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SHORT REPORT

Adipose-derived stem cells from the brown bear (*Ursus arctos*) spontaneously undergo chondrogenic and osteogenic differentiation in vitro

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Abstract In the den, hibernating brown bears do not develop tissue atrophy or organ damage, despite almost no physical activity. Mesenchymal stem cells could play an important role in tissue repair and regeneration in brown bears. Our objective was to determine if adipose tissue-derived stem cells (ASCs) can be recovered from wild Scandinavian brown bears and characterize their differentiation potential. Following immobilization of wild brown bears 7–10 days after leaving the den in mid-April, adipose tissue biopsies were obtained. ASCs were recovered from 6 bears, and shown to be able to undergo adipogenesis and osteogenesis in monolayer cultures and chondrogenesis in pellet cultures. Remarkably, when grown in standard cell culture medium in monolayer cultures, ASCs from yearlings spontaneously formed bone-like nodules surrounded by cartilaginous deposits, suggesting differentiation into osteogenic and chondrogenic lineages. This ability appears to be lost gradually with age. This is the first study to demonstrate stem cell recovery and growth from brown bears, and it is the first report of ASCs spontaneously forming extracellular matrix characteristic of bone and cartilage in the absence of specific inducers. These findings could have implications for the use of hibernating brown bears as a model to study disuse osteoporosis.

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Introduction

Hibernating Scandinavian brown bears (*Ursus arctos*) have no physical activity for 5–7 months while inside their winter dens (Manchi and Swenson, 2005; Friebe et al., 2001). Despite this, hibernating bears do not develop muscle atrophy, coagulopathies, decubitus ulcer (bedsore), or deterioration in heart function and they are not prone to osteoporosis (McGee-Lawrence et al., 2009a, 2009b).

It is largely unknown how the brown bear tolerates the physiological extremes related to hibernation, extremes that would cause tissue loss and injury in humans. Stem cells are central components in tissue regeneration and repair and may play a role in protecting the hibernating bears against disuse osteoporosis. Thus, the purpose of this study was twofold: 1) to determine if adipose tissue-derived stem cells (ASCs) could be recovered from the adipose tissue of wild brown bears, and 2) to compare the differentiation capacities of ASCs from brown bears with those of human origin.

Results

Isolation and growth of ASCs

Adipose tissue samples were obtained from 4 yearling male and 3 adult female bears (9, 14 and 16 years old) and mesenchymal stem cells were isolated. Only few floating cells were observed immediately following the isolation of the stem cells (Fig. 1A, left panel). However, after 4 days, a significant number of cells had attached to the plastic flask (Fig. 1A, middle panel) and displayed the spindle-shaped form characteristic of mesenchymal stem cells. After the cells had attached, the cultures proliferated rapidly and achieved confluency after approximately a week in culture (Fig. 1A, right panel). Six of the 7 samples established stem cell cultures. The established culture from the 14-year old bear proliferated at a very low rate, and was not used in subsequent experiments.

Adipogenic properties of the ASCs

Culturing of the cells in adipogenic induction medium led to accumulation of lipid droplets in the cells as indicated in Fig. 1B, consistent with a conversion into an adipogenic phenotype. As expected, the accumulation of lipids was present in the induced cells and absent in the control cells for both human and bear cells (Fig. 1B).

Osteogenic potential of brown bear ASCs

All bear cultures that had been exposed to osteogenic growth factors underwent osteogenesis in a manner similar to the human cells (Fig. 2). In the control cultures from the three yearlings and the young bear, however, (B1, B3, B6, and B8), cells spontaneously formed nodules that stained positive for Alizarin red, indicating calcium mineral deposition (Fig. 2, nodules indicated by arrows). Similarly to the human cells, bear culture B4 (from a 16-year-old bear) incubated in normal growth medium showed neither nodule formation nor positive staining with Alizarin red.

