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Ovine Cord Blood Accommodates Multipotent Mesenchymal Progenitor Cells

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Abstract. *Background: Stem cells derived from umbilical cord blood display mesenchymal multipotency and can differentiate into osteoblasts, chondroblasts and adipoblasts in vitro under defined stimuli. Although sheep have been used as experimental models for investigations on xenoreactivity after transplantation of stem cells isolated from human umbilical cord blood, the potential of ovine cord blood stem cells to differentiate has been examined to date. Materials and Methods: Mononuclear cells from the placentoms of 3 lambs were isolated via density gradient centrifugation and cultivated. After expansion up to 3 passages, the cells were stimulated to differentiate towards osteogenic (dexamethasone, ascorbic-acid-2-phosphate, β-glycerol-phosphate), chondrogenic (TGF-β3, insulin, transferrin, selenium, dexamethasone, ascorbic-acid-2-phosphate) and adipogenic (indomethacine, insulin, 3-isobutyl-1-methylxanthine, dexamethasone) lines for 20 days. The cells were characterized morphologically by transmission and phase contrast light microscopy during lineage-specific stimulation. Immunocytochemistry and conventional stains were used to detect lineage-typical markers: fat vacuoles and peroxisome proliferation-acitivated receptor γ2 (PPAR) served to detect adipoblasts, whereas osteopontin (OP) was used to characterize osteoblasts. A positive antibody reaction to collagen II and chondrogenic oligomeric protein (COMP) revealed the presence of chondroblasts. Results: The osteogenic line formed bone nodules, adipogenic cells developed lipid droplets and the cells of the chondrogenic line showed typical chondroblast-like morphology. Conclusion: It was demonstrated that ovine mesenchymal stem cells, derived from umbilical cord blood (sheep unrestricted somatic stem cells, S-USSCs), can be isolated via gradient density centrifugation and expanded in vitro. Under lineage-specific stimulation, S-USSCs differentiated into osteo-, chondro- and adipoblasts with typical morphological characteristics. Significant quantitative differences between the stimulated and control groups in lineage-typical immunocytochemical markers verified these findings.*

Stem cell-based therapy is rapidly becoming a promising alternative in the treatment of musculoskeletal defects. As early as the 1970s, Friedenstein *et al.* (1, 2) began investigating bone marrow-derived mesenchymal stem cells (MSCs) and the groundwork for characterizing and understanding MSC differentiation pathways was laid in the following decades (3-7). In addition to donor site morbidity, risks of infection and tumor development, the immunogenic barrier between the host and donor is still a challenging problem in tissue engineering and limited allogene stem cell transplantations.

Human MSCs have been isolated from numerous tissues like bone marrow, cord and cord blood, peripheral blood, liver, lymphatic organs, periosteum and fat (8-12). It has been shown that unrestricted somatic stem cells (USSCs) derived from human cord blood are pluripotent and can differentiate into osteo-, chondro- and adipogenic cells *in vitro* (8, 13). Furthermore, there is evidence that the use of USSCs leads to a significant reduction in transplant rejections after xenotransplantation and allows graft survival rates comparable to those of other MSC types (8, 13-15). Recent reports showed similar results for human umbilical cord perivascular (HUCPV) cells (16) and MSCs harvested from Wharton's jelly (17).

Appropriate experimental models are needed to elucidate the differentiation pathways of MSCs derived from cord blood

Abbreviations: BFU-E, burst forming units erythroid; CD, cluster of differentiation; COMP, chondrogenic oligometric protein; CSD, critical size osseous defect; DAB, 3,3-diaminobenzidine; DAG, dexamethasone, ascorbic-acid-2-phosphate, β-glycerol-phosphate; DMEM, Dulbecco's modified Eagles medium; FCS, fetal calf serum; HS, horse serum; HUCPV, human umbilical cord perivascular; IGF, insulin-like growth factor; IMDM, Iscove's modified Dulbecco's medium; MSC(s), mesenchymal stem cell(s); OP, osteopontin; P, passage; PBS, phosphate-buffered saline; PPAR, peroxisome proliferation-acitivated receptor γ2; S-USSC(s), sheep-derived unrestricted somatic stem cell(s); TCP, tricalcium phosphate; TGF, transforming growth factor.

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Key Words: Stem cell, sheep, osteoblast, chondroblast, adipoblast.

and to evaluate possible immunoreactions *in vivo*. The critical size osseous defect (CSD) in small animals is such a model and has been used to investigate cell therapeutics and local bone healing (13, 18, 19). Other reports emphasize the value of small animals to assess the regeneration of cartilage and fat after stem cell transplantation (20, 21). This model is based on the local placement of MSCs into a tissue defect zone, with or without scaffolds of biomaterial, or on the systemic application of MSCs. The disadvantages of using small animals in these types of investigations include the high technical demands and the lack of feasibility for biomechanical evaluations, for which large animal models would be much more effective. One possible model in which the regeneration of bone and cartilage can be examined is the sheep. Almeida-Porada *et al.* (22-24) showed that the sheep model can be useful for human stem cell research and Windhagen *et al.* (25) demonstrated that it is also feasible for biomechanical studies in fracture callus-distraction investigations.

