Supplementary Material for:

Human Bone Progenitor Cells for Clinical Application: What Kind of Immune Reaction Does Fetal Xenograft Tissue Trigger in Immuno-Competent Rats?

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Materials and Methods

Cell Viability in 2D and in Scaffolds

The hBPCs were seeded either in 96-well plates (2D condition, 10 000 cells/cm^2 per well) or in PLA/5% β-TCP scaffolds (3D condition) as previously described (n=3 per condition). The cells were either cultured in standard medium for hBPCS or in osteogenic differentiation medium for hOBPCs. The cell viability was measured after 0, 3, 7 and 14 days of culture. The Cell-Titer-Glo 3D viability kit (Promega, Fitchburg, WI, USA) was used for 2D and 3D condition analysis. According to the manufacturer's instructions, the ready-to-use reagent was added 1:1 (v/v) to each sample. After 5 min of plate shaking and 25 min of rest at room temperature (RT) in a light free environment, 100 μL of the obtained solution was deposited in a black flat bottom 96-well plate (Greiner bio-one, Germany). The luminescence intensity was then quantified with a Victor Wallac multiplate reader 1420 (PerkinElmer, Waltham, MA, USA). A t-test was performed to examine the statistical difference between hBPCs and hOBPCS at each time point (n=3), and a p-value of less than 0.05 was considered as significant.

Immunofluorescent Staining of Cells in Scaffold

For this experiment, hBPCs and hOBPCs were used and imaged after 3 and 14 days of culture (n=3 per condition). Each cell type was either seeded on thin PLA/5% β-TCP scaffolds (3D sample), which had a thickness of 0.5 mm and a diameter of 3 mm due to imaging limitation of the confocal microscope. After an ethanol oxide sterilization, scaffolds were sonicated during 20 min before adding 20 μL of a cell suspension containing 5 000 cells. Each seeded scaffold was put in a 96-well and left for 10 min at 37°C. Two hundreds μL of standard medium was then added.

Before staining, scaffolds seeded with hBPCs and hOBPCs were washed three times during 5 min in phosphate-buffered saline 1x (PBS, Invitrogen Life Technologies Ltd Thermo Fisher Scientific) and fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS for 1 h at RT.

They were then rinsed three times for 5 min in PBS. 0.2% (v/v) Triton X-100 (AppliChem, Darmstadt, Germany) in PBS was added for 5 min on each sample and washed once for 5 min with 0.02% (v/v) Triton X-100 in PBS. The primary and secondary antibodies as well as the immunofluorescent labels were all diluted in 0.02% (v/v) Triton X-100. The primary antibody, containing rabbit anti-fibronectin (1:400, Sigma-Aldrich), was incubated for 1 h and then washed three times in PBS. In parallel to the secondary antibody (goat anti-rabbit, 1:100, Sigma-Aldrich) which stains fibronectin, 4', 6-Diamino-2-phenylindole dihydrochloride (DAPI, 1:50, Merck Millipore Corporation, Darmstadt, Germany) and Alexa Fluor 488® conjugated Phalloidin (1:1000, Invitrogen) were incubated for 45 min to stain the cell nuclei and actin fibers respectively. The samples were all washed three times for 20 min in PBS before imaging. The images of the fluorescent-labeled 2D and the 3D samples were all obtained using the Zeiss LSM 700 inverted confocal microscope with a 10x magnification objective (Carl Zeiss, Jena, Germany).