Chondrogenic properties of the ASCs

We did not see positive staining in any of the monolayer cultures exposed to chondrogenic induction medium (Fig. 3). Surprisingly, however, we found evidence of chondrogenic differentiation in the control cultures from all three yearling bear cell lines (Fig. 3). In cultures where cells had formed nodules, lakes of glycosaminoglycans were found in the extracellular matrix surrounding the nodules. ASC culture B1 (from a 9-year-old bear) that also formed nodules did not undergo spontaneous chondrogenesis. Similarly to the human cells, bear culture B4 (from a 16-year-old bear) incubated in normal growth medium showed neither nodule formation nor positive staining with Alcian blue (Fig. 3).

When grown in pellet cultures, all cells grown in the presence of chondrogenic inducers showed signs of chondrogenic differentiation as evidenced by deposition of extracellular matrix with glycosaminoglycans. For the bear cells, this deposition was most pronounced around the periphery of the nodules (Fig. 4, edge of nodule indicated by arrows). For the cultures in control medium, the cells in pellets of human origin and from bears B1 and B4 were so loosely connected that the pellets to a large degree disintegrated during the sectioning and staining process (Fig. 4, disintegration of the pellet structure indicated by asterisks). However, the pellets from bears B3, B6, and B8 formed firmer pellets albeit with lower amounts of extracellular matrix compared to the induced cultures (Fig. 4).

Discussion

This study represents the first documentation of stem cells from brown bears. As defined by the International Society for Cellular Therapy some of the characteristics of mesenchymal stem cells are their capacity for plastic adherence during growth and their capacity to differentiate into adipocytes, osteocytes, and chondrocytes (Dominici et al., 2006). While, ideally clonal assays should be performed to assess the multipotential of the stem cells, to minimize the number of cell doublings prior to characterization, it is now commonly used to rather assess the multipotentiality of the entire cell culture (Mosna et al., 2010). ASCs from brown bears were recovered and cultured with a high success rate. The cells were selected on their potential to adhere to plastic and when induced to undergo either adipogenic, osteogenic, or chondrogenic differentiation, all cultures showed characteristics similar to the human cells. Remarkably, however, the cells from yearlings showed remarkable spontaneous cartilage and bone formation capacity. Interestingly, the spontaneous bone and cartilage formation appears to occur in a concurrent manner in and around the nodules, respectively, with mineralization characteristic of bone within the nodules and cartilage formation in the periphery. To our knowledge, this is the first report of spontaneous chondrogenic and osteogenic differentiation of ASCs. We

