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Development of photosynthetic biomaterials for in vitro tissue engineering

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ABSTRACT

Tissue engineering has opened a new therapeutic avenue that promises a revolution in regenerative medicine. To date, however, the translation of engineered tissues into clinical settings has been highly limited and the clinical results are often disappointing. Despite decades of research, the appropriate delivery of oxygen into three-dimensional cultures still remains one of the biggest unresolved problems for in vitro tissue engineering. In this work, we propose an alternative source of oxygen delivery by introducing photosynthetic scaffolds. Here we demonstrate that the unicellular and photosynthetic microalga *Chlamydomonas reinhardtii* can be cultured in scaffolds for tissue repair; this microalga shows high biocompatibility and photosynthetic activity. Moreover, *Chlamydomonas* can be co-cultured with fibroblasts, decreasing the hypoxic response under low oxygen culture conditions. Finally, results showed that photosynthetic scaffolds are capable of producing enough oxygen to be independent of external supply in vitro. The results of this study represent the first step towards engineering photosynthetic autotrophic tissues.

1. Introduction

Although tissue engineering promises unlimited possibilities for regenerative medicine, there are still several issues that limit its application in clinical settings. Appropriate oxygen supply is a key aspect in the field [1], and the insufficient delivery of oxygen into three-dimensional (3-D) cultures is recognized as one of the biggest limitations for tissue engineering approaches in vitro [2].

Because of oxygen's limited diffusion, low solubility and high consumption rate, oxygen gradients created within the engineered construct directly correlate with a decrease in cell proliferation and density [3], and also affect other key processes, such as gene expression and matrix deposition [4]. In some cases, the oxygen concentration in the inner core of the scaffold is as low as 0%, triggering cell death due to hypoxia [5]. Furthermore, as a consequence of insufficient oxygen supply, non-homogeneous cellular distributions within the scaffolds impair the generation of isotropic tissues in vitro [6] and, most importantly, limit the engineered constructs to sizes that in most cases are not clinically relevant [7,8].

The development of new approaches to provide the appropriate oxygen levels in seeded scaffolds has become an intensive issue of research. Among some strategies to increase oxygenation of tissue constructs, the combined use of scaffolds with perfluorocarbon-based synthetic oxygen carriers has been challenged in a broad range of engineered tissues, including bone [9], heart [2] and trachea [10]. Moreover, the design of several types of flow perfusion bioreactors has also shown to be suitable to overcome the limitations of oxygen diffusion in scaffolds in vitro [6]. Finally, the evaluation of scaffolds containing microchannels [11] or capillary-like networks [12] has revealed promising results. However, despite decades of research to improve in vitro oxygenation in tissue engineering, to our knowledge, scaffolds that actively produce oxygen have not been described yet. Here, we created a photosynthetic scaffold that generates oxygen upon exposure to light. This approach, named HULK (from the German abbreviation of *Hyperoxie Unter Licht Konditionierung*), allows us to regulate the oxygen tension in 3-D cell cultures by providing a local and constant source of oxygen supply through a perfusion-independent mechanism: photosynthesis.

With this goal, as a proof of principle we decided to evaluate the effect of photosynthetic cells, seeded in a bioartificial scaffold. Here, we combined the unicellular alga *Chlamydomonas reinhardtii* and a collagen-based scaffold that is approved for dermal repair by the US Food and Drug Administration (FDA).

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2. Material and methods

2.1. Cell co-culture

A cell-wall-deficient *C. reinhardtii* wild-type strain (*cw* = 15) was grown in Tris acetate phosphate (TAP) medium [15]. NIH-3T3 fibroblasts were kept in Dulbecco's modified Eagle medium (DMEM; Biochrom AG, Berlin, Germany) supplemented with 20% fetal calf serum (FCS; PAA, Pasching, Austria). A 1:1 mixture of DMEM-20% and TAP medium was used as co-culture medium. Fibroblasts and *C. reinhardtii* were cultured in a ratio of 1:15 and kept in standard cell incubators at 30 °C and 5% CO_2. Oxygen concentrations were fixed at 21% (normoxia) or 1% (hypoxia) depending on the experimental setting. In order to determine proliferation of *C. reinhardtii* in two-dimensional (2-D) co-culture, the algae were removed and counted in a Casy Counter TT (Roche Diagnostics, Mannheim, Germany). The co-cultured cells were subsequently trypsinized and their proliferation and viability were checked by cell counting in the Casy Counter TT by the exclusion of dead cells via software set-up. For illumination, 1.600 lx was applied by placing a lamp (Nano Light, 11 Watt, Dennerle, Vinningen, Germany) with the full spectrum of white light at a distance of 40 cm from the sample.

