*J. Cell. Mol. Med. Vol 15, No 6, 2011 pp. 1364-1378*

# Directly auto-transplanted mesenchymal stem cells induce bone formation in a ceramic bone substitute in an ectopic sheep model

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*Received: April 7, 2010; Accepted: June 2, 2010*

## Abstract

Bone tissue engineering approaches increasingly focus on the use of mesenchymal stem cells (MSC). In most animal transplantation models MSC are isolated and expanded before auto cell transplantation which might be critical for clinical application in the future. Hence this study compares the potential of directly auto-transplanted *versus in vitro* expanded MSC with or without bone morphogenetic protein-2 (BMP-2) to induce bone formation in a large volume ceramic bone substitute in the sheep model. MSC were isolated from bone marrow aspirates and directly auto-transplanted or expanded *in vitro* and characterized using fluorescence activated cell sorting (FACS) and RT-PCR analysis before subcutaneous implantation in combination with BMP-2 and β-tricalcium phosphate/hydroxyapatite (β-TCP/HA) granules. Constructs were explanted after 1 to 12 weeks followed by histological and RT-PCR evaluation. Sheep MSC were $CD29^+$, $CD44^+$ and $CD166^+$ after selection by Ficoll gradient centrifugation, while directly auto-transplanted MSC-populations expressed CD29 and CD166 at lower levels. Both, directly auto-transplanted and expanded MSC, were constantly proliferating and had a decreasing apoptosis over time *in vivo.* Directly auto-transplanted MSC led to *de novo* bone formation in a heterotopic sheep model using a β-TCP/HA matrix comparable to the application of 60 μg/ml BMP-2 only or implantation of expanded MSC. Bone matrix proteins were up-regulated in constructs following direct auto-transplantation and in expanded MSC as well as in BMP-2 constructs. Up-regulation was detected using immunohistology methods and RT-PCR. Dense vascularization was demonstrated by CD31 immunohistology staining in all three groups. Ectopic bone could be generated using directly auto-transplanted or expanded MSC with β-TCP/HA granules alone. Hence BMP-2 stimulation might become dispensable in the future, thus providing an attractive, clinically feasible approach to bone tissue engineering.

**Keywords:** mesenchymal stem cells • MSC characterization • bone tissue engineering • large animal model • sheep model • BMP-2

## Introduction

Bone grafts are an important part of the armamentarium in different medical specialities like orthopaedic surgery, head and neck surgery or in plastic and reconstructive surgery. Bone grafts are used for the treatment of non-unions and necrotic lesions, for skeletal structural support and for the reconstruction of defects resulting from trauma, tumour excision, osteomyelitis, pseudarthrosis or radiation necrosis. Once the normal bone healing process has been delayed or stopped, it is necessary to provide both stability to the fracture site and a biological stimulus for the fibrocartilagenous callus in order to complete the healing process. Furthermore, although a useful and elaborated technique, autologous bone grafts still come along with donor site morbidity including long lasting pain, paresthesia, haematoma, infections or unaesthetic scares [1]. In the past, numerous different experimental approaches addressing the problem of bone regeneration and replacement have been proposed. A large

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doi:10.1111/j.1582-4934.2010.01131.x

number of different bone graft materials have been developed including the combination with transplantation of cells and growth factors. Beside autologous bone grafts, bone substitutes such as allogenic bone or biomaterials like β-TCP/HA (β-tricalcium phosphate/hydroxyapatite) are currently used to address clinical bone loss pathologies [2]. Calcium phosphate materials such as HA and TCP are classified as bioactive ceramics. They have been widely used for the reconstruction of bone defects and are known as a three-dimensional porous material to induce bone formation and osteoconduction [3]. Growth factors as well as different types of osteogenic cells have been used in combination with bone substitute material in the past [4]. BMP-2 (bone morphogenetic protein-2) is well known as an osteoinductive protein enlarging bone formation in critical size defects and serving for bone tissue engineering purposes [5–8]. Since 2002, rhBMP-2 with a collagen carrier is approved as an iliac crest bone graft substitute for spinal fusion. Multiple centres have published successful results, with a superior fusion rate upon application of rhBMP-2 for spinal fusion [9]. The combination of osteogenic growth factor release (BMP-2) from polymer scaffolds and the addition of preosteogenic cells have further increased the potential to engineer bone [10]. Until now it is unclear which cell type serves best for bone tissue engineering purposes. Osteoblasts, periosteal cells and embryonic or mesenchymal stem cells (MSC) have been used in a large number of studies [4, 11–13]. Bone marrow derived stem cells seemed to be superior to adipose tissue derived stem cells in terms of osteogenic differentiation capacity [14]. MSC have been used either after expansion *in vitro* or as a directly auto-transplanted cell population for tissue engineering purposes in the past (10–13) but their potential for bone tissue engineering has not been evaluated in a large animal model using a clinically approved ceramic bone substitute comparing two different modes of MSC transplantation and in combination with BMP-2 stimulation. In this study we combined directly auto-transplanted or expanded MSC and BMP-2 with an osteoconductive β-TCP/HA graft material in the sheep model. Directly auto-transplanted MSC can be easily obtained and are not associated with limitation such as expanded MSC. Avoiding the expansion could reduce time until surgery and reduce risk of infection in a clinical application in the future. Furthermore, regulatory issues like the necessity of GLP/GMP facilities for *in vitro* expansion would render the use of directly auto-transplanted MSC a more attractive approach. Moreover, the combined effect of human expanded MSC or directly auto-transplanted MSC seeded onto this osteoconductive β-TCP/HA scaffold could enhance co-development of bone and supplying vessels.

# Materials and methods

## Sheep model and experimental group design

For all experiments female merino land sheep with a body weight of 25–30 kg at an age of 4 to 6 months were used. German regulations for the care and use of laboratory animals were observed at all times. The animal care committee of the University of Erlangen-Nuernberg and the government of Mittelfranken, Germany approved all experiments (Az 54.2531.31–23/06). The animals were housed in the veterinary care facility under standardized conditions of 55% air humidity and 20°C room temperature with a 12-hr light/dark rhythm. Sheep were fed once a day with 300 g of standard sheep diet (Altromin GmbH & Co. KG, Ssiff Spezialdiäten GmbH, Soest, Germany) and hay and water *ad libitum*. The animals were deprived of food for 24 hrs prior to operation to limit regurgitation.

Sedation and analgesia of the sheep were induced administering midazolam 0.5–1 mg/kg i.m. (Delta Select, Pfullingen, Germany) and ketamin 10 mg/kg i.m. (Pfizer, Karlsruhe, Germany). A central venous line was inserted into one jugular vein. Subsequently orotracheal intubation (7.5 Ch) was performed under laryngoscopic control. Respirator (Draeger, Luebeck, Germany) was adjusted to controlled artificial respiration IPPV (intermittent positive pressure ventilation) with weight adapted breath volume and breathing rate of 15 per min. Anaesthesia was maintained by inhalation of a gas mixture of 1–2% isoflurane (Baxter, Unterschleissheim, Germany) with air/oxygen. Passage of a stomach tube was performed into the paunch to prevent rumenal bloat. To adjust intra-operative fluid volume losses the animals received weight adapted of Ringer-lactate during the operation. Perioperatively antibiotic therapy (2 mg/kg Cobactan (Cefquinom), Intervet, Unterschleissheim, Germany) was administered i. m., followed by a postoperative application for 3 days. For post-operative analgesia Caprofen (4 mg/kg, Rimadyl®, Pfizer, Berlin, Germany) was given subcutaneously. The surgical site was shaved, prepped and draped for sterility. Subcutaneous pockets were prepared on the back of the sheep. A total of 2 ccm β-TCP/HA granules (TricOs®, Baxter Healthcare S. A., Wallisellen, Switzerland) were mixed with 2 ml fibrinogen–thrombin–matrix (TISSEEL VH S/D Kit, Baxter Healthcare S. A.) and 1,000,000 MSC per ml (groups 2/3/8/9/10).

Fibrinogen–thrombin matrix was prepared according to the manufactures protocol. In brief: fibrin tissue seal was mixed with 5 ml Aprotinin/Penta 3000 solution. This fibrin tissue seal solution was mixed 1:3.5 with dilution buffer. A total of 5 ml of 40 mM $CaCl_2$ solution were added to 500 IE/ml Thrombin. MSC or the BMP-2 were solved in the fibrin tissue seal solution and both components were mixed 1:1 directly before implantation. Human recombinant BMP-2 (Baxter) was added in a concentration of 2.5 μg/ml, 12.5 μg/ml or 60 μg/ml (groups 5/6/7/10). Constructs with β-TCP/HA granules in fibrinogen–thrombin matrix without cells or growth factors were used as control groups (groups 1/4). An overview of the different groups is given in Tables 1 and 2. Always constructs were distributed over different animals and at least two constructs of the same group were implanted into one sheep to be able to estimate intra- and inter-individual differences. The constructs were implanted into the subcutaneous pockets which were closed using resorbable 3–0 interrupted sutures. Animals were anesthetized for the explantation as described above and constructs were explanted.