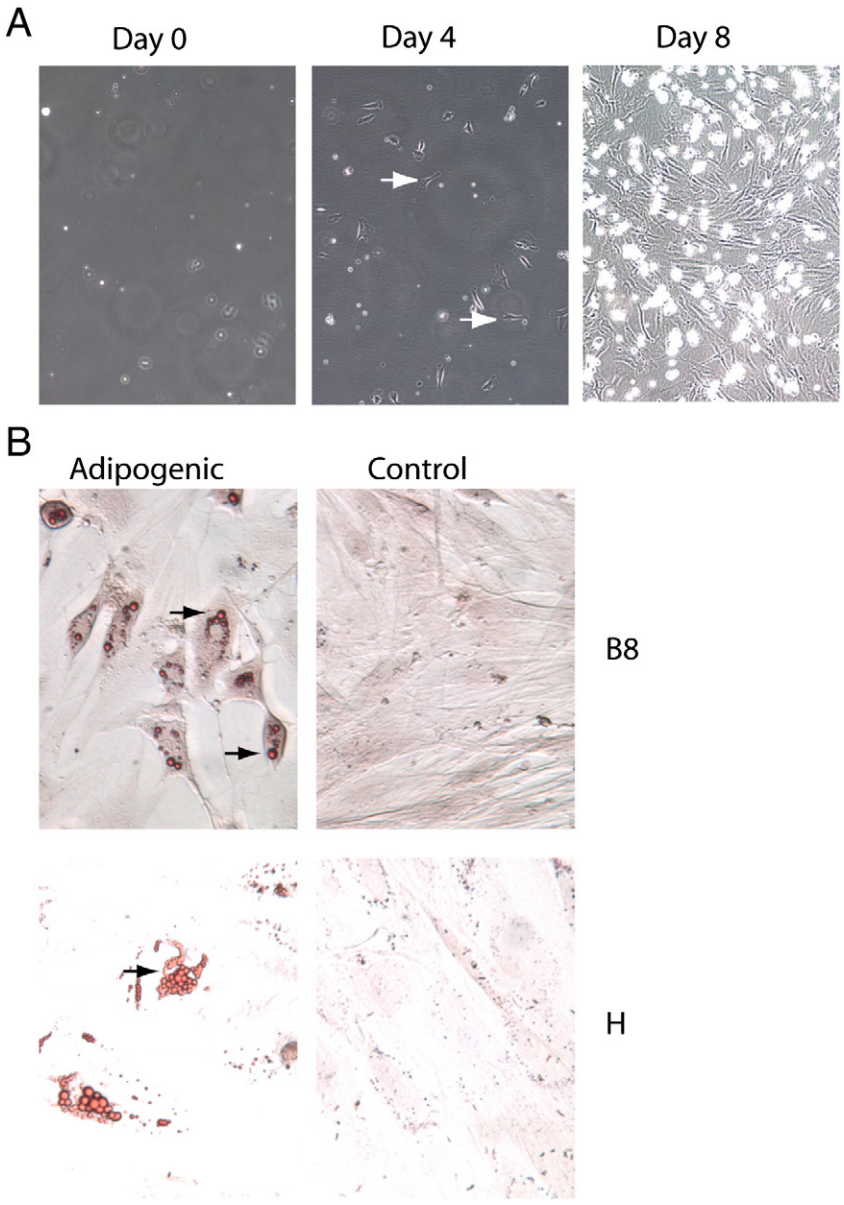

Figure 1 Cell growth and adipogenic differentiation of primary adipose tissue-derived stem cells from wild brown bears. A: Photomicrographs of cells from one representative donor grown for 1, 4, and 8 days after isolation. Arrows indicate spindle shaped cells. B: Cells grown for 2 weeks in standard growth medium or adipogenic induction medium, after which intracellular lipids were stained with Oil Red O. Arrows indicate lipid inclusions. Magnification, ×100.

have previously isolated ASCs of human origin from donors of ages ranging from 22 to 56 and assessed their capacity for osteogenic and chondrogenic differentiation after growth in different basal media and in different types of sera or serum replacers (Fink et al., 2011; Lund et al., 2009; Pilgaard et al., 2009a), however we have never observed the formation of nodules by human cells grown under any of the tested conditions. Neither have we found reports of this phenomenon by other groups. It cannot be excluded that human ASCs from neonates or very young children may display the same characteristics as the ASCs from the bear cubs, however due to the fact, that we receive the adipose tissue from plastic surgeons, we are not in a position to investigate this at present.

As the stem cells were recovered from bears that recently were hibernating, it is possible that circulating factors that protect the bear from bone degeneration during hibernation prime the stem cells. That we did not see any chondrogenic differentiation in the cultures exposed to the chondrogenic induction medium was not surprising, as it is notoriously difficult to achieve chondrogenic differentiation in monolayer cultures, and most protocols call for either micromass pellet culture or culture in alginate or similar scaffolds (Bobick et al., 2009). In the monolayer cultures, it is possible

