping was performed by radioactive polymerase chain reaction (PCR)-amplification




A. B. Cellular outgrowth from one frozen femoral head. The cells were large with high cytoplasm-to-nucleus ratio.
C. MG63 osteosarcoma cells as undifferentiated bone cell reference.

of 4 highly polymorphic microsatellite markers (D8S133, D9S156, D14S67 and D17S786). Screening for Y-chromosome DNA in the cellular outgrowth of retrieved donor material was performed by chromosome-Y-specific PCR.

Study of the persistence of donor cells in the recipient after bone grafting requires very sensitive detection of donor cells in a vast majority of host cells. A unique level of sensitivity was achieved by screening vital cells. In the bone biopsies obtained from the graft site after male donor material was transplanted into a female host, the presence of theoretically one (male) donor cell could be found among thousands of (female) host cells.

### *Frozen bone bŠpsies contain living cells*

7 weeks of culture resulted in cellular outgrowth from bone chips obtained from one of the two frozen femoral heads used in these experiments. The cells were large and showed a high cytoplasm-to-nucleus ratio. MG63 human osteosarcoma cells are shown as an undifferentiated control bone cell

**Cellular outgrowth and culture conditions**

| Collagenase treatment | Cortical chips | | Trabecular chips | |
|---|---|---|---|---|
| | 5% FCS | 20% FCS | 5% FCS | 20% FCS |
| 0 h | +++ | | | +++ |
| 1 h | | | | |
| 3 h | | | +++ | |

reference (Figure). Nucleoli were clearly visible in nuclei and cells showed large cytoplasmic extensions. The general morphology was that of differentiated cells, which was confirmed by the low proliferating capacity of these cells (cultures were lost due to lack of proliferation after 2 passages). Vital cells were obtained from both cortical and trabecular bone-derived chips under various culture conditions (Table). Outgrowing cells were found to originate from the original donor by allelotyping.

### *No vital donor cells are detected 3–18 months after grafting*

No donor signal was obtained from the cellular outgrowth of the biopsies, using a test method with high discrimination capacity.

## Discussion

Bone biopsies, frozen at –80 °C to devitalize bone cells before use of the bone matrix as graft material in orthopedic surgery, can contain living cells. This corresponds with a recent observation that cells may survive standard bone bank freezing protocols (unpublished data reported at 46th Annual Meeting Orthopaedic Research Society 2000, poster 672). These cells may grow from bone chips in vitro independently of collagenase H treatment, indicating that the same processes can occur in vivo. The morphology of the cells cultured from frozen bone grafts combined with the low proliferation capacity points towards a differentiated cell type that had lost its capacity to enter the cell cycle. The cells show long cellular processes; an osteocyte characteristic, although human cells cultured in this study do not have a morphology resembling that of isolated chicken osteocytes (Nijweide and Mulder

1986). Furthermore, the large cytoplasm is indicative of a cell that is actively focusing on protein production. These observations suggest that cells in bone graft material that survive standard freezing protocols and can grow from the grafts in vitro are late osteoblasts or early osteocytes. Osteocytes are notoriously hard to isolate and culture (Tanaka-Kamioka et al. 1998), since bone cell cultures are easily overgrown by fast proliferating fibroblast-like cells or osteoprogenitors. The freezing of bone material before the isolation of cells may be a new method of isolating and culturing specifically differentiated bone cells, because it devitalizes the superficial cell layers while leaving the inner bone cells intact.

3 and 18 months after bone grafting, we found no vital donor cells in the outgrowth of a bone biopsy from the graft site. The combination of male donor material transplanted into a female host permitted very sensitive determination of the presence of any donor (male; chromosome-Y-containing) cells among great amounts of (female) host cells. The absence of vital donor cells in the graft site, 3 months after grafting indicates that viable cells in the graft material do not survive for long in the recipient. However, more grafts need to be studied to be able to exclude entirely that donor cells survive in recipients. Although we do not exclude a role of donor bone cells in contributing to initiation of remodeling, our findings show that viable donor cells from the graft do not form a significant sub-population capable of proliferation and long-term survival in the host. Because of the low persistence and low proliferating capacity of vital cells in a frozen bone allograft, we regard it as highly unlikely that these cells contribute significantly to bone remodeling at the graft site.

The short lifespan of donor cells in the recipient graft site may be caused by hypoxia due to insufficient vascularization of the graft, but also host-versus-graft immune responses may be involved. Antigenicity of the allograft is greatly reduced by freezing (Friedlaender and Horowitz 1992), which correlates with fewer viable cells due to the treatment. Residual immunogenicity of the frozen graft (Deijkers et al. 1999) may be due to the persistence of vital cells that can activate cytotoxic T cells directly. In dogs, histocompatibility-matching and immunosuppressive drugs increase the incorporation of frozen allografts (Goldberg et al. 1984), which would suggest that immune activation negatively influences osteointegration. In man, reports are inconclusive as to the effects of matching donor and recipient for class I or class II antigens. Matching does not significantly change the radiographic outcome of frozen allografts (Muscolo et al. 1996), and no correlation has been found between immune parameters and the clinical outcome of grafting (Aho et al. 1998). Friedlaender et al., however, reported that matching histocompatibility antigens, particularly class II antigens, improved clinical success (Friedlaender et al. 1999). The matching of donor and recipient for HLA antigens for bone transplantation is not included in AATB & EAMST guidelines and is not usually done (AATB 1996, EAMST 1997).

The potential hazard of transmission of viral or bacterial infections from the donor to the recipient is of great concern in allografting. Simian immunodeficiency virus can be transmitted from the bone allograft donor to the recipient even after double freeze-thawing of the graft (Cook et al. 1995). Chemical decontamination in combination with a double freeze-thaw cycle prevents the transmission of SIV from donor to recipient in these monkeys. It is uncertain whether the virus resides in the vital cells that may remain in the graft after this treatment or whether it can survive in the graft even without vital cells. The screening of donors for viral or bacterial infections significantly reduces the risk of transmitting known pathogens. According to general AATB or EAMST guidelines, viral or bacterial infections of potential allograft donors are exclusion parameters for material in bone banks.

The finding that vital cells are present in devitalized, frozen bone used for allografting raises several questions. Are these vital cells involved in detrimental immune responses in the recipient? Do these cells entail a risk of transmission of infections from the donor to the recipient? As long as these questions remain unanswered, protocols for devitalizing bone before allografting should be reconsidered and made more stringent because there is no proof that vital cells contribute significantly to the formation and incorporation of bone.

No competing interests declared.

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