Although the sheep model is a valuable tool for the study of the mechanisms underlying stem cell differentiation after xenotransplantation of human stem cells (24, 26), there are, to date, no studies which reveal the *in vitro* differentiation potential of ovine MSCs derived from cord blood. In the present study, we showed, for the first time, that MSCs isolated from ovine cord blood (sheep-derived unrestricted somatic stem cells, S-USSCs) are multipotent and can differentiate into osteo-, chondro- and adipogenic lines under adequate stimuli.

Materials and Methods

Immediately after natural birth, the blood of 3 lambs (German breed known as Moorschnucken) was obtained by sterile puncture of the cord placentoms (Figure 1a). Samples of 5 to 20 ml were incubated in an equal volume of Dulbecco's modified Eagles medium (low glucose DMEM-LG; PAA Laboratories, Cölbe, Germany) containing 10,000 IU heparin (Liquimine, La Roche) and 1% penicillin/streptomycin/L-glutamine. MSCs were isolated by densitiy gradient centrifugation and cultured in DMEM-LG supplemented with 30% fetal calf serum (FCS; Biochrome, Berlin, Germany), 1% penicillin/streptomycin/L-glutamine in tissue culture T75 polystyrene flasks at 37˚C and 5% CO_2 for 30 days (7).

On day 10, adherent cells, judged 80%-90% confluent by phase contrast microscopy, were passaged using a 0.1% trypsin/EDTA solution (PAA Laboratories) and plated in tissue polystryrene flasks. The second and the third passages followed on days 20 and 30. Furthermore, passage (P) 1 cultures were maintained without any additional passage for 20 days (Figure 1b).

After the third passage, $1x10^5$ cells were cultured in 24-well plates and stimulated for 20 days with osteogenic, chondrogenic or adipogenic mixtures as follows:

Osteogenic mixture: 500 ml DMEM-LG, 50 ml FCS, 625 μl 50 mM ascorbic-acid-2-phosphate (Sigma, Steinheim, Germany), 10 ml 1 M β-glycerol-phosphate (Sigma), 500 μl 10^{-5} M dexamethasone (DAG) (Sigma) and 5 ml penicillin/streptomycin/L-glutamine (PAA Laboratories).

Chondrogenic mixture: 450 ml DMEM-LG, 500 μl insulin-transferrin-selenium (ITS Liquid Media Supplement, Sigma), 562 μl 50 mM ascorbic-acid-2-phosphate, 90 μl TGF-β3 (Sigma), 450 μl 10^{-5} M dexamethasone and 5 ml penicillin/streptomycin/L-glutamine.

Adipogenic mixture: 450 ml DMEM-LG + 80 μl indomethacine (Sigma), 2250 μl insulin (Sigma), 5 ml 3-isobutyl-1-methylxanthine (Sigma), 176 μl 10^{-5} M dexamethasone, 50 ml FCS and 5 ml penicillin/streptomycin/L-glutamine.

Unstimulated cells cultured under corresponding conditions served as control groups. Cell growth was monitored and documented by episcope light microscopy. The culture medium was changed every third day. All cultures were carried out in triplicate.

Osteoblastic differentiation was detected by a positive antigen reaction to osteopontin (OP), whereas chondrogenic differentation was revealed by positive reactions to chondrogenic oligomeric protein (COMP) and collagen II. Adipogenic differentiation was verified by the presence of fat and the reaction product for the peroxisome proliferation-acitivated receptor γ2 (PPAR).

The immunoreactions were carried out on culture monolayers fixed in 5% paraformaldehyde at 4˚C for 30 min, rinsed in phosphate-buffered saline (PBS) and dehydrated in graded alcohols. Endogenous peroxidases were blocked by a 3% solution of perhydrol-isopropanol (Merck, Darmstadt, Germany). After rinsing in Tris-buffer, the samples were incubated with primary antibodies against OP, (Chemicon, Germany), type II collagen (Chemicon Int., Hampshire, UK), COMP (Serotec GmbH, Düsseldorf, Germany) or PPAR (Sigma) and then incubated at 4˚C for an additional period of 12 h. The reaction product was contrast-enhanced with the avidin-biotin complex and 3,3-diaminobenzidine (DAB) (Sigma).

To detect intracellular lipids as a sign of adipoblastic differentiation, monolayers were fixed in 5% paraformaldehyde, rinsed twice in distilled water, immersed in 50% isopropanol for 5 min, and stained with oil red staining solution (Sigma).

The specimens were examined and evaluated in a phase and transmission light microscope (Axiovert 200, Zeiss, Germany) connected to a computer image analysis system (Axiovision, Zeiss).

The Student's *t*-test for independent statistical groups was used. $P<0.01$ was rated highly significant and $p<0.05$ statistically significant, whereas $p>0.05$ was not considered significant. Average values (X) and standard deviations (SD) served as descriptive parameters.