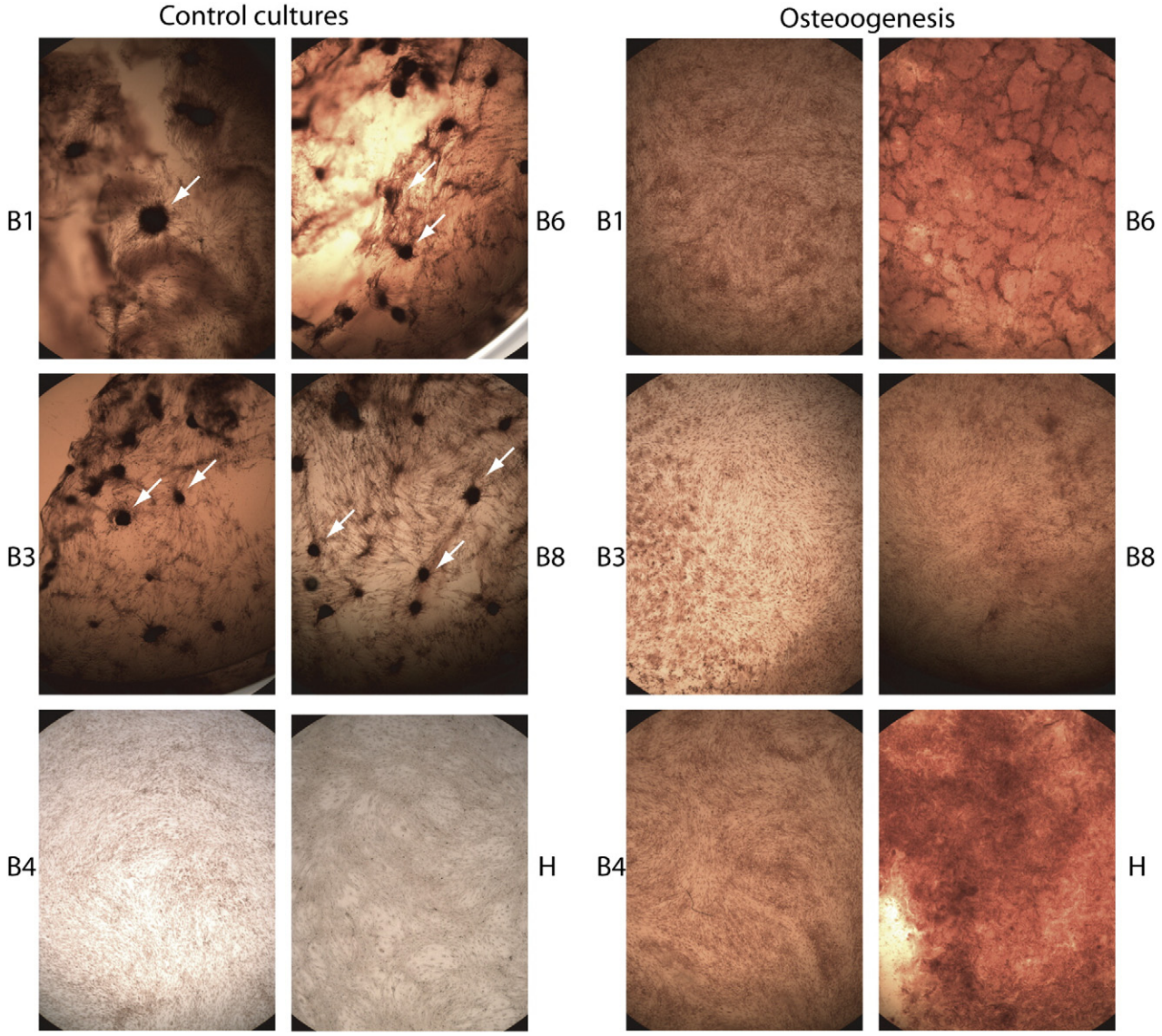

Figure 2 Osteogenic differentiation of ASCs from bears and one human. Bear (B1–B8) and human (H) stem cells were cultured in standard growth medium (control cultures) or osteogenic induction medium for 3 weeks, after which the cultures were stained with Alizarin red. Arrows indicate nodules. Magnification, ×40.

that the control medium allows for a higher differentiation rate of the cells and optimum nodule formation. That we, in fact, did see clear signs of chondrogenesis taking place in all pellet cultures, highlighted the need for close cell-to-cell contact for chondrogenesis to occur.

Bone regeneration is a complex process of mesenchymal stem cell invasion, chondrogenesis, osteogenesis and angiogenesis. However, these normal bone regenerative mechanisms decline and cannot compensate for the bone loss during osteoporosis. Recent findings of circulating multipotent stem cells with mixed characteristics of endothelial and osteogenic cells have raised interest in new therapies for pathologic bone loss (Clines, 2010). We speculate that the spontaneous osteogenesis and chondrogenesis of brown bear ASCs, highlight the potential of hibernating bears and ASCs as model systems to study the prevention of osteoporosis.