Alkaline Phosphatase Activity Assay and Mineralization

The hBPCs were seeded at 10 000 cells/cm^2 in 35 mm plates and incubated for 4 h, 3 and 14 days in a humidified, 5% CO_2 atmosphere (n=3 per condition). For hBPCs, cells were cultured in standard and osteogenic culture media for ALP activity and extracellular matrix mineralization assessment. The hBPCs cultures, for both standard and osteogenic culture media, to be stained for ALP activity were washed 3 times with freshly, sterile, pre-warmed distilled water (dH_2O). One mL of fixative solution (60% (v/v) acetone (pure, Sigma-Aldrich) and 40% (v/v) citrate (1.5 M, Sigma-Aldrich)) was added in each plate and incubated for 30 sec. After rinsing the plates once with dH_2O, 1 mL of revelation solution (0.25 mg Fast Violet (Sigma-Aldrich) in 1 mL dH_2O and 0.04 μL Naphtol (Sigma-Aldrich)) was added for 30 min. After this step was completed, images of the samples were taken using a Leica stereomicroscope (Leica Microsystems).

For mineralization assessment, monolayers of hBPCs cultured in standard and osteogenic culture media were washed with PBS and fixed with 70% ethanol for 1 h at -20°C. The

monolayer was then washed with excess dH_2O prior to the addition of 0.5% (w/v) Alizarin Red S (342.26 M, Sigma-Aldrich) working solution. The samples were incubated at room temperature for 15 min with a gentle shaking. After aspiration and rinsing the plates with dH_2O, images of the stained monolayers were taken using a Leica stereomicroscope (Leica Microsystems).

Results

Cell Growth and Distribution

In 2D condition and PLA/5% β-TCP scaffolds, hBPCs and hOBPCs exhibited an equivalent growth kinetic after a period of 14 days of culture (Fig. S1). The shift in the luminescence curves between the 2D and the 3D conditions is mainly due to the fact that the initial seeded cell concentration was 50 times less in the 2D condition than in the scaffold.

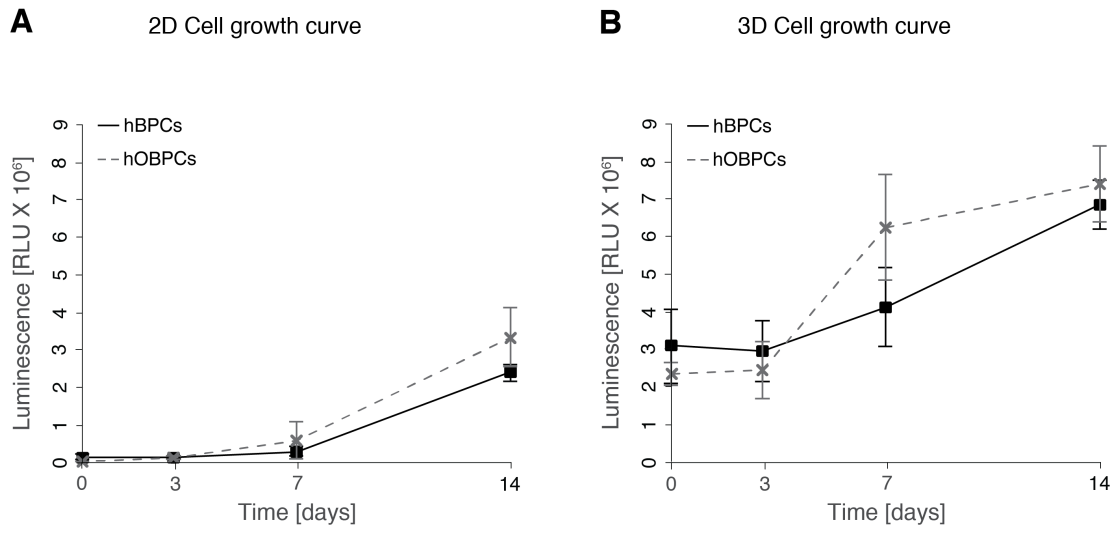

Figure S1. Cell proliferation of human bone progenitor cells (hBPCs) and osteogenic hBPCs (hOBPCs) seeded over 14 days. The cells were cultured over 14 days and were seeded at an initial concentration of 10 000 cells per cm^2 and 0.5 x 10^6 cells per scaffold in 96-well plates (A) and in PLA/5% β-TCP scaffold (B) respectively (with n=3, repeated 2-3-times). Significant *P*-values **P*<0.05.

