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Adipose-derived Stem Cells from the Brown Bear (*Ursus arctos*) Spontaneously Undergo Chondrogenic and Osteogenic Differentiation

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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 adipose tissue of wild Scandinavian brown bears and characterize osteogenic, chondrogenic, and adipogenic differentiation in the cells. Following immobilization of 8 wild brown bears 7-10 days after leaving the den in mid-April, adipose tissue biopsies (5-8 ml) were obtained subcutaneously from 7 bears. ASCs were recovered and characterized. Adipose stem cell cultures were established from 6 of 7 bears. Adipose tissue-derived stem cells 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 differentiating into osteocytes and chondrocytes. These findings could have implications for the use of hibernating brown bears as a model to study osteoporosis.

Key words:

Adipose, osteogenesis, chondrogenesis, differentiation, brown bear

INTRODUCTION

Hibernating Scandinavian brown bears (*Ursus arctos*) have no physical activity for 5-7 months while inside their winter dens [1,2]. 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 [3,4].

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 four 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 (Table 1).

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. 3). 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. 3, nodules indicated by arrows). Bear culture B4 (from a 16-year-old bear) showed

characteristics similar to the human control cell line, without nodule formation or positive staining with Alizarin red (table 1).

Chondrogenic properties of the ASCs

We did not see positive staining in any of the cultures exposed to chondrogenic induction medium (Fig. 2). Surprisingly, however, we found evidence of chondrogenic differentiation in the control cultures from all three yearling bear cell lines (Fig. 3, table 1). In cultures where cells had formed nodules, lakes of glycosaminoclycans 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. Bear culture B4 (from a 16-year-old bear) showed characteristics similar to the human control cell line, with neither nodule formation nor positive staining with Alcian Blue.

DISCUSSION

This study represents the first documentation of stem cells from brown bears. ASCs were recovered and cultured with a high success rate and 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. 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 [5]. It is possible that the control medium allows for a higher differentiation rate of the cells and optimum nodule formation.

The mineral content of femurs has been found to increase with age in black bears (*Ursus americanus*) [4]. In this context, it would be interesting to determine if there is a correlation between the propensity of stem cells from bears to spontaneously form bone cells and the mineral content of bone. In conclusion, the spontaneous osteogenesis and chondrogenesis of the ASCs, highlight the potential use of hibernating bears and ASCs therefrom as model systems to study 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 tiletamine-zolazepam and medetomidine [6]. Adipose tissue biopsies (1.5-3 ml) were obtained subcutaneously during intra abdominal implantation of tracking devices [7]. 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 hours 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 two hours. The digests were centrifuged at 400g 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 10x 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 CO₂ incubator overnight, after which non-adherent cells were removed. The media was 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. All subsequent experiments were performed on cells in passage 4 in duplicates in two independent experiments.

Human ASCs for use as control samples were isolated and propagated as previously described after informed written consent [8]. The regional Committee on Biomedical Research Ethics of Northern Jutland, Denmark approved analysis of human stem cells from persons undergoing elective liposuction (project no. 2005054).

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 [8]. In brief, to induce adipogenesis, cells were incubated for two weeks in adipogenic induction medium consisting of α -MEM supplemented with FCS, isobutylmethylxanthine (IBMX) insulin, and indomethacin, after which the adipogenic differentiation was visualised through staining of intracellular lipid accumulation with Oil Red O.