Materials and methods

Collection of adipose tissue samples from brown bears

All procedures involving the animals were in compliance with Swedish laws and regulations and approved by the Uppsala animal ethics committee (Uppsala Djurförsöksetiska Nämnd, nr C47/9, 2009-03-27). In mid-April 2009, approximately 7–10 days after leaving the den, wild brown bears were immobilized from a helicopter by darting with a mixture of

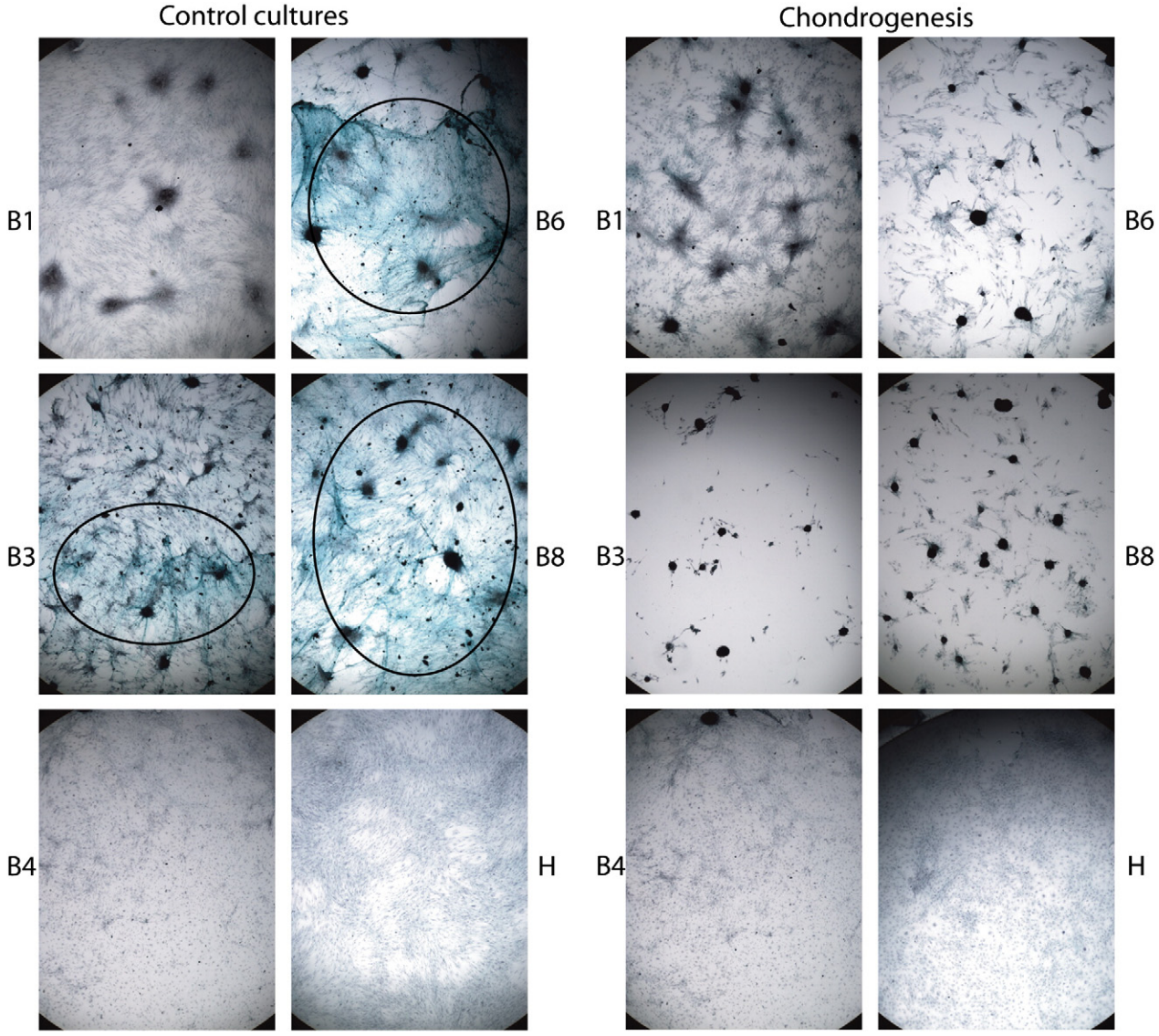

Figure 3 Chondrogenic differentiation in monolayer cultures of ASCs from bears and one human. Bear (B1–B8) and human (H) stem cells were cultured in standard growth medium (control cultures) or chondrogenic induction medium for 3 weeks, after which the cultures were stained with Alcian blue. The cyan-colored glycosaminoglycan deposits are indicated by circles. Magnification, ×40.