## Bone marrow harvesting/mesenchymal stem cell isolation

After sedation and analgesia of the sheep with midazolam, ketamin and local anaesthetics, a small incision was made near the spina iliaca anterior of the iliac crest. An 11 G needle was used to puncture the iliac crest and about 20 ccm of bone marrow were aspirated. Bone marrow was diluted with PBS (phosphate-buffered saline), and bone marrow stem cells were harvested by Ficoll gradient centrifugation. Directly auto-transplanted MSC were immediately re-transplanted without any interim expansion or storage procedure. Expanded MSC were cultured in DMEM with 1% Penicillin/Streptomycin and 10% FCS (foetal calf serum). Before

**Table 1** Overview of the different groups for evaluation of apoptosis and proliferation implantation time: 2 days, 1/2 /4/6/8 weeks, *n* per group: 2

| Experimental group | β-TCP/HA + fibrinogen–thrombin matrix | Expanded MSC | Directly auto-transplanted MSC |
|---|---|---|---|
| Group 1 | X | - | - |
| Group 2 | X | X | - |
| Group 3 | X | - | X |

**Table 2** Overview of the different groups for evaluation of bone formation implantation time: 12 weeks, *n* per group: 6

| Experimental group | β-TCP/HA + fibrinogen–thrombin matrix | Expanded MSC | directly auto-transplanted MSC | BMP-2 |
|---|---|---|---|---|
| Group 4 | X | - | - | - |
| Group 5 | X | - | - | 2.5 μg/ml |
| Group 6 | X | - | - | 12.5 μg/ml |
| Group 7 | X | - | - | 60 μg/ml |
| Group 8 | X | X | - | - |
| Group 9 | X | - | X | - |
| Group 10 | X | - | X | 60 μg/ml |

cell implantation, both cell populations were labelled with DiI (Cell tracker CM –DiI, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

## FACS analysis

Flow cytometry was performed on a FACS Canto II Cytometer with a FACSDiva Sofware (BD Biosciences, San Jose, CA, USA) and analysed with FlowJo Software (Tree Star, Inc., Ashland, OR, USA). For more detailed description of applied method, please see appendix of this manuscript.

## RT-PCR analysis

For RNA extraction cells were harvested as described above. RNA of all probes was extracted using the RNeasy-mini-kit (Qiagen, Hilden, Germany) with corresponding QiaShredder. The probes were reverse-transcribed into cDNA with Omniscript®-RT-kit, oligo-dT primers for cDNA synthesis and RNase Inhibitor (Qiagen). For quantitative PCR ABsolute™ QPCR SYBR® Green kit was used (Thermo Fisher Scientific, Waltham, MA, USA) with a Light Cycler (Bio-Rad iCycler iQ5, Bio-Rad Inc., München, Germany). All kits were used according to the manufacturers' protocols. For more detailed description of applied method, please see appendix of this manuscript.

## Assessment and quantification of bone formation

After explantation constructs were fixed in formalin, de-calcificated by ethylenediaminetetraacetic acid (EDTA) treatment and paraffin embedded. Five micrometer cross-sections were obtained from six standardized planes using a Leica microtome (Leica Microsystems, Wetzlar, Germany). Paraffin sections were stained for haematoxylin and eosin. Microphotographs were taken using a Leica microscope and Leica digital camera (Leica Microsystems). For histomorphometic analysis six sections per group (groups 8/9/10) were examined by two blinded observers. Statistical analysis was performed with two-tailed unpaired Student's t-test. The critical level of statistical significance chosen was $P < 0.05$.

## Immunohistochemistry

For identification of endothelial cells CD31 immunohistochemistry was performed with a biotin-free tyramide signal amplification system (CSAII-System; Dako Cytomation, Carpinteria, CA, USA). For the collagen I immunohistochemical staining for detection of newly formed bone an ABC reagent was used. In the explanted constructs a Ki67 (mouse anti-human Ki67 Clone MIB-1, Dako, Glostrup, Denmark) staining to determine cell viability and a terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (FragEL™ DNA Fragmentation Detection Kit, Calbiochem, Darmstadt, Germany) was used for detection of apoptotic cells. For more detailed description of applied method, please see appendix of this manuscript.

# Results

## Directly auto-transplanted and expanded MSC had a different marker expression profile

Sheep MSC were successfully isolated from sheep bone marrow by puncture of the iliac crest. MSC could be isolated using Ficoll gradient centrifugation and plastic adherence selection. *In vitro* MSC showed a sufficient proliferation. Further characterization by RT-PCR analysis revealed expression of sheep MSC markers CD29, CD44 and CD166 (Fig. 1A). FACS analysis confirmed this observation on the protein level (Fig. 1B–D). Expanded MSC showed a different marker expression profile compared to directly auto-transplanted MSC. Whereas expanded MSC had a strong CD29, CD44 and CD166 expression on protein level, directly auto-transplanted MSC seemed to have only a strong CD44 expression and a weak CD29 and CD166 expression. Differences in expression of CD29, CD44 and CD166 in directly auto-transplanted MSC compared to expanded MSC were less evident on mRNA level. As indicated by increased CD45 expression, ratio of hematopoietic cells was higher in directly auto-transplanted MSC as compared to expanded MSC.

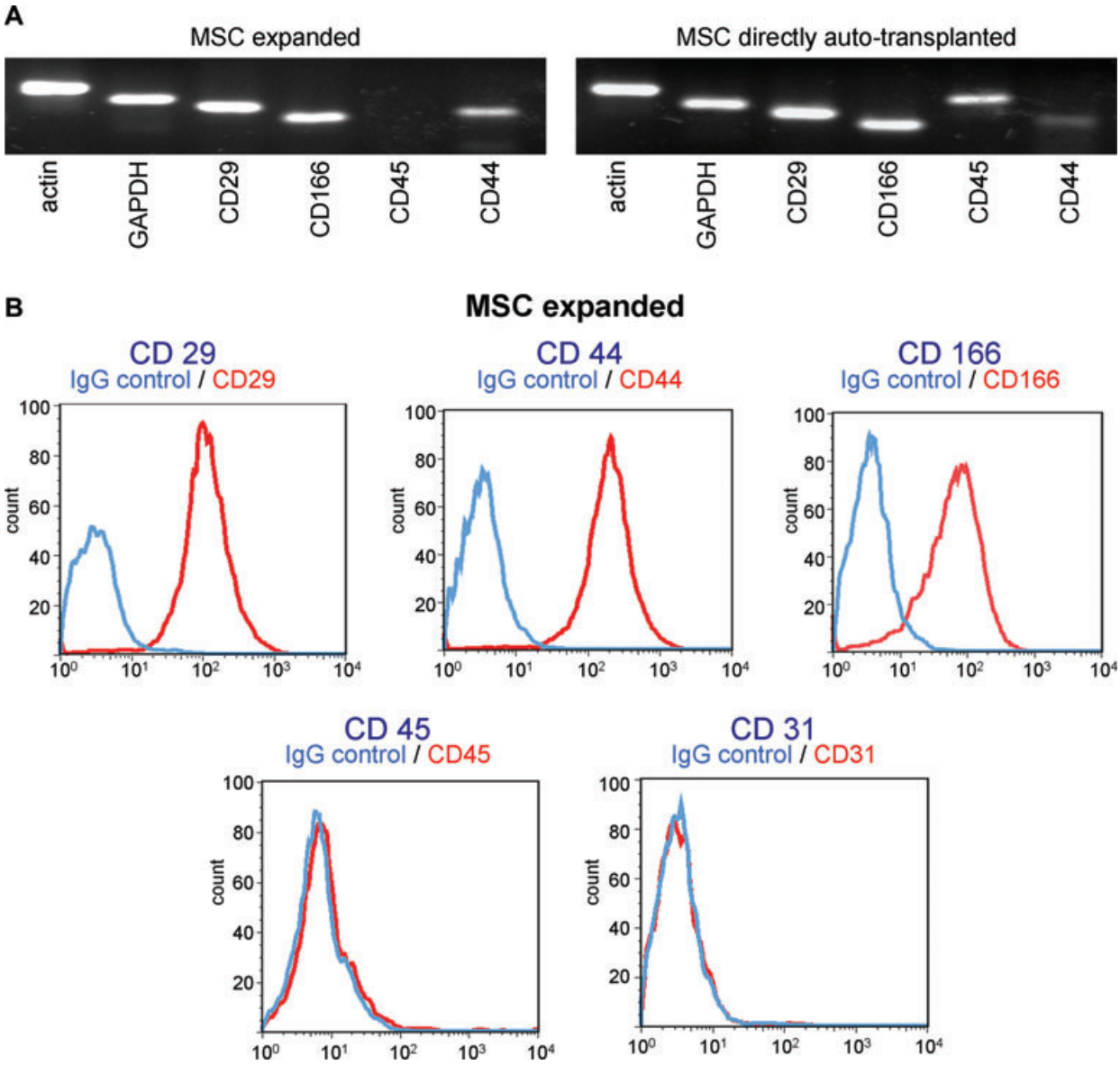


**Fig. 1** Sheep MSC were characterized using FACS and RT-PCR analysis. (**A**) With RT-PCR analysis CD29, CD44 and CD166 expression of MSC could be proofed on mRNA level. As indicated by increased CD45 expression, ratio of hematopoietic cells was higher in directly auto-transplanted MSC as compared to expanded MSC. (**B**–**D**) FACS analysis revealed sheep MSC to express CD29, CD44 and CD166. Expanded MSC (**B**) were negative for the hematopoietic markers CD31 and CD45. Directly auto-transplanted cells (**C**) had a different expression pattern than expanded MSC. The directly auto-transplanted MSC had a weaker CD29 and CD166 but a stronger CD45 expression. Mean fluorescent indices are shown in (**D**).

