Porous Bioprinted Constructs and Non-viral Gene Therapy Combined for Bone Tissue Engineering

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Disclosures:

Introduction:
A well-known stimulus of bone formation in the field of regenerative medicine and tissue engineering is bone morphogenetic protein-2 (BMP-2). It has already been used extensively in clinical applications. Doses of BMP-2 that are used in the clinic are supraphysiologic and may result in side effects. BMP-2 gene therapy might provide a more physiologic protein production.[1] Therefore, in previous work, a non-viral gene delivery system for BMP-2 has been developed, which resulted in a sustained level of BMP-2 production in vitro.[2] Small constructs containing this gene therapy have led to good bone formation when implanted in rodents, but upscaling to clinically relevant-sized, i.e. cm-sized grafts remains challenging.

The bioprinting technology might overcome this problem, as it enables relevant sized bone constructs to be printed, in combination with application of the BMP-2 gene. Layers of cell-laden hydrogel strands are deposited according to a computer design. The resulting 3D scaffolds are accurate and reproducible in size, shape, porosity, and pore geometry. Pores reduce diffusion distances and allow blood vessel ingrowth and therefore enhance nutrient supply and waste product removal, which is highly desired for constructing clinically relevant sized bone implants.

In this study, we aim to improve the efficiency of the non-viral gene therapy and stimulate vascularization and osteogenesis by printing 3D constructs in a porous fashion.

Methods:
Goat multipotent stromal cells (MSCs) were suspended in alginate hydrogel with various concentrations cDNA encoding BMP-2 (pBMP-2), to determine optimal plasmid DNA concentration. After seven days of incubation BMP-2 production in medium and gel was analyzed by ELISA. MSCs and cDNA coding for green fluorescent protein (pGFP) as a tracer gene were suspended in alginate hydrogel, ranging from 1-10% (w/v). Expression of GFP positive cells was scored using a fluorescence microscope and given as percentage of total cells.

To enable 3D printing at low alginate concentration, the hydrogel was precrosslinked and basic rheology was performed on these constructs.

After selection of optimal cDNA and alginate concentrations, 3D constructs (10x10x5 mm) were printed, either in a porous (fig. 1A) or a solid fashion (fig. 1B). Constructs consisted of alginate hydrogel (3% w/v) supplemented with MSCs (107 cells/ml) and ceramic particles (biphasic calcium phosphate, < 200 μm). pBMP-2 was included in porous and solid constructs and porous constructs without pBMP-2 were printed as controls. For in vitro experiments bioprinted constructs were cultured for 7 and 14 days, where after BMP-2 production and osteogenic differentiation were determined by ELISA (fig. 2) and ALP activity measurement, respectively. Measurements were corrected for the differences in weight between the constructs. Osteocalcin immunohistochemistry was performed on isolated cells of every construct to support results on osteogenic differentiation.

In vivo performance of bioprinted constructs was investigated after subcutaneous implantation in nude mice (n=6). Each mouse received all constructs. Implants were retrieved after 6 weeks, decalcified and processed for paraffin embedding and staining with haematoxylin and eosin to evaluate tissue morphology and bone formation.

Results:
Construct optimization:
BMP-2 production was the highest in the plugs with 10 μg/ml pBMP-2 resulting in a BMP-2 protein production of 8.4 ng/ml. A low alginate concentration resulted in the highest transfection efficiency. For 1% (w/v) alginate the transfection efficiency was 40.8%, for 2% (w/v) alginate this was 35.7% and for 3% (w/v) alginate it was 31.2%. At higher concentrations of alginate transfection efficiency further decreased to <4%. Precrosslinked constructs of 3% alginate or more remained intact during printing and in vitro incubation for at least 2 weeks. The Young's moduli of porous and solid constructs were 5.63±0.31 kPa and 7.27±0.40 kPa, respectively.

Bioactivity of produced BMP-2 in the bioprinted constructs:
Optimized porous and solid constructs containing the BMP-2 gene were bioprinted and remained intact for at least 14 days (fig. 1). Cells were efficiently transfected by the pBMP-2 in both porous and solid constructs, and differentiated towards the osteogenic lineage as shown by elevated BMP-2 production and ALP activity. Porous constructs performed better in producing BMP-2 than solid constructs at day 7 (p<0.05), which was not significantly different on day 14 (fig. 2). After 14 days, MSCs encapsulated in porous constructs exhibited a higher degree of early osteogenic marker expression than cells in solid scaffolds, measuring around 70% and 50% osteocalcin-positive cells, respectively. In the porous control samples only a small amount of cells <2% was positive.
Tissue development in vivo:
Analysis of the histological sections after an implantation period of 6 weeks showed matrix deposition around the ceramic particles in the porous constructs with or without pBMP-2, which was not seen in the solid constructs. The solid constructs contained large areas of alginate remnants and some of the solid constructs were partially encapsulated. In none of the constructs bone formation could be observed.

Discussion:
Here, we demonstrated that porous constructs can be printed and remain intact for 14 days in culture in vitro. Cells survived the printing process, were efficiently transfected by plasmid DNA, and kept their ability to differentiate towards the osteogenic lineage as is shown by BMP-2 and ALP production. Porous constructs performed significantly better in producing BMP-2 than solid constructs. This is probably caused by a higher cell metabolism due to better nutrient supply and waste removal caused by shorter diffusion distances. After 14 days it was noted that also the solid constructs started producing physiological levels of BMP-2, which is in accordance with previous studies.[2] After six weeks in vivo implantation in mice, matrix deposition was seen around ceramic particles although no bone formation could be observed in the bioprinted constructs. This could be caused by the relatively high percentage of alginate, required for the bioprinting process. Lowering the alginate concentration by copolymerization with different hydrogels might solve this. In conclusion, we showed for the first time a bioprinted 3D model in which non-viral gene expression of BMP-2 is accomplished.

Significance:
Upscaling tissue engineered constructs to a clinical relevant size is major hurdle in the field of bone regeneration. 3D bioprinting enables the fabrication of customized constructs that are highly porous, thereby promoting vascular ingrowth, which is essential for bone formation. Our work shows that 3D bioprinting has potential for bone regeneration applications. Secondly, the physiological levels of BMP-2 protein, which are generated using gene-activated matrices, might preclude unwanted side effects associated with high protein dosage.

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References:
Figure 1: Biporous constructs containing HAp, ceramic particles with/without plasmid DNA encoding BMP-2. A porous construct (A) and a solid construct (B), shortly after printing. Porous constructs with and without DNA have the same macroscopic appearance. Scale bars A and B: 500 μm. C) Detail of a porous construct.

Figure 2: BMP-2 production in medium and gel of biporous alginate constructs, measured by ELISA. The results represent mean ± SD. * indicates a significant difference (p < 0.05).

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