tiletamine–zolazepam and medetomidine (Fahlman et al., 2011). Adipose tissue biopsies (1.5–3 ml) were obtained subcutaneously during intra abdominal implantation of tracking devices (Arnemo et al., 2011). Each biopsy was placed into a 15 ml v-bottomed centrifuge tube with phosphate buffered saline (PBS) with 10 IU/ml of penicillin, and 10 μg/ml of streptomycin. All samples were kept at room temperature and transported by courier to be processed within 48 h of harvest.

Isolation of adipose tissue-derived stem cells

The tissue samples were minced finely and digested by incubation in a 0.14 Wünsch units/ml Liberase Blendzyme 2 (Roche Applied Science, Hvidovre, Denmark) solution at 37 °C for 2 h. The digests were centrifuged at 400×*g* for 10 min. The pellet was briefly resuspended in sterile water to lyse contaminating erythrocytes, after which the salt concentration was adjusted through addition of 10× PBS. The cells were filtered through a 100 μm cell strainer, centrifuged and resuspended in 5 ml growth medium, consisting of minimum essential medium alpha (α-MEM) (GIBCO/Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (FCS), and penicillin (10 IU/ml), streptomycin (10 μg/ml) and gentamicin (5 μg/ml) (all from GIBCO/Invitrogen). The cells were seeded in a T25 flask and transferred to a CO_2 incubator overnight, after which non-adherent cells were removed. The media were changed twice a week during expansion of the cells. When cells were 90% confluent, they were detached from the culture flasks using 0.125% trypsin/0.01% EDTA and transferred to new flasks. When cells were in passage three, they were frozen in aliquots of approximately 0.5×10^6 cells.