2.2. Cell seeding and culture in the scaffold

Integra matrix bilayer skin (Integra® DRT, Integra Life Science Corporation, Plainsboro, NJ, USA) was used as scaffold. For seeding of *C. reinhardtii*, scaffolds were dried and rehydrated with a suspension of *C. reinhardtii* in TAP medium. For co-cultures, 4×10^6 3T3 cells were seeded in DMEM 10% FCS as described previously [13] and incubated for 24 h for attachment. Then 3T3-seeded scaffolds were dried with sterile gauze and rehydrated in co-culture medium containing different densities of *C. reinhardtii* according to the experimental setting. For imaging, nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA). Images were then taken with a fluorescence microscope (Axio Observer, Carl Zeiss AG, Oberkochen, Germany) and analyzed with the software Axiovision (Carl Zeiss AG, Oberkochen, Germany). The sizes of all the scaffolds used in this work were 10 mm in diameter.

2.3. Scanning electron microscopy (SEM)

Scaffolds were seeded with 1×10^4 *C. reinhardtii* cells and incubated for 4 days at 30 °C under light exposition. After fixation in 3% glutaraldehyde and dehydration with graded ethanol, samples were air-dried and sputtered with gold to 20 nm thickness (Baltec, SCD 005; Leica Microsystems, Wetzlar, Germany). A voltage of 5 kV was used for the scanning electron microscope (Hitachi, S-3500-N, Tokyo, Japan) analysis.

2.4. Chlorophyll measurements

Scaffolds were seeded with 3×10^5 *C. reinhardtii* cells and at different times in culture (days 1, 7 and 14) were placed in 250 μl of dimethyl sulfoxide (DMSO) and shock frozen in liquid nitrogen. The samples were then defrosted and 1 ml of DMSO was added to each sample. Next, samples were mechanically disrupted with a pestle and the optical density of the DMSO was measured at 435 nm (Nanodrop, Thermo scientific, Waltham, MA, USA).

2.5. Oxygen release measurements

Oxygen concentrations in vitro were constantly measured by the SensorDish® Reader (SDR; PreSens GmbH, Regensburg, Germany) in Oxodishes® according to the manufacturer's instructions. For light stimulation, a lamp with the full spectrum of white light (Nano Light, 11 Watt, Dennerle, Vinningen, Germany) was placed 40 cm from the sample. The oxygen concentration was measured as the percentage to saturation.

2.6. Quantification of HIF-1α

Co-cultures were grown with a 3T3:*C. reinhardtii* seeding ratio of 1:10 for the experiments performed in the scaffold (3-D) and a ratio of 1:5 for the 2-D measurements. After 22 h of incubation in hypoxic conditions (1% oxygen), samples were quickly placed in liquid nitrogen. Total protein extracts and HIF-1α ELISA were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The protein content was quantified by BCA assay (Thermo Scientific, Waltham, MA, USA). For immunostaining, cells were quickly fixed with ice-cold acetone. After permeabilization, cells were first incubated with a rabbit anti-mouse HIF-1α antibody (Novus, Littleton, CO, USA) and next with goat anti-rabbit antibody coupled to Alexa-488 (Invitrogen, Carlsbad, CA, USA). Additionally, cells were counterstained with DAPI (Invitrogen, Carlsbad, CA, USA). Staining was performed according to the manufacturer's instructions.

2.7. Statistic analysis

All assays were repeated in at least three independent experiments. Data were expressed as mean ± SD. The GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA) was used for statistical analyses. Two-tailed Student's *t*-tests were performed to evaluate the differences between two groups. Differences between means were considered significant at $p \leqslant 0.05$.