Cell adhesion in thin scaffolds was verified by immunohistochemistry. The actin fibers of hBPCs and their deposed fibronectin, as well as the cell nuclei, were stained after 3 days and 14 days of culture. The osteogenic differentiation of hBPCs started after the third day of culture, the osteogenic condition is then only shown after 14 days of culture, as we can observe in Fig. S2. Using immunohistochemistry, one can distinguish the pores of the scaffold around which the cells grew. The staining also showed a homogeneous distribution of the fibronectin all over the surface of the scaffold.

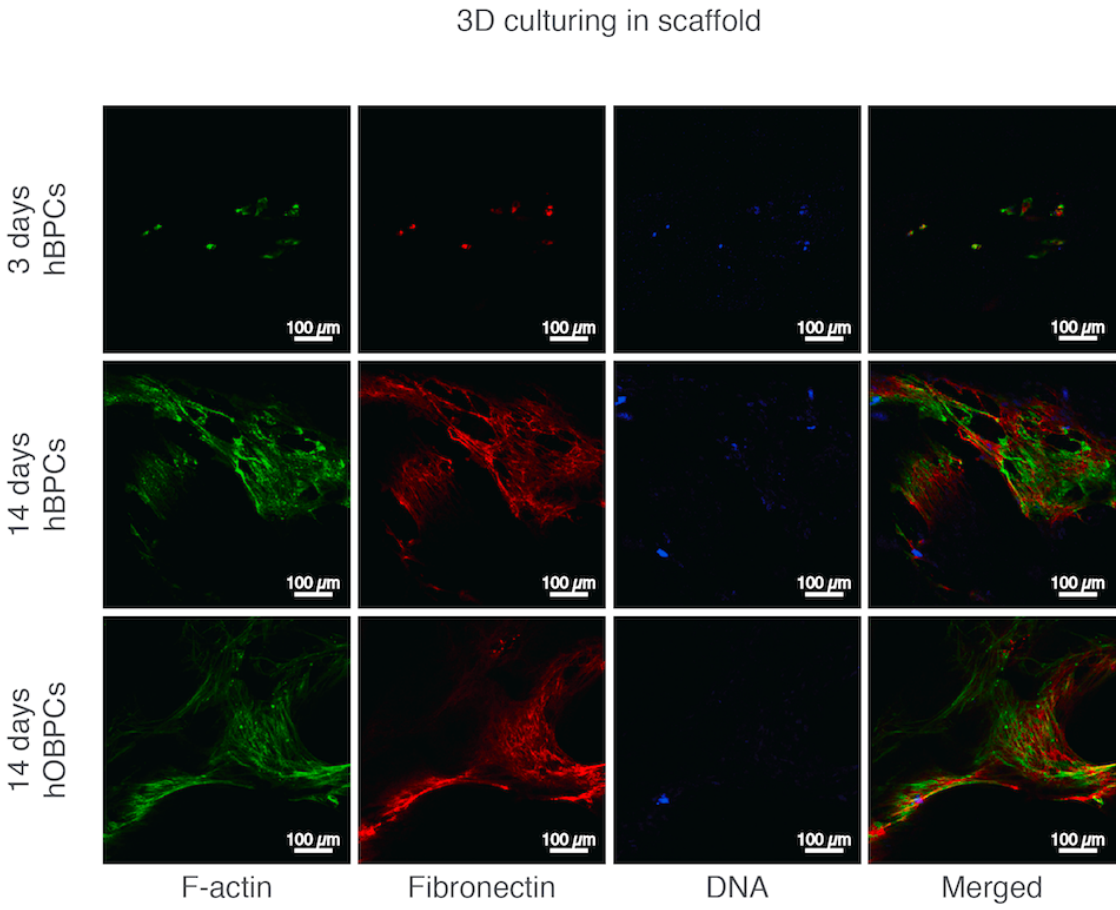

Figure S2. Immunohistochemistry of human bone progenitor cells (hBPCs) and osteogenic hBPCs (hOBPCs) seeded in PLA/5% β-TCP scaffolds after 3 days and 14 days of culture**.** The actin fibers (F-actin) are shown in green, the fibronectin is in red and the nuclei (DNA) are stained in blue. The cells were seeded at an initial concentration of 0.5 x 10^6 cells per scaffold.