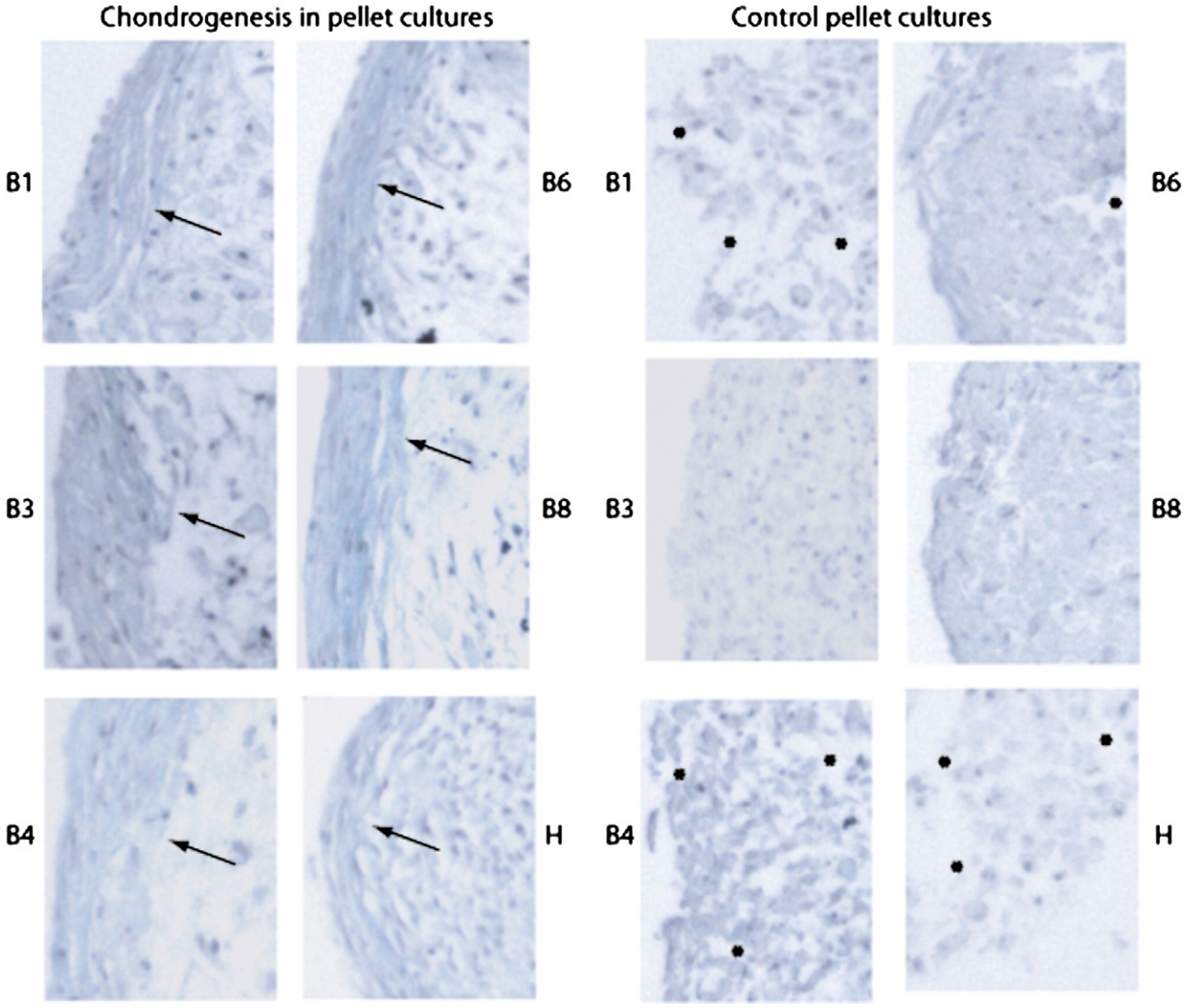

Figure 4 Chondrogenic differentiation in pellet cultures of ASCs from bears and one human. Bear (B1–B8) and human (H) stem cells were cultured in standard growth medium (control cultures) or chondrogenic induction medium for 3 weeks, after which the pellets were sectioned and slices were stained with Alcian blue. In the induced cultures the cyan-colored glycosaminoglycan deposits in the periphery of the pellets are indicated by arrows. In the control cultures, areas of disintegration of the pellet are indicated by asterisks. Magnification, ×200.

All subsequent experiments were performed on cells in passage 4 in duplicate in two independent experiments.

Human ASCs for use as control samples were isolated and propagated as previously described after informed written consent (Fink et al., 2008). The regional Committee on Biomedical Research Ethics of Northern Jutland, Denmark approved the analysis of human stem cells from persons undergoing elective liposuction (project no. 2005054). The donor was a 42 year old female, and the sample was obtained from inner thigh.

Induction of adipogenic, osteogenic, and chondrogenic differentiation

Cells from 5 bears and one human were used for the induction experiments. The induction of the cells into the different lineages was carried out as previously described (Fink et al., 2008). In brief, to induce adipogenesis, cells were incubated for 2 weeks in adipogenic induction medium consisting of α-MEM supplemented with FCS, isobutylmethylxanthine (IBMX) insulin, and indomethacin, after which the adipogenic differentiation was visualized through staining of intracellular lipid accumulation with Oil Red O.

To induce osteogenesis, cells were maintained for 3 weeks in osteogenic induction media consisting of α-MEM supplemented with FCS, dexamethasone, L-ascorbic acid 2-phosphate, calcitriol, and glycerol 2-phosphate. After 3 weeks the degree of osteogenesis was evaluated by staining of calcium deposits with Alizarin red.

To induce chondrogenesis in monolayer cultures, the cells were incubated in chondrogenic induction media consisting of high-glucose (4.5 g/l) Dulbecco's modified Eagle's medium supplemented with 10 transforming growth factor β3

(TGFβ3), dexamethazone, L-ascorbic acid 2-phosphate, L-proline, and 1× ITS$^+$ Premix. After 3 weeks in culture, the chondrogenic differentiation was assessed by staining extracellular deposition of glycosaminoglycans with Alcian blue.

The chondrogenesis in pellet cultures were carried out as described previously (Pilgaard et al., 2009b). In brief, 2×10^5 cells were pelleted by centrifugation and either induced to undergo chondrogenesis by incubation in chondrogenic induction media or grown in normal growth medium for 3 weeks. After 3 weeks the pellets were sectioned and stained with Alcian blue.