3. Results

3.1. Seeding, distribution and proliferation of the microalgae in the scaffold

We first evaluated whether the microalga *C. reinhardtii* could be incorporated in a FDA-approved collagen-based scaffold commonly used for dermal repair. Algae were efficiently seeded and created a sort of "green skin" (Fig. 1A). SEM revealed that *C. reinhardtii* were located even in the inner cavities of the scaffold and did not actively attach to the material (Fig. 1B). To further assess the viability of the microalgae in the scaffold, *C. reinhardtii* were seeded in low concentrations and macro- and microscopic pictures were taken over time. Surprisingly, the microalgae did not only survive in the scaffold but also proliferated and created clusters of photosynthetic cells, which were distributed homogeneously along the scaffold (Fig. 2, left). In order to quantify the growth of the algae, we evaluated the chlorophyll content of the scaffold at different times, and results revealed a significant increase in chlorophyll content at days 7 and 14 when compared to day 1 ($p \leqslant 0.005$; Fig. 2, right).

3.2. Oxygen release from seeded scaffolds

The next consideration was whether the scaffold containing *C. reinhardtii* could generate photosynthetic oxygen in response to light stimulation and, if so, whether this oxygen production would correlate with the algae concentration. For that, scaffolds were placed under hypoxic conditions (1% oxygen) and subjected

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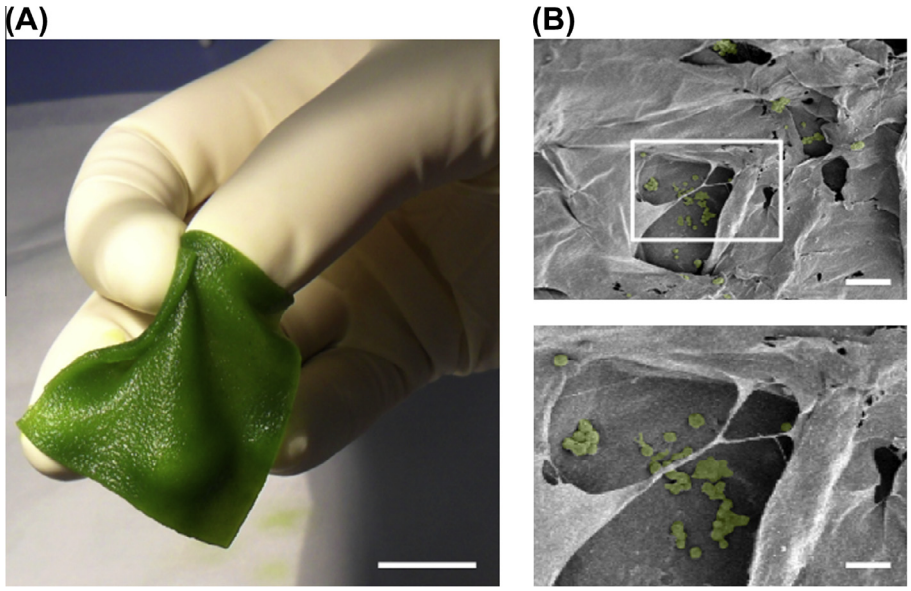

Fig. 1. Seeding of the microalgae into the scaffold. *C. reinhardtii* algae were seeded at a high cell density (2.5×10^7 cells ml^{-1}) in a collagen-based scaffold (5×10 cm). The overall green color shows that the microalgae were homogeneously distributed after seeding (A). SEM analysis shows that *C. reinhardtii* were incorporated even in the inner cavities of the scaffold (B). Scale bars represent 1 cm in (A), 200 μm in the upper panel of (B) and 50 μm in the lower panel of (B).