Alkaline phosphatase activity and mineralization

Qualitative analysis of the ALP enzymatic activity and extracellular matrix mineralization in hBPCs and hOBPCs after 4 h and 14 days of culture in standard and osteogenic culture medium are shown in Fig. S3. On day 0, only the ALP staining of hBPCs is shown, as the osteogenic differentiation was induced only 3 days after cell seeding. As expected, the presence of active ALP activity was visible in hOBPCs after 14 days of culture in osteogenic medium compared to hBPCs cultured in standard medium (Fig. 3). As for ALP staining, only the mineralization of hBPCs is shown on day 0, as the osteogenic differentiation was induced only 3 days after cell seeding. As expected, for hBPCs, the amount of mineralization in the extracellular matrix increased over time when cultured in an osteogenic differentiation medium compared to the cells in standard culture medium (Fig. S3).

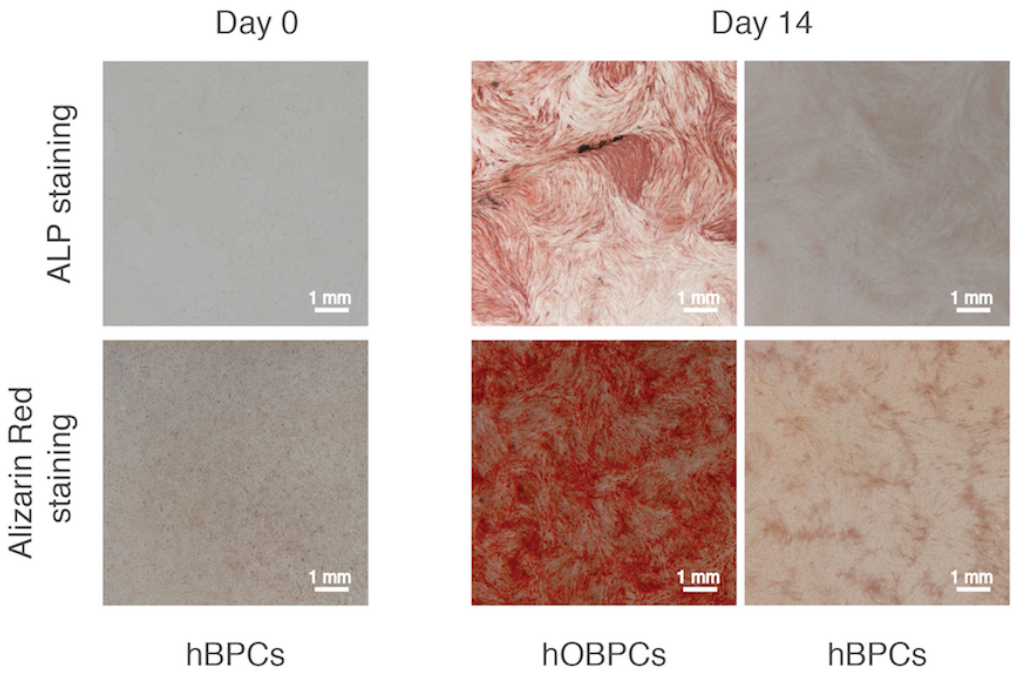

Figure S3. Human bone progenitor cells (hBPCs) and osteogenic hBPCs (hOBPCs) after 4 h and 14 days of culture for alkaline phosphatase (ALP) activity and mineralization determination. The hBPCs were cultured either in standard culture medium or in an osteogenic differentiation medium to obtain hOBPCs. The cells were stained with ALP to assess the ALP activity and they were stained with Alizarin Red for mineralization determination. The cells were seeded at an initial concentration of 10 000 cells per cm^2 in 35 mm plates.