For all differentiation experiments, control cells were plated and incubated in growth medium until completion of the experiment. All experiments were carried out twice, each in duplicate.

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References

Arnemo, J.M., Evans, J.F., Fahlman, A., 2011. Biomedical Protocols for Free-Ranging Brown Bears, Gray Wolves, Wolverines and Lynx. Hedmark University College, Evenstad, Norwayhttp://www1.nina.no/RovviltPub/pdf/Biomedical%20Protocols%20Carnivores%202011.pdf.

Bobick, B.E., Chen, F.H., Le, A.M., Tuan, R.S., 2009. Regulation of the chondrogenic phenotype in culture. Birth Defects Res. C Embryo Today 87, 351–371.

Clines, G.A., 2010. Prospects for osteoprogenitor stem cells in fracture repair and osteoporosis, Curr Opin Organ Transplant, 15 73–78.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., Horwitz, E., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8, 315–317.

Fahlman, A., Arnemo, J.M., Swenson, J.E., Brunberg, S., Pringle, J., Nyman, G., 2011. Physiologic evaluation of capture and anesthesia with medetomidine–zolazepam–tiletamine in brown bears (*Ursus artcos*), J Zoo Wildl Med, 42, 1–11.

Fink, T., Lund, P., Pilgaard, L., Rasmussen, J.G., Duroux, M., Zachar, V., 2008. Instability of standard PCR reference genes in adipose-derived stem cells during propagation, differentiation and hypoxic exposure. BMC Mol. Biol. 9, 98.

Fink, T., Rasmussen, J.G., Lund, P., Pilgaard, L., Soballe, K., Zachar, V., 2011. Isolation and expansion of adipose-derived stem cells for tissue engineering, Front Biosci (Elite Ed), 3 256–263.

Friebe, A., Swenson, J.E., Sandegren, F., 2001. Denning chronology of female brown bears in central Sweden. Ursus 12, 37–46.

Lund, P., Pilgaard, L., Duroux, M., Fink, T., Zachar, V., 2009. Effect of growth media and serum replacements on the proliferation and differentiation of adipose-derived stem cells. Cytotherapy 11, 189–197.

Manchi, S., Swenson, J.E., 2005. Denning behavior of Scandinavian brown bears *Ursus Arctos*. Wildl. Biol. 11, 123–132.

McGee-Lawrence, M.E., Wojda, S.J., Barlow, L.N., Drummer, T.D., Bunnell, K., Auger, J., Black, H.L., Donahue, S.W., 2009a. Six months of disuse during hibernation does not increase intracortical porosity or decrease cortical bone geometry, strength, or mineralization in black bear (*Ursus americanus*) femurs. J. Biomech. 42, 1378–1383.

McGee-Lawrence, M.E., Wojda, S.J., Barlow, L.N., Drummer, T.D., Castillo, A.B., Kennedy, O., Condon, K.W., Auger, J., Black, H.L., Nelson, O.L., Robbins, C.T., Donahue, S.W., 2009b. Grizzly bears (*Ursus arctos horribilis*) and black bears (*Ursus americanus*) prevent trabecular bone loss during disuse (hibernation). Bone 45, 1186–1191.

Mosna, F., Sensebe, L., Krampera, M., 2010. Human bone marrow and adipose tissue mesenchymal stem cells: a user's guide, Stem Cells Dev, 19 1449–1470.

Pilgaard, L., Lund, P., Duroux, M., Fink, T., Ulrich-Vinther, M., Soballe, K., Zachar, V., 2009a. Effect of oxygen concentration, culture format and donor variability on in vitro chondrogenesis of human adipose tissue-derived stem cells. Regen. Med. 4, 539–548.

Pilgaard, L., Lund, P., Duroux, M., Lockstone, H., Taylor, J., Emmersen, J., Fink, T., Ragoussis, J., Zachar, V., 2009b. Transcriptional signature of human adipose tissue-derived stem cells (hASCs) preconditioned for chondrogenesis in hypoxic conditions. Exp. Cell Res. 315, 1937–1952.