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Towards autotrophic tissue engineering: Photosynthetic gene therapy for regeneration

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ABSTRACT

The use of artificial tissues in regenerative medicine is limited due to hypoxia. As a strategy to overcome this drawback, we have shown that photosynthetic biomaterials can produce and provide oxygen independently of blood perfusion by generating chimeric animal-plant tissues during dermal regeneration. In this work, we demonstrate the safety and efficacy of photosynthetic biomaterials *in vivo* after engraftment in a fully immunocompetent mouse skin defect model. Further, we show that it is also possible to genetically engineer such photosynthetic scaffolds to deliver other key molecules in addition to oxygen. As a proof-of-concept, biomaterials were loaded with gene modified microalgae expressing the angiogenic recombinant protein VEGF. Survival of the algae, growth factor delivery and regenerative potential were evaluated *in vitro* and *in vivo*. This work proposes the use of photosynthetic gene therapy in regenerative medicine and provides scientific evidence for the use of engineered microalgae as an alternative to deliver recombinant molecules for gene therapy.

1. Introduction

Oxygen is an essential molecule for cell metabolism. It plays a key role in tissue survival and regeneration, yet paradoxically, its appropriate delivery is one of the major problems for the clinical translation of non-vascularized tissue engineering approaches [1]. As a result, cells within bioartificial tissue constructs are stringently dependent on oxygen diffusion, which barely reaches few hundred micrometers and limits the construction of artificial tissues to non-clinically relevant sizes [2]. Besides the basic oxygen requirements, tissue regeneration relies on several bioactive molecules, such as growth factors, that control key processes including inflammation, angiogenesis and tissue remodeling [3–5]. Scaffold bioactivation with growth factors has been intensively investigated as strategy to enhance the regenerative potential of engineered tissues [6]. However, direct growth factor administration has major limitations related to the short biological half-life of the molecules, and the consequent need for repeated administration of large and potentially toxic bulk doses [7,8]. Conversely, traditional gene therapy

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represents a better strategy to provide a constant growth factor supply. Yet, this approach raises ethical and technical concerns that affect their translation into clinical settings. For instance, the delivery of target genes often creates risk of oncogenicity, insertional mutagenesis and viral vector-related immune reactions [9,10], while target tissue-specificity and high transduction efficiency are still unrealized for non-viral gene delivery methods [11,12]. Additionally, sustained transgene expression is not guaranteed if the transgenic cell survival depends of local oxygen tension at the injection site.

Recently, we have introduced HULK (from the German *Hyperoxie Unter Licht Konditionierung*) as a novel concept to deliver oxygen into biomaterials independently of blood vessel perfusion. The main idea behind the HULK approach is that, by incorporating photosynthetic microalgae such as *Chlamydomonas reinhardtii* (*C. reinhardtii*) into artificial constructs, the local induction of photosynthesis could be able to supply the metabolic needs of bioengineered tissues *in vitro* [13] and *in vivo* [14]. To take HULK one step forward, in this work we evaluated the feasibility of implanting photosynthetic scaffolds in fully immunocompetent mice. Then, we explored the idea of using genetically modified microalgae to engineer photosynthetic scaffolds that, in addition to oxygen, could provide other pro-regenerative molecules to the wounded tissue. As a proof of concept, we created a gene modified *C. reinhardtii* strain that constitutively secretes the human vascular endothelial growth factor VEGF-165 (VEGF).

In this work we propose that the activation of biomaterials with gene modified microalgae could be used to locally deliver oxygen and other pro-regenerative molecules into bioartificial tissue constructs.

2. Materials and methods

2.1. Cell culture of C. reinhardtii

The cell-wall deficient, cw15-30-derived UVM4 *C. reinhardtii* strain [15] was grown photomixotrophically at 20 °C on either solid Tris Acetate Phosphate (TAP) medium or in liquid TAPS-medium supplemented with 1% (w/v) sorbitol [16]. For light stimulation, a lamp with the full spectrum of white light (Nano Light, 11 Watt, Dennerle, Vinningen, Germany) was used to provide constant illumination (2500 lux, eq. 72.5 $\mu E/m^2 \cdot s^1$). Cell concentration in the culture was determined using a Casy Counter TT (Roche Diagnostics, Mannheim, Germany).