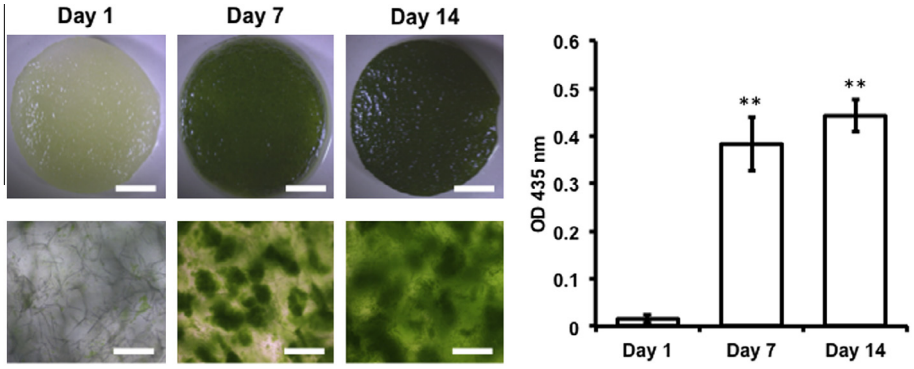

Fig. 2. Biocompatibility of *C. reinhardtii* with the scaffold. After seeding in low density (3×10^5 per scaffold), survival and proliferation of the microalgae was evaluated. The left panel shows a strong increase in the green color intensity within the days. The graph shows a significant increase in the chlorophyll content of the scaffolds at days 7 and 14 compared to day 1. Scale bars represent 2.5 and 500 μm in the upper and lower panels, respectively. $^{**}p \leqslant 0.005$; $n \geqslant 3$. Results are shown as mean ± SD.

to cycles of light and darkness and the oxygen concentration in the culture well was continuously recorded during the experimental setting. Results showed a high photosynthetic capacity of the scaffolds, releasing oxygen immediately after light stimulation, reaching peaks of ~14% of oxygen after 2 h of illumination (Fig. 3A). As expected, the amount of oxygen released by the seeded scaffold was significantly increased by time and at higher numbers of microalgae (Fig. 3B). As expected, oxygen concentrations decreased when the scaffolds were exposed to darkness.

3.3. Cell co-cultures in two dimensions

After confirming that microalgae can be seeded in the scaffold remaining photosynthetically active, we decided to evaluate whether *C. reinhardtii* and mammalian cells are compatible with each other. For this purpose, *C. reinhardtii* and murine fibroblasts were seeded as described in Section 2 (Fig. 4A) and the number of both cells was counted after 7 days in co-culture. Results showed an increase in the number of algae and fibroblasts of 6.1 ± 2.56 and 1.5 ± 0.32 times, respectively (mean ± SD; Fig. 4B). Next, 2-D cultures of fibroblasts or *C. reinhardtii*, or co-cultures of both cell types, were placed in a hypoxic incubator and the oxygen concentration in each independent culture well was quantified. Compared to only fibroblasts, oxygen measurements showed a significant increase of 26.1% in the co-cultures ($p \leqslant 0.005$) and reached values above the limit of detection in wells containing only *C. reinhardtii* ($\geqslant$50% oxygen; Fig. 5A). Consistent with the increased oxygen levels in the co-culture, a significant decrease ($p \leqslant 0.05$) in the expression of the hypoxia-inducible factor-1α (HIF-1α) was observed in co-culture visualized by immunocytochemistry and quantified by ELISA, where a decrease of about six times was detected (Fig. 5B). In this setting, all the experimental groups were exposed to the same light source.

3.4. Cell co-cultures in 3-D

Finally, we assessed the effect of the photosynthetic oxygen in scaffolds co-seeded with *C. reinhardtii* and fibroblasts (Fig. 6A). The 3-D co-cultures were placed in a hypoxic incubator and the oxygen concentration in the well was measured after exposure to light. A significant increase in the oxygen concentrations was observed by increasing the number of seeded microalgae, reaching