## Directly auto-transplanted and expanded MSC displayed a constant proliferation and a decreasing apoptosis over time in subcutaneous implants

MSC in a fibrinogen–thrombin matrix were implanted subcutaneously on the sheep's back. Explants were harvested after 2 days, 1, 2, 4, 6 and 8 weeks to investigate proliferation, apoptosis and sufficient DiI labelling of the implanted MSC (groups 1–3). MSC DiI labelling was effective and was stable over several passages in cell culture (Fig. 2). $DiI^+$ cells were identified at all time-points in the subcutaneous implants. The staining intensity of expanded MSC was stronger in comparison to directly auto-transplanted MSC.

A TUNEL assay was performed to analyse apoptosis. In the first and second week a higher apoptosis could be detected compared to later explantation time-points (Fig. 3A–D, A/B 2 weeks,

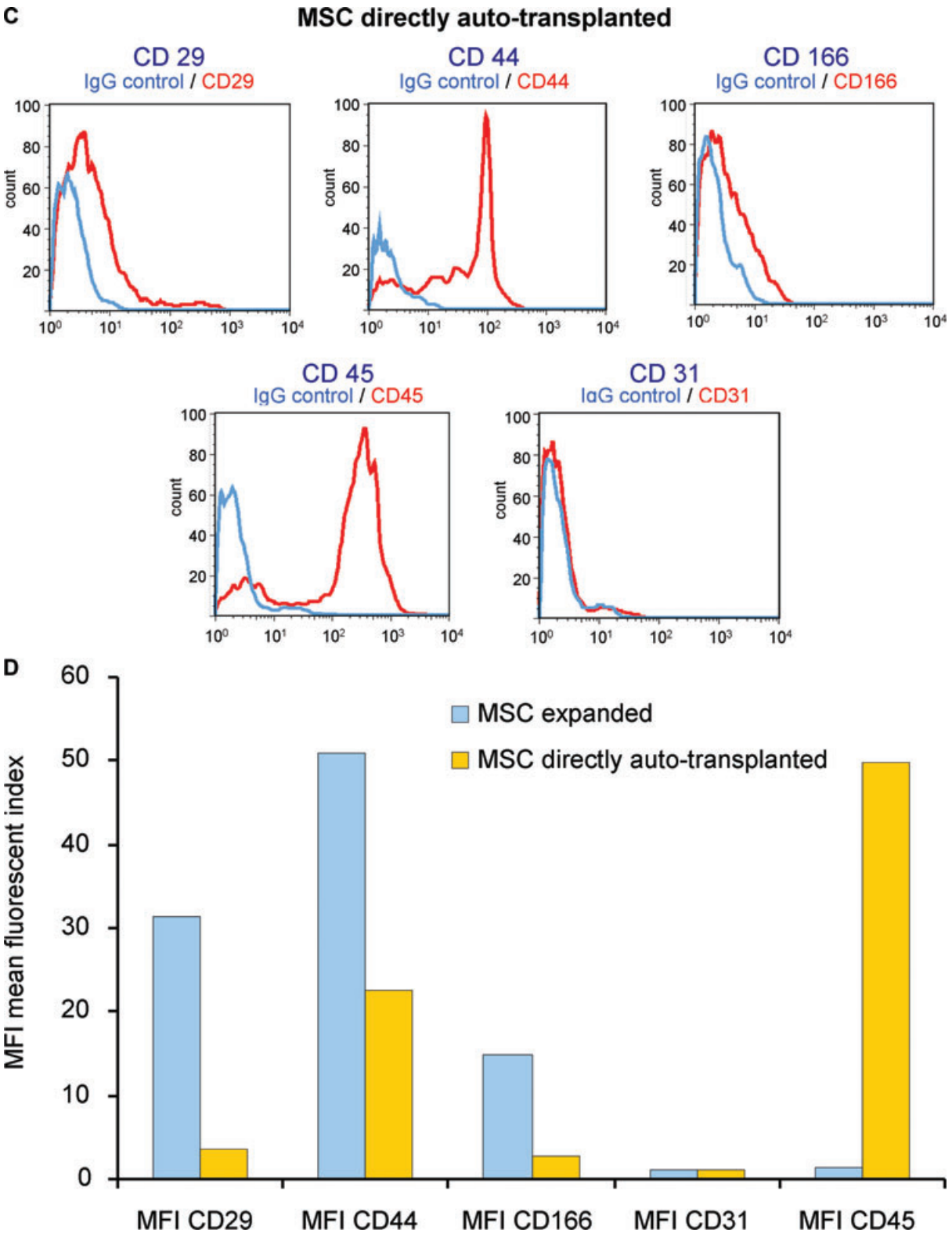


**Fig. 1** Continued.

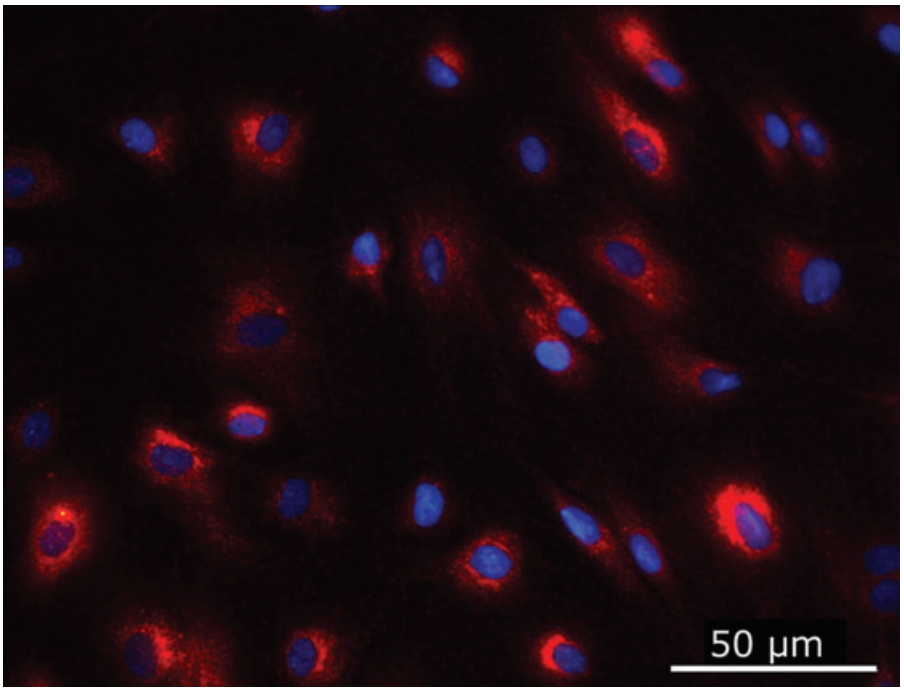


**Fig. 2** DiI-labelled MSC at passage 5. MSC DiI labelling was effective and was stable over several passages in cell culture. Nuclei are counterstained with DAPI (blue).

C/D 4 weeks, A/C expanded MSC, B/D directly auto-transplanted MSC), while the apoptosis decreased from week 1 to week 2. At later time-points only single apoptotic cells were visible. Proliferation was assessed by Ki67 staining. A constant proliferation could be detected over all explantation time-points (Fig. 4A–D, A/B 2 weeks, C/D 4 weeks, A/C expanded MSC, B/D directly auto-transplanted MSC). Despite different marker expression profiles, both cell isolation protocols, directly auto-transplanted or expanded MSC, displayed a constant proliferation and low apoptosis. Therefore, both populations were regarded as suitable for the following implantation studies comparing directly auto-transplanted *versus* expanded MSC aiming at bone tissue engineering.