Results

Density gradient centrifugation of cord blood yielded a suspension of mononuclear cells highly contaminated with erythrocytes. However, with each change of culture medium, the number of erythrocytes was reduced and, by the seventh day, they could only occasionally be found. The cells were passaged after 10 days (P1) when a confluent monolayer had developed. Ten days later, this culture was characterized by large numbers of mesenchymal-fibroblastoid cells and showed some bone nodule formation corresponding to spontaneous osteoblastic differentiation. The second passage (P2) was carried out at this time. Ten days later (total culture period of 30 days), this P2 culture consisted of a mixture of round-to-oval cells intermingled

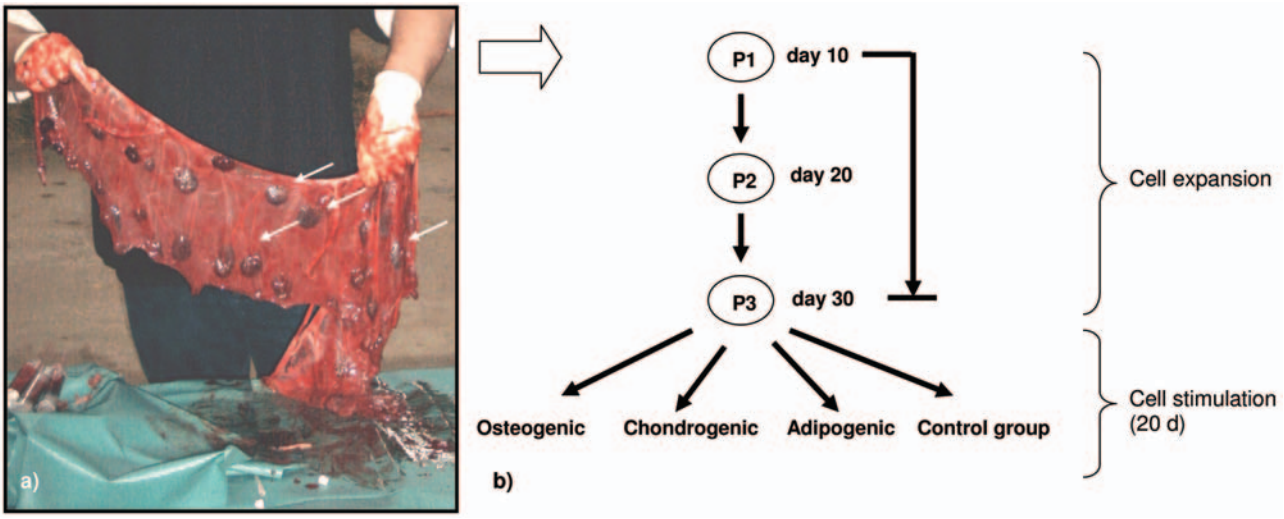


Figure 1. *a) In contrast to the dicoid type of placenta in humans, the ovine placenta is characerized by multiple cavernous placentoms (arrows). The ovine placenta includes about 100 of these deep-dish like fetomaternal units. In ruminants these placentomes attach the cotyledons of the fetal placenta with the maternal placental caruncula. The cotyledon is the basic functional unit, so the thin and less-branched cotyledon leads to the shortage of a blood supply to the fetus and then influences fetal growth and development. b) The figure shows the study design after isolation of ovine cord blood-derived mononuclear cells. P: passage.*

with mesenchymal-fibroblastoid cells. The proliferation rate of the cultured cells showed a linear trend during expansion from passages 1 to 3 (Figure 2).

Figures 3 and 4 show images of characteristic cell morphology at different kinetic points (12 h, 4 days, 8 days, 12 days, 16 days and 20 days) during lineage-specific stimulation. Distinct differences between the three lines could be recognized as early as the fourth day of culture. At this time, the chondrogenic cultures consisted predominantly of small round cells with a central nucleus, whereas fibroblastoid cells were much more numerous in the adipo- and osteogenic cultures. On the eigth day of stimulation, the adipogenic cells contained multiple intracellular vacuoles and a peripheral nucleus. The osteogenic culture, however, showed no marked morphological changes from the fourth to the eigth day of stimulation. Both osteo- and, to a lesser degree, chondrogenic cells tended to aggregate by the eigth day (osteogenic clusters: 5-25 cells; chondrogenic clusters: 4-6 cells). At this time, the cells of the chondrogenic culture were morphologically more uniform than those of the other two cultures. Most cells were small and round.

On day 12, all three stimulated cultures showed areas of cell accumulation (cell aggregates). Among the osteogenic cells the mesenchymal-fibroblastoid type predominated, but a few single roundish cells were also present. By contrast, the adipogenic culture contained predominantly aggregates of 4-8 round cells interposed by isolated mesenchymal-fibroblastoid cells. Furthermore, the number of vacuoles increased continuously with time in the adipogenic cells.

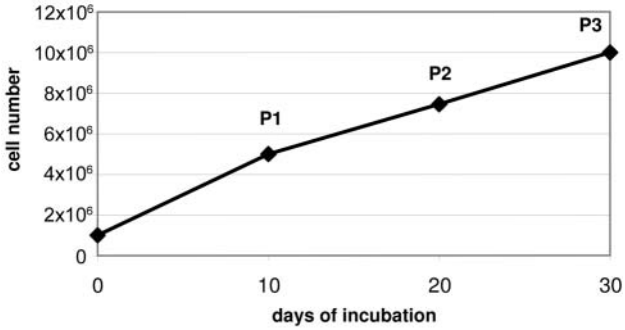


Figure 2. *Expansion of ovine cord blood-derived mononuclear progenitor cells without lineage-specific stimuli over three passages (P1-3). As shown by the graph, the S-USSCs demonstrated a linear proliferation rate with a confluent monolayer of adherent cells after 10 days.*

On days 16 and 20, all stimulated cultures showed further cell differentiation. There were signs of bone nodule formation in the osteogenic cultures (clusters of rounded cells surrounded by mesenchymal cells, see Figure 3) and intracellular, multiple vacuoles in adipogenic cells. Cells in the nodules had the morphological characteristics of mature osteoblasts and contained dense intracellular granula at 20 days. Both the control and the osteogenic cultures showed signs of osteoblastic differentiation. The morphology of the chondrogenic cells remained more uniform than the other two, even on the twentieth day of stimulation.