Histological evaluation

The percentage of fibrous tissue as well as osteoblast activation and number of vessels evaluated on histology slides are summarized in Tab. S1. Osteoblast activation and number of vessels were estimated to be the same for all scaffold conditions for time points at days 3, 7 and 14. Concerning the percentage of fibrous tissue, an increase was observed between the different scaffold conditions. In the case of CF scaffold, 5-20% of fibrous tissue was estimated, whereas for CS scaffolds it was 5-50% and for OCS it was 10-95% of fibrous tissue regarding the first 14 days of implantation. Furthermore, we saw a slight increase of fibrous tissue for all scaffold conditions from day 3 to day 7, and a slight decrease from day 7 to day 14. After 12 weeks of implantation, the percentage of fibrous tissue seemed to stay stable for CF scaffolds and to decrease for CS and OCS scaffolds.

Table S1. Qualitative evaluations of cellular events based on histology slides. The osteoblastic activation was evaluated as rare, few, some or many (n=3).

Scaffold	Time post-implantation	Osteoblast activation (rare, few, normal range)	Number of vessels approximation	Fibrous tissue approximation (%)
CF	**Day 3**	Few	< 5	5-20%
	Day 7	Few	< 5	10-20%
	Day 14	Few	< 5	10-15%
	Week 12	Few	< 20	5-15%
CS	**Day 3**	Few	< 5	5-50%
	Day 7	Few	< 5	25-50%
	Day 14	Few	< 5	25-30%
	Week 12	Some	20-30	5-10%
OCS	**Day 3**	Few	< 5	10-90%
	Day 7	Few	< 5	30-95%
	Day 14	Few	< 5	25-75%
	Week 12	Some	> 20	10-75%

CF: Cell-free. CS: Cell-seeded. OCS: osteogenic cell-seeded

The qualitative evaluation of the immune response is summarized in Tab. S2. The presence of immune cells (neutrophils, macrophages, lymphocytes, plasma cells and multinuclear giant cells) were quantified from rare to some in all scaffold conditions at time points 3, 7 and 14 days post-implantation, where in general more immune cells were noticed after 7 and 14 days of implantation in CF scaffolds compared to CS and OCS scaffolds. For all scaffold conditions and time points, the amount of observed immune cells were in a normal range for all four time points.

Table S2. Qualitative evaluations of the immune response based on the histology slides. The immune response was evaluated as rare, few, some or many (n=3).

Scaffold	Time post-implantation	Neutro-phils	Macro-phages	Lympho-cytes	Plasma cells	Multinuclear giant cells
CF	**Day 3**	Rare to some	Rare to some	Rare to some	Rare to some	Rare to some
	Day 7	Some	Some	Some	Some	Rare to some
	Day 14	Some	Some	Some	Some	Rare to some
	Week 12	-	-	-	-	Few
CS	**Day 3**	Few to some	Rare to some	Rare to some	Rare to some	Rare to some
	Day 7	Rare to some	Rare to some	Rare to some	Rare to some	Rare to some
	Day 14	Rare	Rare	Rare	Rare	Rare
	Week 12	-	-	-	-	Few
OCS	**Day 3**	Rare	Rare	Rare to some	Rare to some	Rare to few
	Day 7	Rare to few	Rare to few	Rare to some	Rare to some	Rare to some
	Day 14	Rare	Rare	Rare	Rare	Rare to some
	Week 12	-	-	-	-	Few

CF: Cell-free. CS: Cell-seeded. OCS: osteogenic cell-seeded