## Directly auto-transplanted or expanded MCS and BMP-2 contributed to bone formation in subcutaneous sheep implants

Different BMP-2 concentrations were tested to determine optimal concentration for osteogenic stimulation effects (groups 4–7). In the control group using β-TCP/HA granules and a fibrinogen–thrombin matrix only, no bone formation could be detected. While using 2.5 μg/ml or 12.5 μg/ml BMP-2 early bone formation could be detected. Around the β-TCP/HA granules a small margin of osteoblast-like cells and small parts of bone matrix was found (Fig. 5A/B). Using higher concentration of BMP-2 with 60 μg/ml, trabecular, osteon-like, well-vascularized bone formation occurred within 12 weeks (Fig. 5C). The newly formed bone parts were located close to the β-TCP/HA granules. We could demonstrate the expression of collagen type I (COL1) in the newly formed bone in the 2.5, 12.5 and 60 μg/ml explants using immunohistological staining (Fig. 5D–F, D 2.5 μg/ml, E 12.5 μg/ml and F 60 μg/ml). Expression of genes specific for bone-like COL1, osteocalcin, osteonectin, osteopontin as bone matrix proteins and the osteoblast differentiation marker runt-related-transcription factor (RUNX) 2 in the 60 μg/ml explants were demonstrated using RT-PCR. Osteocalcin, osteonectin, osteopontin and COL1 were up-regulated in 60 μg/ml constructs compared to the control group using β-TCP/HA granules with fibrinogen–thrombin matrix without growth factors or cells (Fig. 6A–C).

To identify the most suitable cell type for bone tissue engineering purposes, different groups (expanded *versus* directly auto-transplanted MSC, groups 8–10) were investigated. Cells were either expanded over five passages following plastic adherence or only purified with Ficoll gradient centrifugation without interim plastic adherence selection. In both groups cells were DiI labelled prior to implantation and implanted subcutaneously with or without BMP-2. Based on the clinical application and because expanded MSC have been evaluated in combination with BMP-2 previously we decided to perform only the directly auto-transplanted MSC in combination with the BMP-2. In all three groups bone formation was observed after standard histology haematoxylin and eosin and immunohistology COL1 staining (Fig. 7A–F, A–C haematoxylin and eosin, D–F COL1 staining, A/D expanded MSC, B/E directly auto-transplanted MSC, C/F directly auto-transplanted MSC in combination with BMP-2). Formation of new bone parts was in particular taking place adjacent to the β-TCP/HA granules. Newly formed bone parts commenced to interconnect the granules into one continuous bony block. Sizes of areas with newly formed

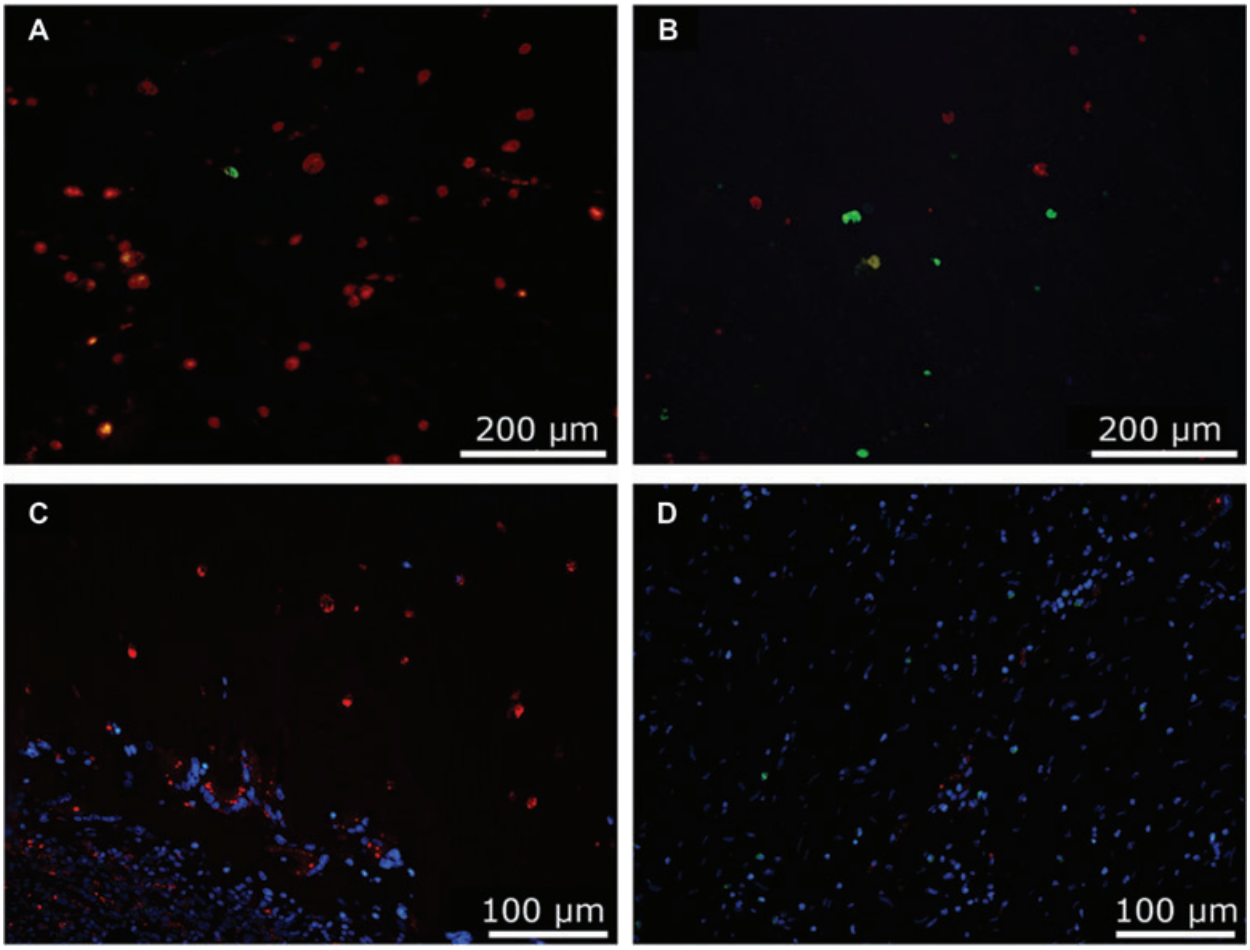


**Fig. 3** MSC in a fibrinogen–thrombin matrix were implanted subcutaneously on the sheep's back (groups 1–3). Explants were harvested after 2 weeks (shown in **A** and **B**) and 4 weeks (shown in **C** and **D**) to investigate apoptosis and sufficient DiI labelling of the implanted MSC. Constructs with expanded MSC are shown in (**A**) and (**C**) *versus* constructs with directly auto-transplanted MSC shown in (**B**) and (**D**). $DiI^+$ cells (red) were identified at all time-points in the subcutaneous implants. The staining intensity of expanded MSC was stronger in comparison to directly auto-transplanted MSC. Apoptotic cells (TUNEL assay) are shown in green. Both expanded and directly auto-transplanted cells had a decreasing apoptosis during the implantation period.

bone were analysed semi-automatically: no significant difference between the three groups could be detected. No significant differences between groups with directly auto-transplanted and expanded MSC were detected, neither did combination with BMP-2 induce a significant higher amount of bone formation (Fig. 7G). The expression of bone-specific genes was demonstrated in groups 8–10 as shown by RT-PCR analysis (Fig. 8A–C). Osteocalcin and osteopontin were up-regulated in groups 8–10 in comparison to β-TCP/HA granules with fibrin matrix without growth factors or cells (Fig. 8D). DiI-labelled MSC were found particularly close to β-TCP/HA granules contributing to the new formation of bone (Fig. 9A–C, A expanded MSC, B directly auto-transplanted MSC, C directly auto-transplanted MSC in combination with BMP-2). In the explants with directly auto-transplanted MSC a higher section of the DiI-labelled cells was found in the connective tissue parts of the constructs compared to the explants with expanded MSC or directly auto-transplanted MSC with BMP-2. The constructs in all groups were well vascularized as shown by CD31 immunohistochemistry (Fig. 9D–F, A expanded MSC, B directly auto-transplanted MSC, C directly auto-transplanted MSC in combination with BMP-2). There seemed to be no difference with regards to the used cell type.