Figure 3.

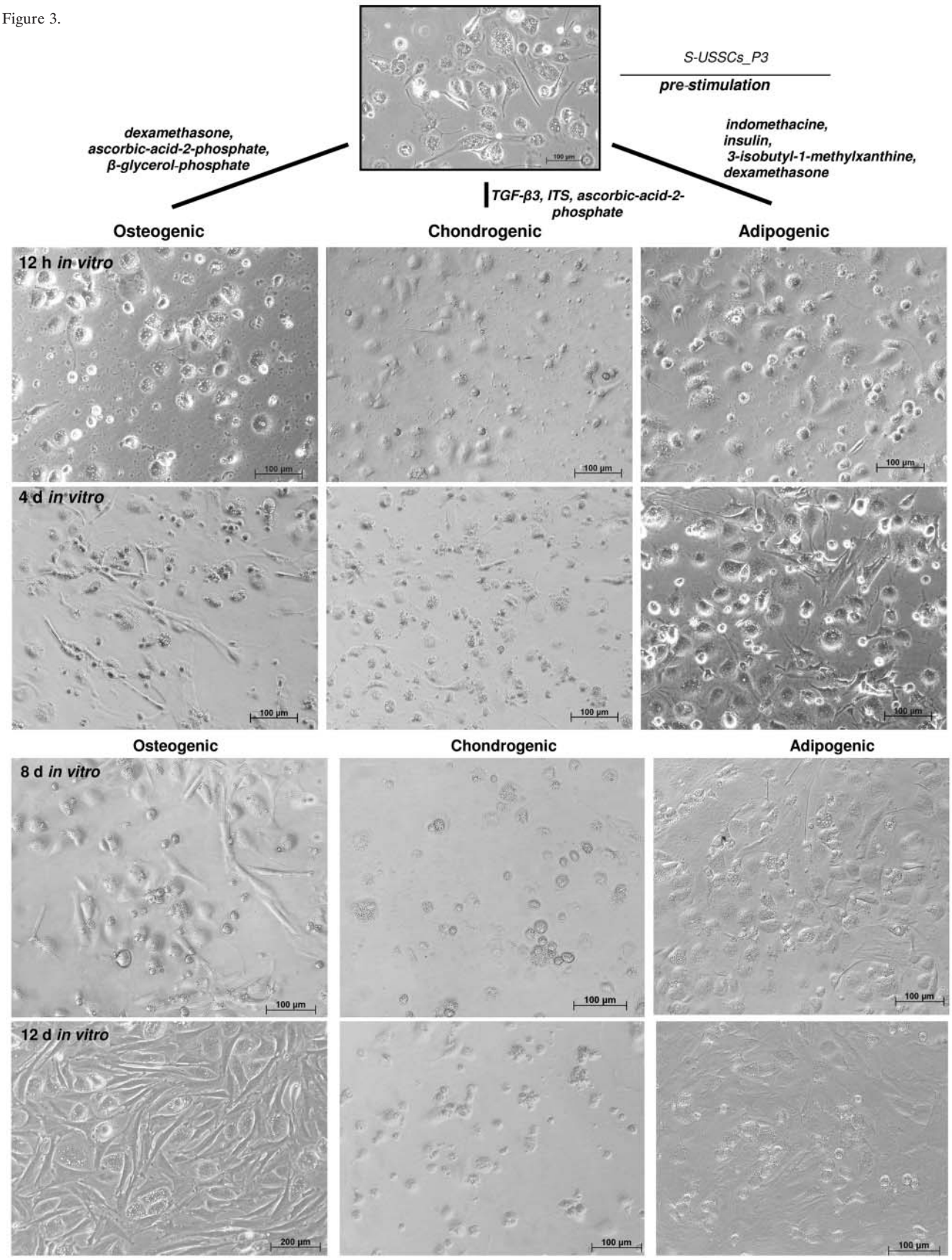

S-USSCs_P3
pre-stimulation
dexamethasone,
ascorbic-acid-2-phosphate,
β-glycerol-phosphate
indomethacine,
insulin,
3-isobutyl-1-methylxanthine,
dexamethasone
TGF-β3, ITS, ascorbic-acid-2-phosphate
Osteogenic
Chondrogenic
Adipogenic
12 h in vitro
4 d in vitro
8 d in vitro
12 d in vitro
100 µm
200 µm