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

To induce chondrogenesis, the cells were incubated in media consisting of high-glucose (4.5 g/l) Dulbecco's modified Eagle's medium supplemented with 10 transforming growth factor β 3 (TGF β 3), dexamethazone, L-ascorbic acid 2-phosphate, L-proline, and $1 \times$ ITS⁺ Premix. After three weeks in culture, the chondrogenic differentiation was assessed by staining extracellular deposition of glycosaminoglycans 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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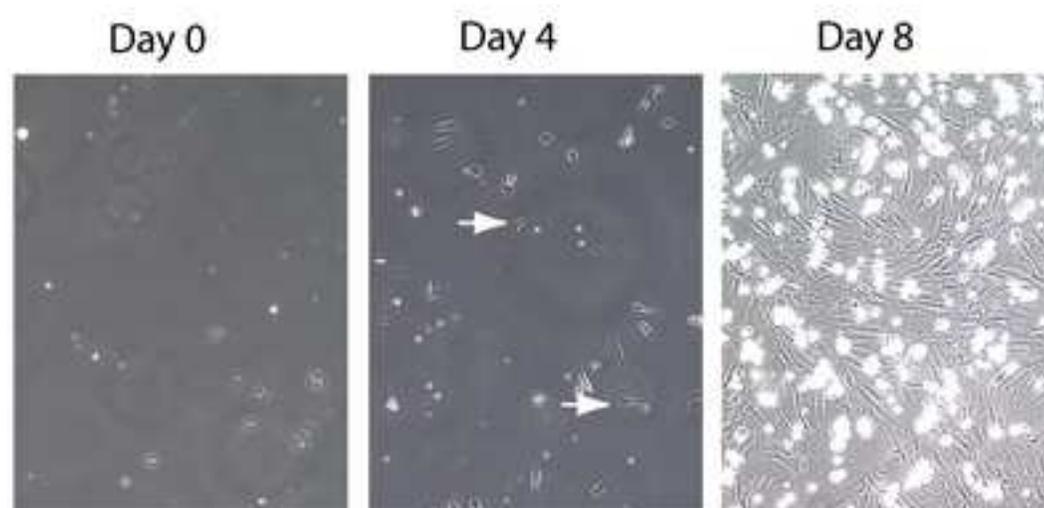
FIGURE LEGENDS

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 two weeks in standard growth medium or adipogenic induction medium, after which intracellular lipids were stained with Oil red O. Arrows indicate lipid inclusions. Magnification, x 100.

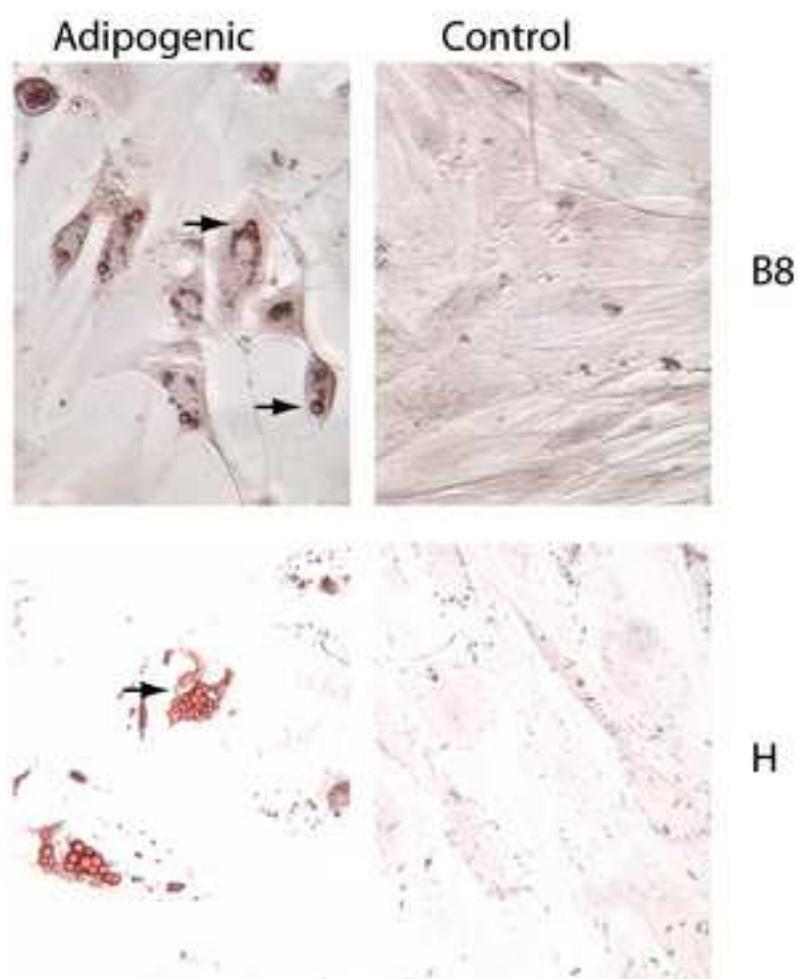
Figure 2. Osteogenic differentiation of ASCs from bears and one human. Bear (B1-B8) and human (H) stem cells were cultured in standard growth medium (control cultures) or osteogenic induction medium for three weeks, after which the cultures were stained with Alizarin red. Arrows indicate nodules. Magnification,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

Figure 3. Chondrogenic 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 chondrogenic induction medium for three weeks, after which the cultures were stained with Alcian blue. The cyan-colored glycosaminoglycan deposits are indicated by circles. Magnification, x 40.