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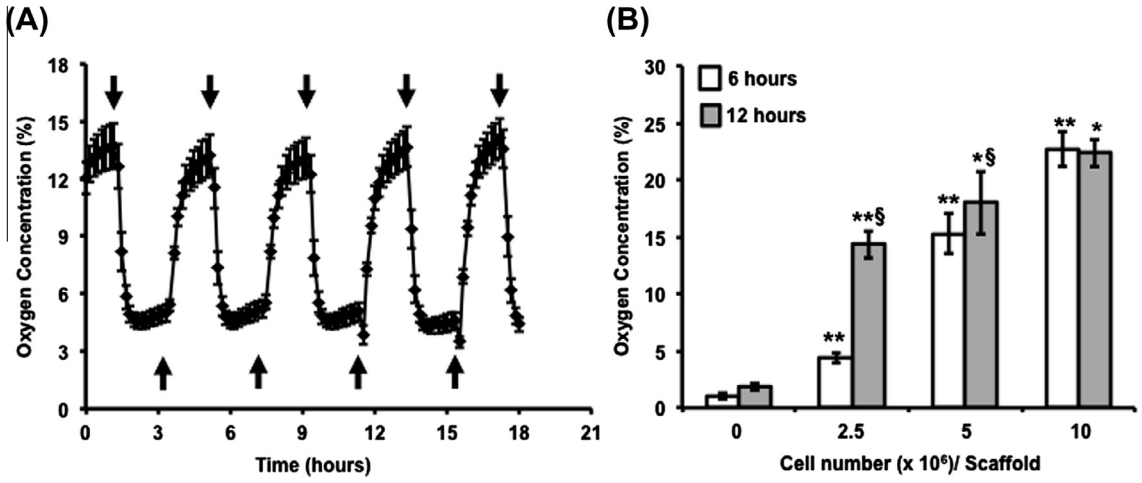

Fig. 3. Oxygen release from photosynthetic scaffolds. Scaffolds seeded with microalgae showed a strong light-dependent capacity to deliver oxygen after stimulation. The upper arrows show the time point when the light was turned off, while the lower arrows show the time point when the light was turned on (A). The amount of oxygen released by the scaffold was significantly increased over time (§: 6 vs. 12 h) and in correlation with the density of the microalgae (*: compared to concentration directly below at their respective time) (B). $^{\S}p \leqslant 0.001$; $^{*}p \leqslant 0.005$; $^{**}p \leqslant 0.001$; $n \geqslant 3$. Results are shown as mean ± SD.

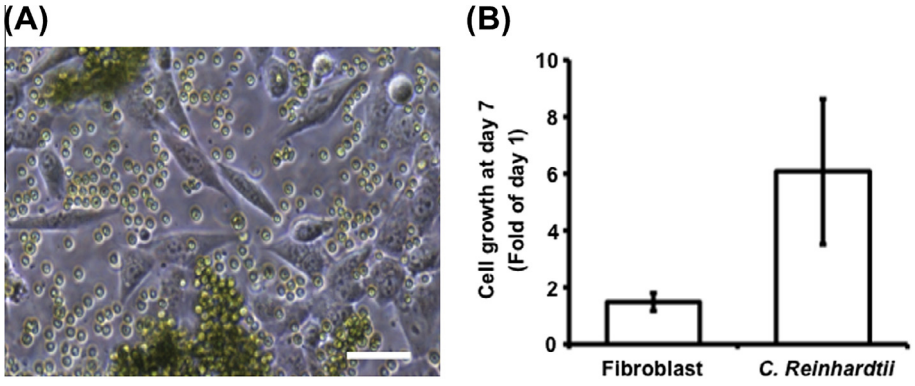

Fig. 4. Cell co-culture. *C. reinhardtii* was co-cultured with fibroblasts in standard culture wells. A representative picture of the co-culture is shown (A). An increase in the number of both microalgae and fibroblasts was observed from day 1 to day 7 (B). Scale bar represents 100 μm. Results are shown as mean ± SD.