Figure 3.*continued*

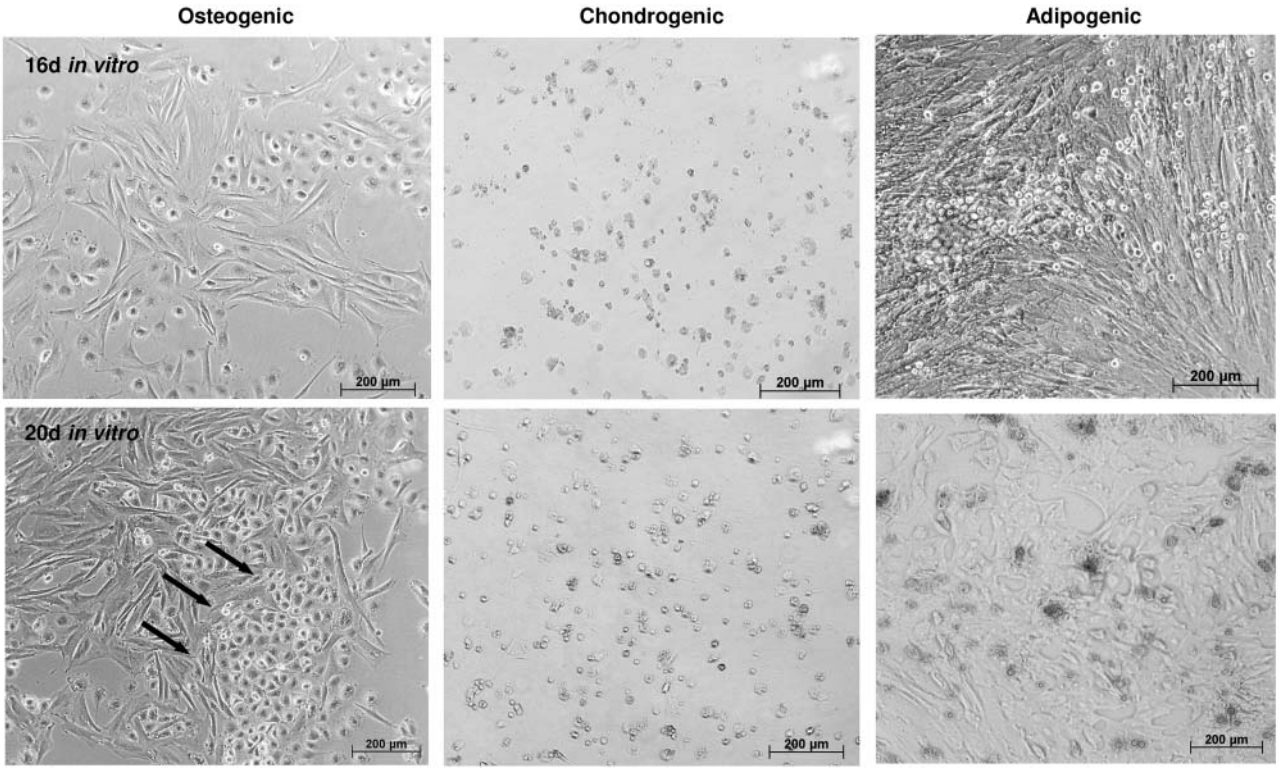


Figure 3. *Morphological differentiation of cord blood-derived stem cells at different kinetic points with a maximum follow-up of 20 days under specific stimuli. Significant differences were demonstrated in cellular morphology and formation between the differently-stimulated cultures from day 4 in vitro. In the osteogenic-stimulated culture, bone nodule formation was found at the end of the study (arrows on day 20).*