A

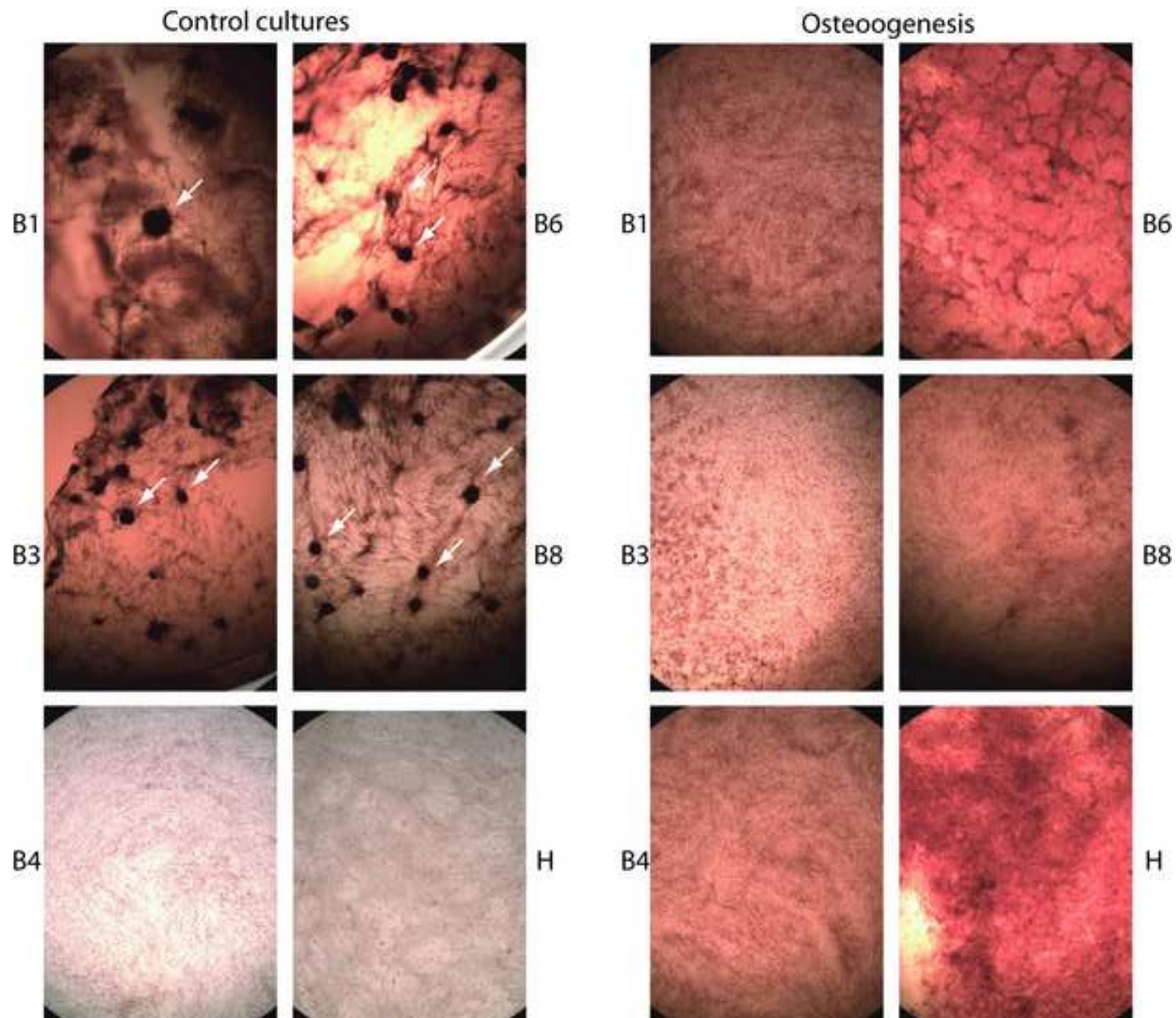


B



Figure

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Figure

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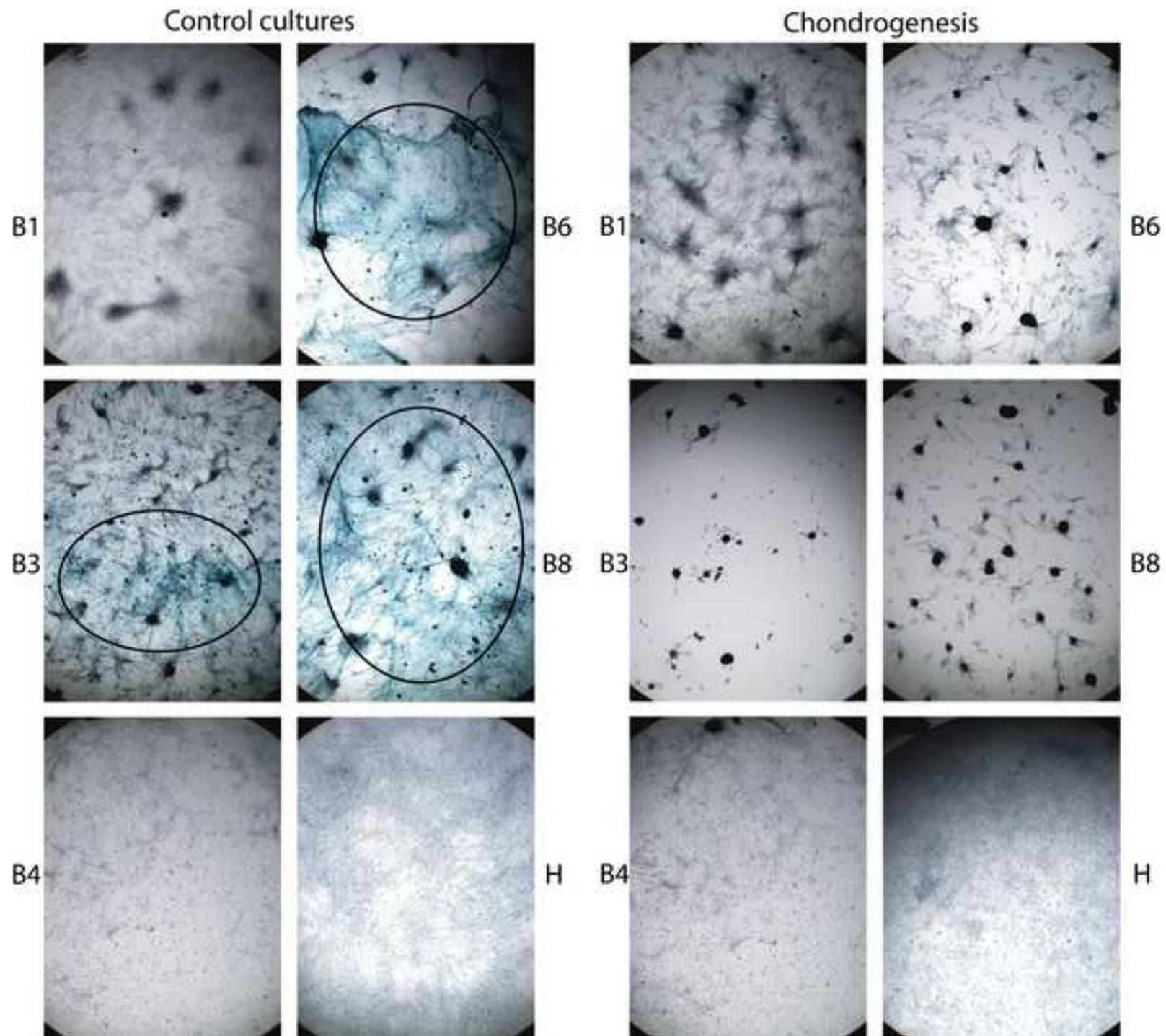


Table 1. Summary characteristics of brown bear-derived cell cultures

Bear No	Sex	Year	Establishment	Optimal	Spontaneous	Spontaneous
		of Birth	of cell cultures	cell growth	osteogenesis	chondrogenesis
B1	F	2000	Yes	Yes	Yes	No
B3	M	2008	Yes	Yes	Yes	Yes
B4	F	1993	Yes	Yes	No	No
B5	F	1995	Yes	No	-	-
B6	M	2008	Yes	Yes	Yes	Yes
B7	M	2008	No	-	-	-
B8	M	2008	Yes	Yes	Yes	Yes