## Discussion

Critical size bone defects are still a challenging problem which is addressed by several disciplines in medical science. Current strategies are based on autologous transplants or a large range of bone scaffolds. In the last years attempts were made to combine bone scaffolds with osteogenic cells like MSC, osteoblasts or

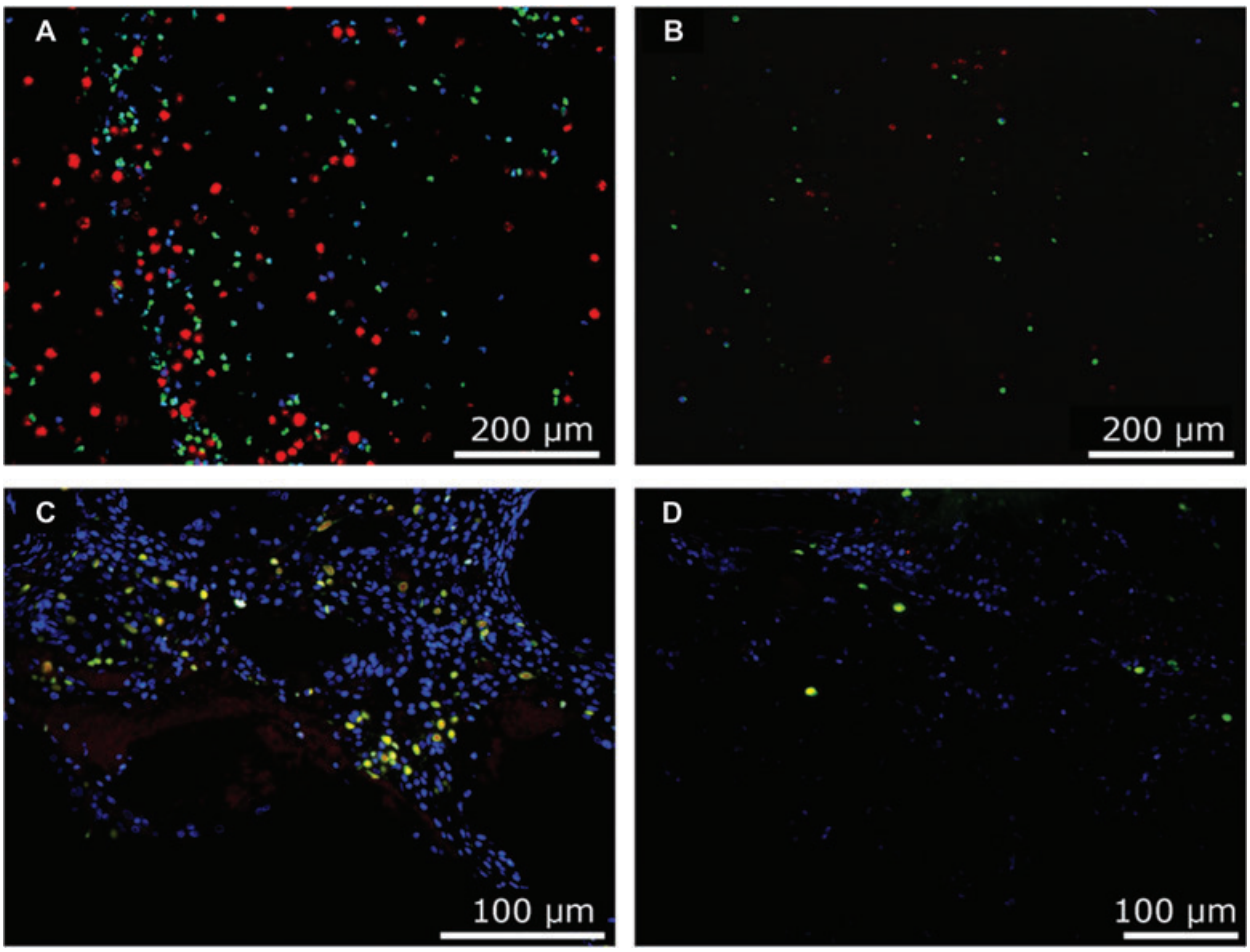


**Fig. 4** MSC in a fibrinogen–thrombin matrix were implanted subcutaneously on the sheep's back (groups 1–3). Explants were harvested after 2 weeks (shown in **A** and **B**) and 4 weeks (shown in **C** and **D**) to investigate proliferation of the implanted MSC. Constructs with expanded MSC are shown in (**A**) and (**C**) *versus* constructs with directly auto-transplanted MSC shown in (**B**) and (**D**). DiI-labelled MSC are shown in red. Proliferating cells (Ki67) are shown in green. Both expanded and directly auto-transplanted cells had a constant proliferation during the implantation period.

growth factors to abbreviate the time for *de novo* bone formation. Also allotransplantation of MSC was propagated as possible means for cell-based tissue engineering therapy. Critical size defects pose a clinical challenge when osteoinductive and conductive properties of the bone scaffold and/or the remaining bone are not sufficient for proper bone formation. Our study aimed to engineer ectopic bone constructs in a large animal model to meet this challenge by using directly auto-transplanted MSC and to compare this clinically feasible approach to established techniques like the use of expanded MSC or the application of BMP-2. In this study we were able to engineer ectopic bone constructs in a large animal model using directly auto-transplanted or expanded MSC or BMP-2 within 12 weeks. The bone constructs were well vascularized and cells inside express bone-specific markers on protein and mRNA level.

Other groups have previously studied the application of sheep MSC intensively. Sheep MSC seemed to have a different marker expression profile compared to human MSC. Whereas human MSC, beside CD29, CD44 and CD166, mostly express the markers like CD90, CD105 and stromal precursor antigen (STRO)-1, sheep MSC were described to express CD29, CD44, CD166 and Vimentin, but they had only a weak CD105 expression [15–17]. Kon and colleagues could show that sheep MSC had different alkaline phosphatase activity as compared to human MSC [18]. Therefore it is unclear yet if findings in the sheep are transferable into human beings. Sheep MSC could also be differentiated in *in vitro* and *in vivo* assays into the chondrogenic, adipogenic and osteogenic lineage [19]. The cells isolated from sheep bone marrow aspirates in this study using Ficoll gradient centrifugation and plastic adherence also expressed the described ovine MSC markers, but not CD31 or CD45. Hence we abdicated experiments on differentiation induction into the chondrogenic, adipogenic and osteogenic line and presumed that cells with the same marker expression profile behave as descript previously [19].

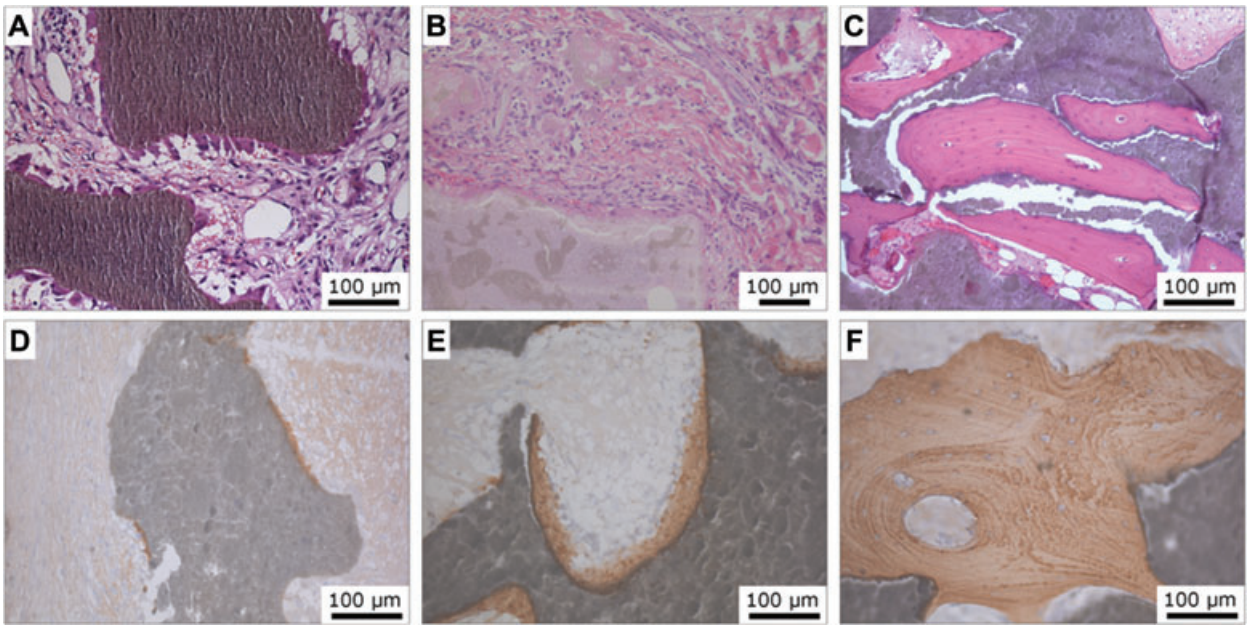


**Fig. 5** β-TCP/HA granules were implanted subcutaneously in sheep with different BMP-2 concentrations (groups 5–7). Haematoxylin and eosin (**A**–**C**) and collagen I (**D**–**F**) staining were performed. A/D 2.5 µg/ml BMP-2, B/E 12.5 µg/ml BMP-2, C/F 60 µg/ml BMP-2. Only initial signs of bone formation could be observed while using 2.5 or 12.5 µg/ml BMP-2 nearby the β-TCP/HA granules. Trabecular, osteon-like bone formation could be detected in combination using 60 µg/ml BMP-2.