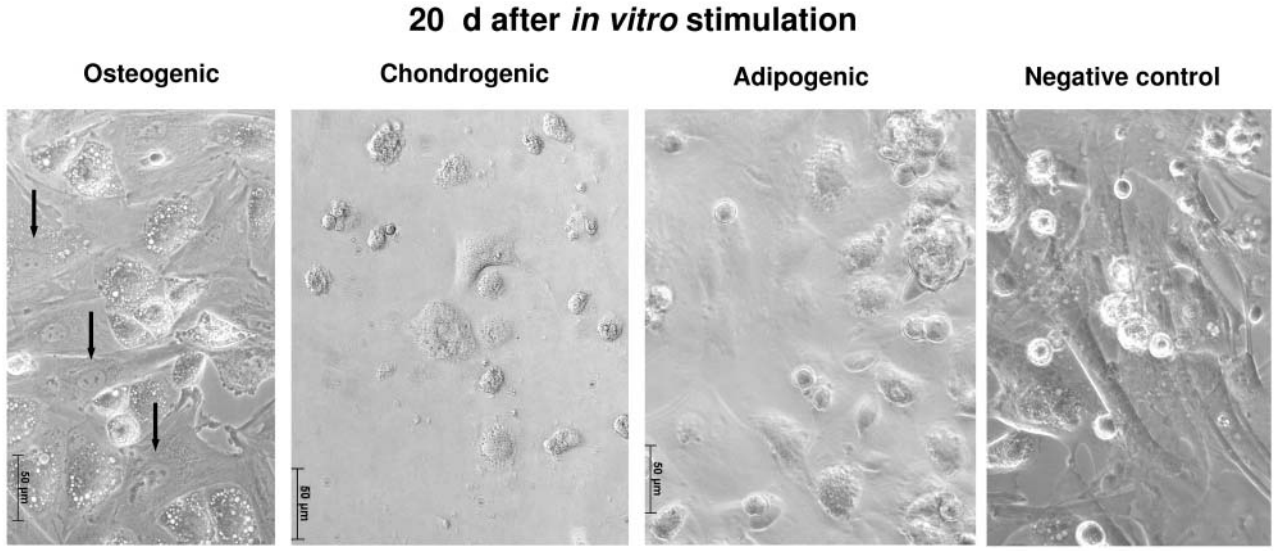
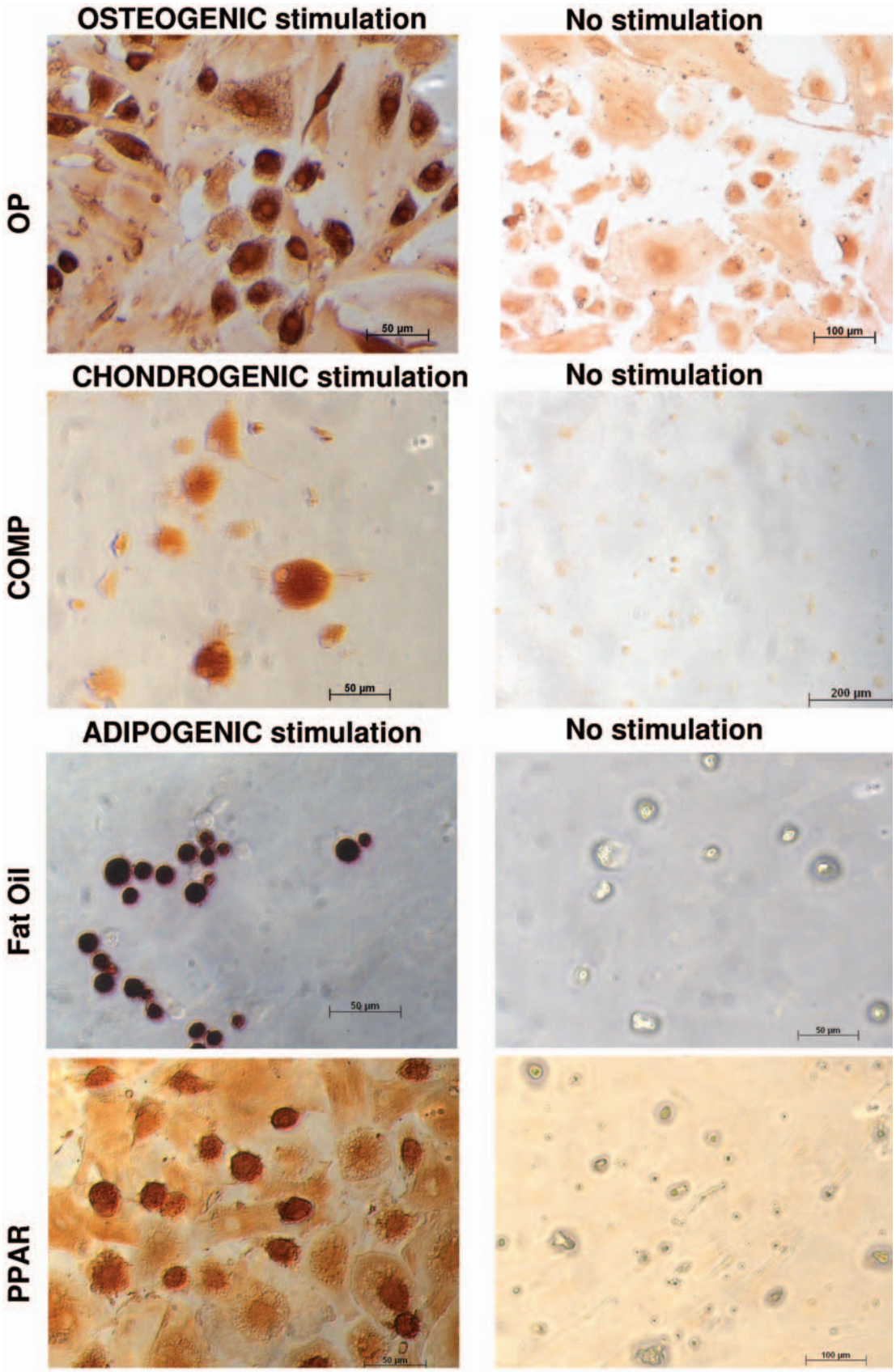


Figure 4. *The figure shows the characteristic cellular morphology 20 days after lineage-specific stimulation. In contrast to the adipogenic- and chondrogenic-stimulated cultures, there were morphological similarities between the osteogenic-stimulated cells and the control group. In both cultures, a flat-cuboidal cell type predominated, but osteogenic-stimulated cells showed an increased rate of mitosis (arrows) compared to the control group.*


OSTEOGENIC stimulation
No stimulation
OP
50 µm
100 µm
CHONDROGENIC stimulation
No stimulation
COMP
50 µm
200 µm
ADIPOGENIC stimulation
No stimulation
Fat Oil
50 µm
50 µm
PPAR
50 µm
100 µm

Figures 5 and 6 illustrate the qualitative and quantitative results of antigen expression and markers. There were significant differences between stimulated and unstimulated cultures in all three lineages (osteo-, chondro- and adipogenic). The reaction product for OP marked most cells in the dexamethasone, ascorbic-acid-2-phosphate, β-glycerol-phosphate (DAG)-stimulated osteogenic cultures clearly, but was only faintly expressed in unstimulated cultures. This was also true for the differences between the large number of cells expressing COMP under chondrogenic stimulation and the lack of them in the unstimulated control cultures. Both fat and PPAR reactions were more intense and more numerous in the adipogenic cultures than in the unstimulated control cultures.