2.2. Cell seeding in the scaffolds

For *in vitro* experiments, if not stated otherwise, Integra® matrix single layer (Integra Life Science Corporation, Plainsboro, NJ, USA) was used as a scaffold. For seeding, a *C. reinhardtii*, suspension was mixed with fibrinogen (Tissucol-Kit 2.0 Immuno, Baxter GmbH, Unterschleiβheim, Germany) in a 1:1 ratio to the respective final concentration. Pieces of IM (ø 12 mm, 1 mm thick) were cut using a biopsy punch and dried with sterile gauze. 50 μl thrombin-solution (Tissucol-Kit 2.0 Immuno, Baxter GmbH, Unterschleiβheim, Germany) followed by 100 μl of the algae-fibrinogen solution were pipetted over each scaffold. Control scaffolds were prepared by adding 50 μl thrombin and 100 μl TAPS-fibrinogen 1:1 solution. After 1 h, the scaffolds were covered with TAPS-buffer and incubated over the desired period of time at room temperature and constant illumination. Pictures of the scaffolds at different time points were taken with a stereomicroscope (Stereomicroscope Stemi 2000-C, Carl Zeiss AG, Oberkochen, Germany) or an inverted phase-contrast microscope (Axiovert 25, Carl Zeiss AG, Oberkochen, Germany) and analyzed with the Axiovision software (Carl Zeiss AG, Oberkochen, Germany).

2.3. Construction of transformation vector

The coding sequence for the human vascular endothelial growth factor A isoform l (accession number NP_001165097) was adapted to the codon bias of *C. reinhardtii*. The synthetic gene pBC1-VEGF165 was assembled from synthetic oligonucleotides and PCR-products. The sequence was inserted into an expression cassette derived from the endogenous *PsaD* gene 5′ and 3′ UTRs regulatory elements and behind the export sequence encoding the 21 amino acid leader peptide of the *C. reinhardtii* extracellular enzyme arylsulfatase (ARS2) to target the transgenic proteins for secretion into the culture medium. The pBC1-CrGFP_J131 basis vector [15] contained the *APHVIII* resistance gene for selection on paromomycin, whose expression is controlled by the constitutively active HSP70/RBCS2 promoter regions and the first intron of the RBCS2 gene. The transgene fragment was cloned into the vector backbone pBC1-CrGFP_J131 using NdeI and EcoRI cloning sites. The final constructs were verified by sequencing. The plasmid DNA was replicated and purified from transformed *E. coli* K12 (dam + dcm + tonA rec-) bacteria. All this was performed by Gene Art AG, Life Technologies, Regensburg, Germany.

2.4. Transformation of C. reinhardtii

$1 \cdot 10^7$ UVM4 *C. reinhardtii*-cells were suspended to a volume of 300 μl and vortexed with ø 0.5 mm glass beads for 20 s in the presence of 5 μg plasmid DNA. Then, the transformants were seeded in TAPS-liquid medium and incubated overnight protected from light under continuous shaking. Next, the algae were seeded over TAP-Agar plates containing 10 μg/ml paromomycin and incubated for the first three days under weak illumination. Upon successful transformation, the new created strains integrated an antibiotic-resistance gene and were therefore able to grow selectively in the presence of paromomycin. The plates were then moved to conditions with standard light exposition (2500 lux), until the colonies were large enough to be picked and platted into a fresh plate. Clones were subsequently maintained in solid TAP-medium under selective conditions.

2.5. Southern blot

Genomic DNA from *C. reinhardtii* cells was extracted using the DNeasy Plant Mini Kit (Qiagen N.V., Lumburg, Netherlands) according to the manufacturer's instructions. 10 μg DNA were digested using the restriction enzymes HindIII and BamHI at 37 °C for 48 h. Samples were separated by 0.8% agarose-TBE gel electrophoresis for 16 h at 25 V. Following depurination (15 min, 0.25 M HCl), denaturation (30 min, 0.4 M NaOH, 0.6 M NaCl) and neutralization (30 min, 1 M Tris−HCl pH 8.0, 1.5 M NaCl) genomic DNA was transferred to a nylon membrane (Roti-Nylon plus,; Carl-Roth, Karlsruhe, Germany) in 20× SCC (3 M NaCl, 0.3 M Trisodiumcitrate) overnight. Digoxigenin-nucleotide labeled DNA-probes (Digoxigenin-11-dUTP alkali-labile, Roche, Basel, Switzerland) were obtained using the same primer pairs and conditions as for the PCR (see below). After blocking unspecific binding sites, immobilized genomic DNA was incubated with the labeled probes for 14 h at 68 °C for hybridization. Signals were detected using an alkaline phosphatase conjugated anti-DIG antibody (Roche, Basel, Switzerland) and CDP* (Roche, Basel, Switzerland) as reaction substrate.