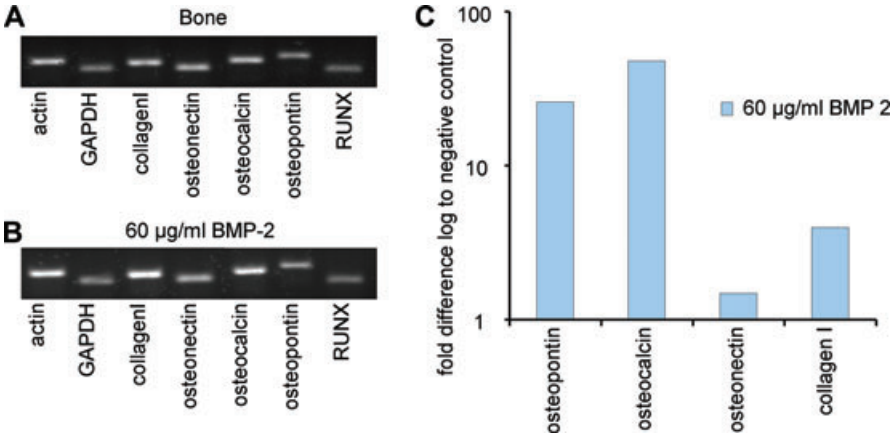


**Fig. 6** β-TCP/HA granules were implanted subcutaneously in sheep with different BMP-2 concentrations (groups 4–7) (**A** and **B**). The expression of genes specific for bone can be detected in the 60 µg/ml constructs. Expression levels in bone serve as controls. (**C**) Osteocalcin, osteonectin, osteopontin and collagen I are up-regulated in 60 µg/ml constructs compared to the control group using β-TCP/HA granules with fibrinogen–thrombin matrix without growth factors or cells.

Both directly auto-transplanted as well as expanded MSC have been used for tissue engineering purposes in the past, but most studies worked with expanded MSC, most likely because of their good expansion capacity and their known differentiation potential in the osteogenic, chondrogenic and adipogenic cell line. We focused on the assessment of directly auto-transplanted MSC in comparison to expanded MSC based on the following considerations: working with directly auto-transplanted cells has advantages concerning an aspired clinical application in the future. Beside a reduction costs and regulatory issues related to cell culture and good manufacturing practice, the time frame necessary for *in vitro* cell expansion is a critical factor in most clinical scenarios, which can be avoided by direct auto-transplantation.

Vincentelli and colleagues reported distinct behaviour using directly auto-transplanted or expanded MSC for heart valve tissue engineering purposes [20]. We could show different expression profile of CD markers on directly auto-transplanted *versus* expanded MSC, but in our experimental setting both cell populations behaved similarly concerning proliferation, apoptosis and potential to induce bone formation. The different expression profile of directly auto-transplanted MSC compared to expanded MSC was possibly due to remaining hematopoietic cells which

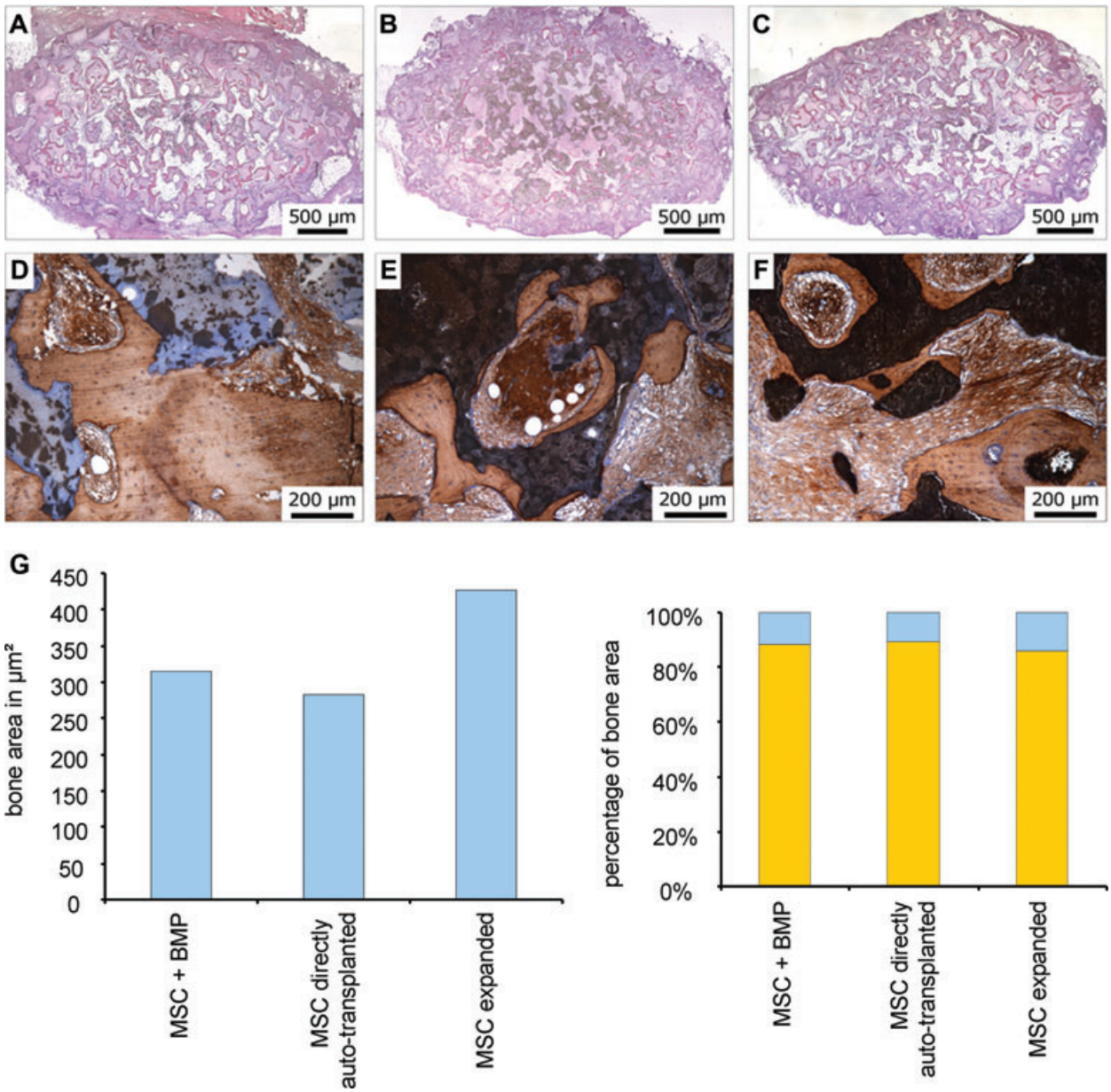


**Fig. 7** For determination of the cell type which is qualified best for bone tissue engineering purposes, different groups (expanded *versus* directly auto-transplanted MSC, groups 8–10) were investigated. In both groups cells were DiI labelled prior to implantation and implanted subcutaneously with or without BMP-2. Bone formation is shown in haematoxylin and eosin (**A**–**C**) and collagen I staining (**D**–**F**). Directly auto-transplanted (**B**, **E**) or expanded MCS (**A, D**) and BMP-2 in combination with directly auto-transplanted MSC (**C, F**) contribute to bone formation in subcutaneous sheep implants. Bone formation was observed in all groups. (**G**) There is no difference between the groups with expanded or directly auto-transplanted MSC. Even the combination with BMP-2 in this setting did not improve the bone mass.

were not separated by the isolation process. Another reason could be the missing plastic adherence selection while working with directly auto-transplanted MSC. The aberrant expression levels in FACS and RT-PCR analysis of directly auto-transplanted *versus* expanded MSC could be attributed to the following reasons: designing primers for RT-PCR analysis is problematic because complete sheep sequences are missing. Alternatively bovine or human sequences with incomplete specificity for the unknown sheep gene sequence were used here, which could be the reason for the weaker CD44 signal in the RT-PCR as compared to the FACS results. Working with sheep is also a challenge if specific antibodies are needed. Possibly the relatively weak