Discussion

In this study, it was shown that mononuclear cells isolated from ovine cord blood can be expanded and undergo osteo-, chondro- and adipogenic differentiation under specific stimuli *in vitro*. In agreement with earlier work on USSCs from human cord blood (8, 27), which are able to differentiate into these same lineages, we named the ovine cells S(sheep)-USSCs.

Several studies have investigated the engraftment, homing and differentiation of human cord blood stem cells after *in utero* transplantation in pre-immune fetal sheep (24, 26). However, there is still a lack of information on stem cells derived from ovine cord blood.

Francey *et al.* (28) demonstrated that ovine bone marrow cells are able to differentiate into mononuclear phagocytes and macrophages. Kelly *et al.* (29) showed that early erythroid progenitors (burst-forming units erythroid, BFU-E) can be isolated from sheep peripheral blood and cultured under defined conditions. Marsicano *et al.* (30) described the cultivation technique for long-term ovine marrow cultures able to sustain hemopoiesis cells. These authors used Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FCS, 10% horse serum and $5x10^{-7}$ M hydrocortisone. In an earlier study, we showed that the culture conditions had a significant influence on the differentiation of MSCs, whether derived from cord blood or from bone marrow (27).

In contrast to numerous studies examining the function and differentiation of hematopoietic ovine stem cells, Jessop *et al.* (31) found the first indications for the existence of MSCs within the bone marrow of sheep. Their results corresponded to Perry *et al.* (32), who isolated MSCs from sternal bone marrow from sheep to develop a trileaflet heart valve.

Rhodes *et al.* (33) isolated MSCs from ovine bone marrow and found no significant correlation either between donor age and proliferation potential, or between age and the initial number of MSCs. The proliferation rate of S-USSCs from P1 to P3 showed a close to linear curve. Moreover, there tended to be a negative correlation between the proliferation rate and concentration of MSCs in the samples. Comparable studies on the growth capacity of human MSCs, derived either from cord blood or from bone marrow, have shown that there are significant differences between the two cell types: the initial cell density of cord blood MSCs must be much higher for successful expansion (34). In our experiments, the S-USSCs proliferated well at densities of $1x10^5$ cells per 3.2 cm^2 petri dish under lineage-specific stimulation.

According to Rhodes *et al.* (33), we supplemented the MSC cultures with 10% FCS and used DAG for osteogenic stimulation. For chondrogenic stimulation, TGF-β3 instead of TGF-β1 was performed. In contrast to our study, Rhodes *et al.* (33) verified and characterized lineage-specific cells only by phenotype, but not by antigen expression.

Besides cord blood-derived MSCs from sheep, there is also a lack of information in the literature about the differentiation potency of ovine MSCs from other tissues. Pisansarakit *et al.* (35) cultivated monolayer outgrowths from mesenchymal components of the skin and vibriss follicles of sheep. They showed a good cellular proliferation under FCS, epidermal growth factor and fibroblast growth factor exposure.

Guo *et al.* (36) seeded bone marrow-derived and culture-expanded autologous MSCs from sheep into bioceramic scaffolds (β-TCP) to repair ovine articular cartilage defects. They demonstrated that MSCs from sheep were able to differentiate into chondroyctes *in vivo*. These results corresponded to the data of Chen *et al.* (37), who used a polylactide scaffold for autologous transplantation of MSCs obtained from the iliac crest of male sheep and emphasized the importance of synovial fluid for chondrogenic differentiation.

Zhang *et al.* (38) elucidated the *in vitro* differentiation of sheep-derived MSCs from bone marrow and recommended the use of DMEM medium supported by TGF-β3, IGF-I, dexamethasone and ascorbic-acid-2-phosphate.

In a previous study, we showed large numbers of CD34+ / CD45+ cells after density gradient centrifugation in human cord blood samples compared to bone marrow-derived cells (27). Corresponding to these results, extensive contamination by ovine erythrocytes was found after cord blood density gradient centrifugation. We decided to

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Figure 5. *Immunocytochemical stainings of S-USSCs after lineage-specific stimulation on day 20. The figure demonstrates the qualitative and quantitative differences between the stimulated cultures and the control groups. OP: osteopontin, COMP: chondrogenic oligomeric protein, PPAR: peroxisome proliferation-activated receptor γ2.*