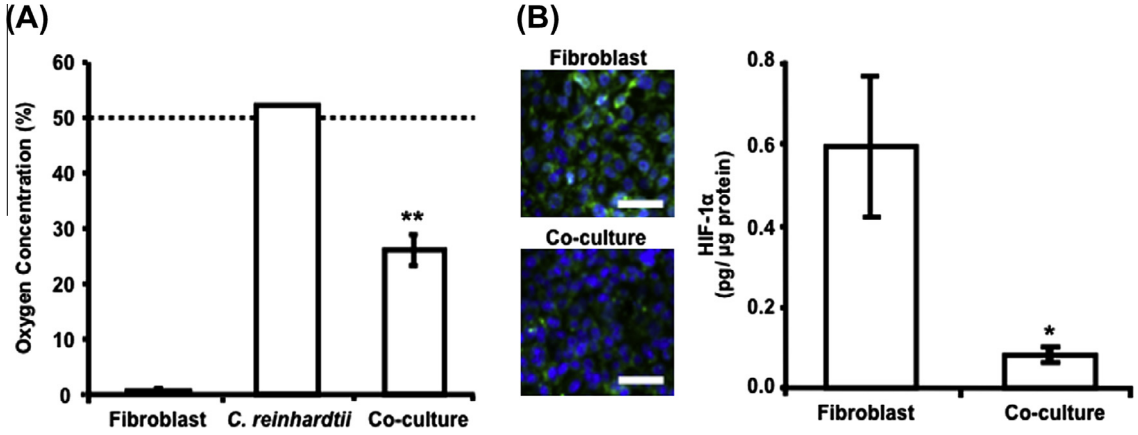

Fig. 5. 2-D cell co-culture under hypoxic conditions. Oxygen concentrations in 2-D co-cultures were measured under hypoxic conditions (1% oxygen) and constant illumination for 22 h. When fibroblasts were seeded alone the oxygen concentration was barely above zero. In contrast, the oxygen concentration in the *C. reinhardtii* cultures reached values above the limit of detection (⩾50%). Compared to seeded fibroblasts, a significant increase of oxygen was observed in co-cultures (A). Moreover, a significant decrease in the levels of the hypoxic marker HIF-1α was detected in co-cultures by immunofluorescence (B, images) and ELISA (B, graph). Scale bars represent 25 μm. $^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.005$; $n \geqslant 3$. Results are shown as mean ± SD.

up to 40% ($p \leqslant 0.05$; Fig. 6B). As consequence, the level of the hypoxia marker HIF-1α was significantly decreased when the co-seeded scaffolds were placed in hypoxia but under constant light stimulation ($p \leqslant 0.05$; Fig. 6C). In this setting, all the experimental groups were exposed to the same light source.

4. Discussion

Critical hypoxia is not compatible with life [14] and represents one of the main problems in tissue engineering in vitro and in vivo. In order to provide an alternative source of oxygen, we seeded microalgae in a collagen scaffold to generate a photosynthetically activated scaffold.

After seeding, scaffolds presented an overall green color, showing a homogeneous distribution of microalgae along the whole 3-D structure (Fig. 1A and B). *C. reinhardtii* were able not only to survive, but also to proliferate inside the scaffold (Fig. 2) and maintain a strong photosynthetic capacity to release oxygen in the presence of light (Fig. 3A). Such light dependency may allow controlling the oxygen concentration in the scaffolds by modulating the light intensity and therefore the spatiotemporal generation of oxygen by differential stimulation of the scaffold. Interestingly, although more algae produced more oxygen, the increase was not directly proportional to the number of *C. reinhardtii* seeded (Figs. 3B and 6B). Among other possible explanations, this unexpected issue could be partially explained by the lower penetration of light at higher densities of the microalgae, thus generating a shade-like effect.

Although any photosynthetic cell could be a candidate for being used in similar approaches, the use of *C. reinhardtii* is particularly attractive because it is easy to grow and handle, its biology is well known and since it does not harbor any known pathogenic viruses or other harmful molecules [15] it is generally recognized as safe by the FDA.

Our results also show that *C. reinhardtii* can be co-cultured with mammalian cells in both 2-D standard plastic culture wells (Fig. 4) and 3-D collagen scaffolds (Fig. 6A). To our knowledge, these data provide the first evidence that murine and photosynthetically active cells can be co-cultured in a potentially symbiotic manner. However, such co-culture systems have to be further optimized to avoid, for instance, light toxicity effects on the mammalian cells or heat shock problems that may affect the microalgae, as their optimum growth temperature lies below those of mammalian cells. Approaches such as the use of specific light wavelengths, according to chlorophyll's absorption spectrum, and the use of thermoresistant algae strains, could be respectively considered in future approaches.