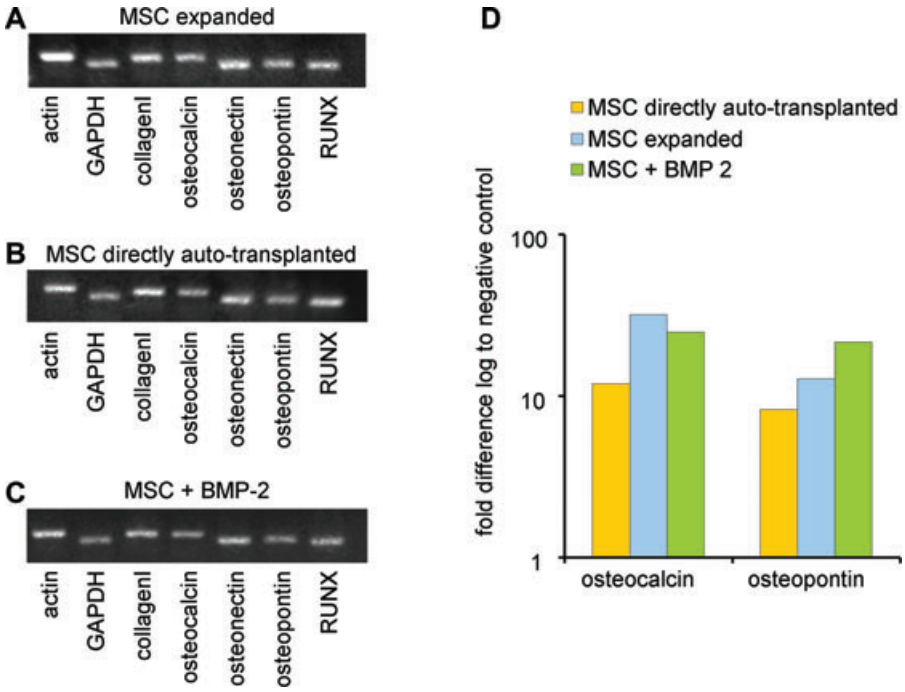


**Fig. 8** For determination of the cell type which is qualified best for bone tissue engineering purposes, different groups (expanded *versus* directly auto-transplanted MSC, groups 8–10) were investigated. In both groups cells were implanted subcutaneously with or without BMP-2. (**A**–**C**) The expression of bone-specific genes is proofed by RT-PCR analysis. (**D**) Osteocalcin and osteopontin are up-regulated in all three groups in comparison to the control group using β-TCP/HA granules with fibrinogen–thrombin matrix without growth factors or cells.

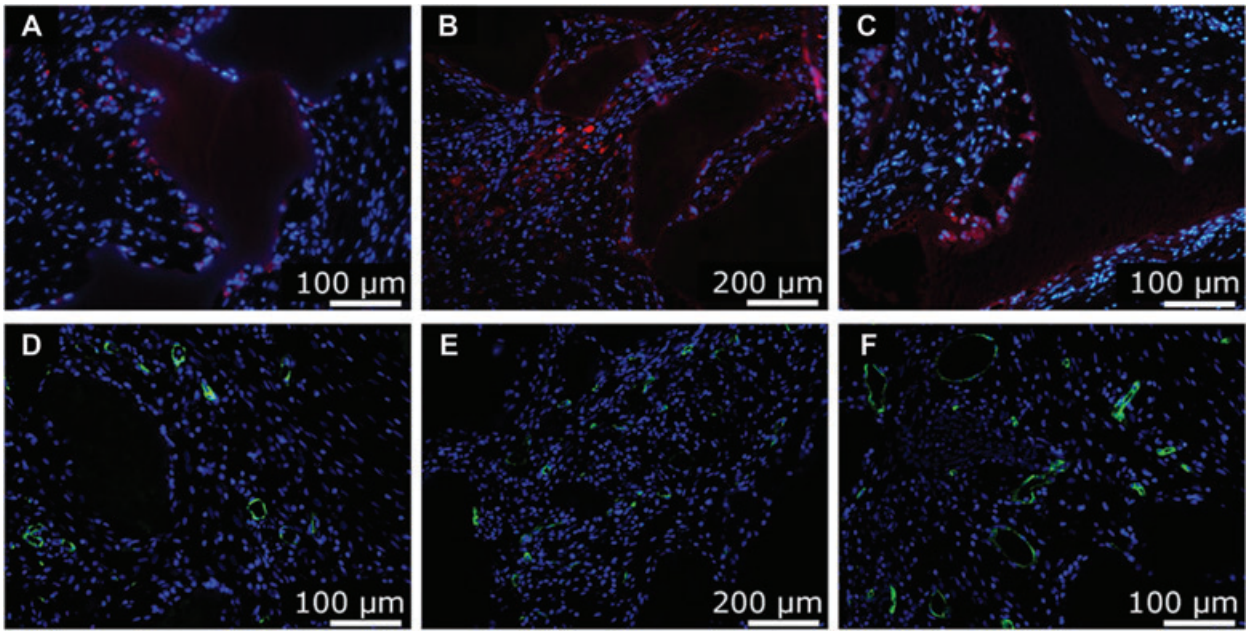


**Fig. 9** For determination of the cell type which is qualified best for bone tissue engineering purposes, different groups (expanded *versus* directly auto-transplanted MSC, groups 8–10) were investigated. In both groups cells were DiI labelled prior to implantation and implanted subcutaneously with or without BMP-2. (**A**–**C**) Expanded MSC (**A**), directly auto-transplanted MSC (**B**), BMP-2 in combination with directly auto-transplanted MSC (**C**). DiI-labelled MSC (red) could be found close to β-TCP/HA granules contributing to the newly formed bone parts. In the explants with directly auto-transplanted MSC a higher section of the DiI-labelled cells were found in the connective tissue parts of the constructs compared to the explants with expanded MSC or directly auto-transplanted MSC with BMP-2. (**D**–**F**) Sections of constructs of the groups with expanded MSC (**D**), directly auto-transplanted MSC (**E**), BMP-2 in combination with directly auto-transplanted MSC (**F**) were evaluated for vascularization. The constructs in all three groups are well vascularized as shown by CD31 immunohistochemistry (green). Nuclei are counterstained with DAPI (blue).

CD166 signal in the FACS analysis might be explained by weaker binding of the antibody, since those antibodies used here were primarily designed for detection of human or bovine antigens, while sheep protein sequences might differ slightly from human or bovine sequences.

We were able to show that directly auto-transplanted as well as expanded MSC alone could induce *de novo* bone formation in a β-TCP/HA matrix. DiI labelled directly auto-transplanted as well as expanded MSC were detected in the newly formed bone parts. While expanded MSC were predominantly located in close proximity to the matrix granula, directly auto-transplanted cells were also distributed within the connective tissue and the blood vessels between the granula. It could be speculated that directly auto-transplanted MSC might have contribute to connective tissue and/or vessel formation by non-osteogenic differentiation. One might presume that this phenomenon could be due to the more heterogenic cell population within the auto-transplanted cells as compared to expanded MSC, since the latter ones underwent the pre-selection process of Ficoll gradient and plastic adherence.

Expanded MSC can be cultured over several passages, while not losing their osteogenic potential as shown previously [21]. It was demonstrated before that expanded sheep MSC improve bone formation in other experimental settings. Kalia and colleagues, *e.g.* were able to show that sprayed autologous MSC on HA-coated collars of segmental bone tumour implants were able to improve the bone growth and bone contact area to the collars [22]. Autologous expanded sheep MSC loaded onto a porous HA ceramic were also able to accelerate bone repair in critical size defects of sheep long bones [18]. Giannoni and colleagues were able to show that the utilization of expanded sheep MSC increased the bone healing and the vascularization process by using a ceramic biomaterial in combination with MSC in a sheep with tibia defects [23]. These settings are delicate to compare with a fully ectopic model with *de novo* bone as used in the study presented here. In comparison to the known evidence for bone formation by expanded MSC, our finding of directly auto-transplanted MSC leading to comparable bone formation in an ectopic sheep model could be an improvement towards clinical application in the future.

Positive CD31 staining revealed dense vascularization of the constructs. It has been shown that proper vascularization is a prerequisite for bone formation and that there is an interplay between angiogenesis and osteogenesis [24, 25]. Possibly the addition of cytokine secreting of MSC contributes not only to osteogenesis, but also to angiogenesis [14, 24, 26–28]. However, in the study presented here MSC contribution to angiogenesis within the implanted matrices as well as dependence of degree of vascularization on use of different cell sources was not subject of detailed investigation, but should be elucidated in upcoming studies.

BMP-2 was able to induce bone formation in our ectopic setting in relevant amount using 60 μg/ml. Previously other authors could demonstrate the bone inductive effect of BMP-2 in other experimental settings. Maus and colleagues could show that BMP-2 incorporated in a β-TCP bone substitute cement enhances bone remodelling in sheep [29]. They observed similar bone formation comparable to usage of autologous bone grafts. Other authors could prove in the rat model that selective combination of BMP-2 and FGF-2 (fibroblast growth factor 2) enhances the osteoblastic differentiation of MSC [30]. BMP-2 and pulsed electromagnetic field also had a positive effect on osteoblastic cell proliferation and gene expression *in vitro* [31, 32]. It could be promising to combine bone and vascular growth factors or application of electromagnetic fields to enhance bone formation also in our experimental setting.

Bone scaffolds today are able to replace autologous bone transplantation under a lot of indications, but fail in special cases when defects exceeded a certain size or the transplantation bed is not adequately vascularized due to infection or radiation. For example, it has been shown by our group that vascularization arising from an arteriovenous loop, *de novo* formation of axially vascularized tissue and successful axial vascularization of a clinically approved bone substitute with a significant volume in a large animal model can be achieved [33, 34]. Furthermore our group was able to demonstrate that axial pre-vascularization increases the survival of implanted osteoblasts in porous matrices in a small animal model [35]. With the study presented here the basis is provided for further experiments aiming to engineer vascularized bone grafts in clinically relevant dimensions in a large animal model. The insertion of an arterio-venous (AV) loop into innovative biomaterials with improved biocompatibility in combination with secondary MSC injection may eventually allow the generation of axially vascularized bone tissues for complex reconstructions of critical size defects.