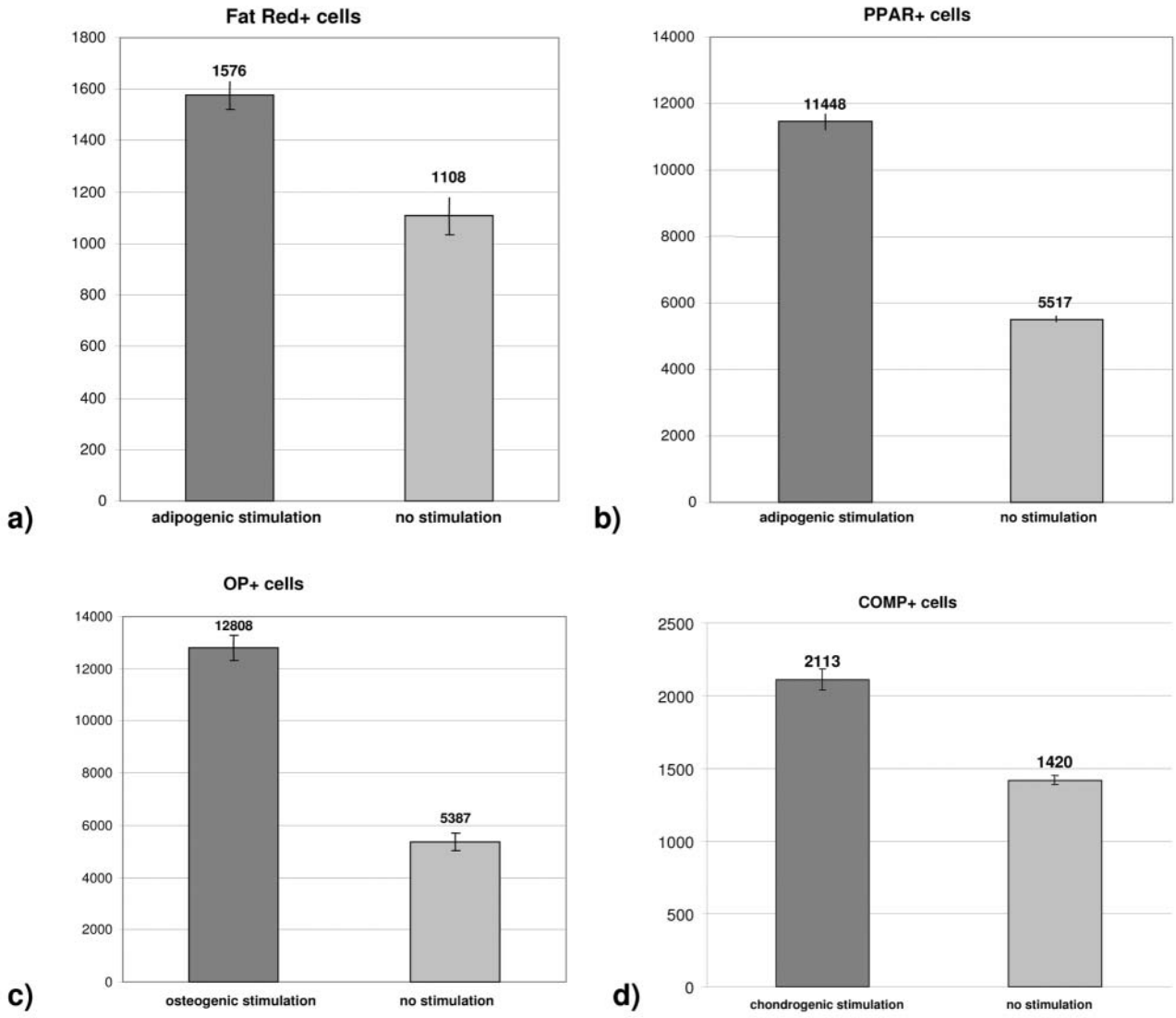

Figure 6. *a-d. Quantification of antigen expression and fat oil-positive cells under defined, lineage-specific stimuli in S-USSCs. The graph shows significant differences between stimulated cultures and ovine stem cells without lineage-specific stimulation. Figures 6a and b show the results after adipogenic stimulation, cells in Figure c were stimulated with osteogenic mixture and Figure d demonstrates the results after chondrogenic stimulation. Although there were morphological similarities between the osteogenic-stimulated cultures and control groups, as shown in Figures 3 and 4, there was a significant increase in OP expression for the osteogenic-stimulated culture. OP: osteopontin, COMP: chondrogenic oligomeric protein, PPAR: peroxisome proliferation-acitivated receptor γ2.*

dispense with cellular lysis of these red cells by β-mercaptoethanol, since some authors reported that erythrocyte lysing techniques were critical for cellular differentiation in cord blood-derived cells and might alter non-hematopoietic cell antigens and structures (39). McNiece *et al.* (40) showed that *ex vivo* expansion of human CD34+ cells generated an increased number of mature and progenitor cells that were capable of more rapid engraftment in fetal sheep compared to unexpanded CD45+ cells. Furthermore, Wang *et al.* (41) showed that cord blood-derived MSCs had the potential to support *ex vivo* expansion of hematopoietic stem cells and allow for chondrogenic differentiation. Koc *et al.* (42) demonstrated advantages in clinical outcome by co-infusion of culture-expanded MSCs with hematopoietic stem cells in patients treated with high-dose chemotherapy for solid tumors. One problem with the immunohistochemical identification of ovine antigens is the limited availability of appropriate antibodies. For example, esterases and the osteoblast marker alkaline phosphatase differ significantly between

species (43). However, the antibodies which were used in this study showed significant binding differences dependent on lineage-specific stimulation.

Conclusion

This is the first study to show that postpartal umbilical cord blood-derived MSCs from sheep allow for osteo-, chondro- and adipogenic differentiation *in vitro* under cell line-specific stimuli. The mesenchymal population of ovine cord blood should be the target for future investigations.

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