## Acknowledgements

We thank Ilse Arnold, Stefan Fleischer, Marina Roschkow and Katja Schubert for excellent technical support. This work contains parts of J.L.'s and G.D.'s doctoral theses.

## Conflict of interest

This study was funded by the 'ELAN Fonds für Forschung und Lehre', the Baxter Innovations GmbH, Vienna, Austria, which is the producer of the β-TCP/HA–matrix (TricOs®) used in this study, the 'Deutsche Forschungsgemeinschaft' (DFG) and the Xue Hong and Hans Georg Geis foundation. One of the authors, Heinz Gulle, Ph.D., is an employee of Baxter Innovations GmbH. He was involved in the design of the studies. All other authors confirm that there are no conflicts of interest.

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# Appendix

## Material and methods

### FACS analysis

Cells were rinsed twice with PBS (Biochrom AG, Berlin, Germany) and harvested using 4 ml trypsin-EDTA. Cells were centrifuged at 1500 rpm for 4 min. and the supernatant was discarded. After resuspending the cell pellet in 5 ml PBS with 5% FBS for a blocking period of 15 min., cells were centrifuged and picked up in 100 μl primary antibody dilution and incubated for 30 min. at 4°C while using the directly labelled antibodies and at room temperature while using unlabelled primary antibodies. The following primary antibodies and concentrations were used: mouse anti-sheep CD31 1:10 (AbD Serotec, Oxford, UK), FITC mouse anti-sheep CD44 1:10 (AbD Serotec), phycoerythrin (PE) mouse anti-human CD166 1:10 (BD Biosciences, San Jose, CA, USA), allophycocyanin (APC)/Cy7 anti-human CD29 1:50 (BioLegand, San Diego, CA, USA) and mouse anti-sheep CD45 1:10 (Acris, Herford, Germany). Cells were washed twice with FACS buffer (PBS containing 2% FBS and 0.1% $NaN_3$) and were resuspended in 100 μl secondary antibody dilution and incubated for 30 min. at 4°C (APC rat antimouse IgG1 1:10 (BD Pharmingen, San Jose, CA, USA); APC goat antimouse IgG2a 1:10 (Invitrogen, Karlsruhe, Germany). After centrifugation cells were picked up in FACS buffer for further flow cytometry analysis. Unstained cells were used as negative control. As isotype controls FITC mouse IgG1, PE mouse IgG1, APC/Cy7 mouse IgG1, mouse IgG1 and mouse IgG2a (all BD Biosciences) were used in the same concentration as the primary antibodies.

### RT-PCR analysis

Samples were tested as triplicates and only variations of less than 1.5 threshold cycles were tolerated and threshold cycles after cycle 35 were defined as invalid. Data evaluation was performed with the $\Delta\Delta C_T$-method.

Following primers were used for real time PCR analyses: CD29 (left primer 'agagaagctgcagccagaag', right primer 'gatagtcttcagcccgcttg'), CD44 (left primer 'catctaccccagcaacccta', right primer 'actgtcttcgtctgggatgg'), CD166 (left primer 'cttgcacagcagaaaaccaa', right primer 'gcctggtcattcacctttc'), CD45 (left primer 'aaggtcccagggatgaaact', right primer 'cttccattgacggccagtat'), CD 31 (left primer 'agcacagtggcaactacacg', right primer 'cagttcgggcttggaaaata').

Real time PCR was also performed to asses the up-regulation of genes important for osteogenesis-like collagen I (left primer 'aagacatcccaccagtcacc', right primer 'taagttcgtcgcagatcacg'), osteocalcin (left primer 'tgagctcaaccctgactgtg', right primer 'gtcctggagagaagccagag'), osteonectin (left primer 'acgggtacctgtctcacacc', right primer 'gtccagggcgatgtacttgt') osteopontin (left primer 'tcccactgacattccaacaa', right primer 'ctgtggcatctggactctca') and RUNX2 (left primer 'cgcattcctcatcccagtat', right primer 'gcctggggtctgtaatctga') within the constructs. As control housekeeping gene actin (left primer 'gtccaccttccagcagatgt', right primer 'atctcgttttctgcgcaagt') and GAPDH (left primer 'tgaccccttcattgacctts', right primer 'gatctcgctcctggaagatg') were used. One part of each construct of groups 4/7/8/9/10 was dissected and frozen in liquid nitrogen immediately. Constructs were powdered using the Mixer Mil MM200 (Retsch, Haan, Germany). Trizol (Invitrogen), chloroform and ethanol were added to the powder to isolate RNA according to the phenol chloroform extraction protocol. Further RNA purification was performed with the RNeasy-mini-kit (Qiagen) according to the manufacturer's protocol. cDNA synthesis and real time PCR analysis were performed as described above.

### Immunohistochemistry

For all immunohistochemical staining slides were deparaffined and rehydrated.

Collagen I: Slides were incubated for 5 min. with 3% hydrogen peroxide solution in distilled water (30% hydrogen peroxide, Merck KGaA, Darmstadt, Germany). Unspecific binding was blocked using the Avidin/Biotin Blocking kit (Avidin/Biotin Blocking kit, Vector laboratories, Burlingame, CA, USA) for 15 min. and 30 min. 10% goat serum (PromoCell GmbH, Heidelberg, Germany) in PBS (PBS-Dulbecco 1×, Biochrom AG). The sections were covered with the primary antibody against COL1 1:100 diluted in 10% goat serum in PBS (polyclonal rabbit anti-bovine; biologo, Kronshagen, Germany) and incubated for 1 hr at room temperature. Thirty minutes incubation with the secondary antibody (biotinylated anti-rabbit IgG (H + L), Vector laboratories) 1:500 diluted in PBS followed. ABC reagent (ABC R.T.U. Vectastain® Kit, Vector laboratories) was incubated for 30 min. For development the slides were incubated with 3,3′diaminobenzidine tertahydrochloride (DAB, liquid DAB$^+$ substrate, Dako Cytomation, Carpinteria, CA, USA) and counterstained with hemalaun solution (Mayers Hämalaunlösung, Merck KGaA).

CD31 staining: After antigen retrieval with pH 6 solution (Target Retrieval Solution; Dako Cytomation) in a pressure cooker for 10 min. (Pascal; Dako Cytomation) peroxidase block (CSAII-System; Dako Cytomation) was applied for 15 min., followed by incubation with 10% goat serum (PromoCell GmbH) in PBS (PBS-Dulbecco 1×, Biochrom AG) for 30 min. and protein block with the CSA II-System for 30 min. Then sections were incubated with the primary monoclonal mouse anti-ovine antibody CD31 (Anti-CD31/PECAM-1, MorphoSys UK Ltd., Kidlington, Oxford, UK) at 1:100 diluted in 10% goat serum in PBS for 1 hr. Secondary antimouse immunoglobulin–HRP antibody (CSAII-System) and the fluorescyl-tyramide hydrogen peroxide amplification reagent (CSAII-System) were applied for CD31 staining, followed by counterstaining for 5 min. with diamidine-phenylindole-dihydrochloride (DAPI) 1:1000 in distilled water (Applied Science/Roche, Indianapolis, IN, USA). Slides were covered with mounting media (Fluoprep, Biomérieux, Marcy l'Etoile, France).

Ki67 staining: Antigen retrieval was performed with the DAKO Retrieval Solution pH6 for 20 min. in the microwave. Peroxidase blocking, protein blocking (both CSA II Kit, DAKO) and blocking with 10% goat serum (PromoCell, Heidelberg, Germany) were performed. Mouse anti-human Ki67 antibody (Dako) was incubated at 1:50 in antibody diluent at room temperature for 1 hr. Isotype controls using IgG1 (BD Biosciences) and negative control have been performed. As secondary antibody, HRP antimouse-immunoglobulin (CSA II-biotin-free Tyramide Signal Amplification System, DAKO) was used. Nuclei were counterstained using DAPI (Applied Science/Roche) 1:1000 in aqua dest. for 5 min. Slides were mounted using Fluoprep (Biomérieux).

## TUNEL assay

In the explanted constructs a TUNEL assay (FragEL™ DNA Fragmentation Detection Kit, Calbiochem) was used for detection of apoptotic cells. TUNEL is a method for detecting DNA fragmentation by labelling the terminal end of nucleic acids. Permeabilization was performed with 2 mg/ml proteinase K incubation 10 min. at room temperature. According to the manufacturer's protocol, sections were incubated with TdT equilibration buffer and TdT labelling reaction mixture. Nuclei were counterstained using DAPI as described above. Sections were mounted using Fluorprep (Biomérieux).