When co-cultures were placed in hypoxia, the presence of the algae decreased the expression of HIF-1α (Fig. 5B and 6C), which is considered the master regulator of hypoxia [16]. This proves that the amount of oxygen generated by photosynthesis is enough to circumvent the lack of oxygen under insufficient supply. The work presented here could be seen as a downscaled analogous approach of the famous experiment performed by Joseph Priestley [17], where he demonstrated that in a closed system, an animal survived in the presence of a plant but died in its absence, showing that under such conditions photosynthesis is able to provide enough oxygen to replace its external supply. In fact, the difference in the oxygen concentration observed between the dishes seeded with only microalgae or microalgae in co-cultures might represent the fraction of oxygen being metabolized by the fibroblasts (Fig. 5A). This observation might have a tremendous impact in the field of tissue engineering not only by improving cell survival and distribution within the scaffold, but also by influencing other several key issues related to the insufficient oxygen supply in 3-D cultures. As an example, hypoxia seems to regulate Oct4 and Notch signaling, which are key regulators of stemness [18], thus several studies have shown that proper oxygen tensions are critical to modulate the stem cell differentiation niche in 3-D environments [19]. In this context, future approaches may consider the

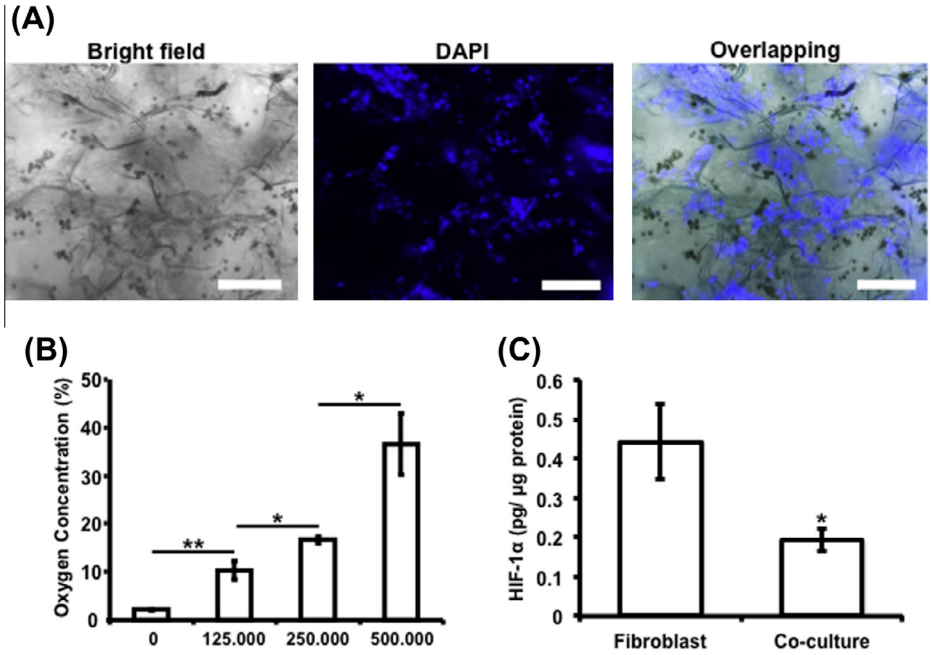

Fig. 6. Fibroblast seeded in photosynthetic scaffolds. *C. reinhardtii* were co-cultured with fibroblasts in a collagen-based scaffold. A representative picture is shown where the microalgae (dark dots in bright field) and murine cells (blue, DAPI) are co-distributed homogeneously all over the matrix (A). Co-cultures with a fixed number of fibroblasts but increasing algae densities resulted in an increasing oxygen release from the scaffold (B). Consistently, after 22 h of light stimulation, ELISA measurements show that the presence of *C. reinhardtii* led to decreased levels of the hypoxic marker HIF-1α in the scaffold (C). Scale bars represent 25 μm. $^{*}p \leqslant 0.05$; $^{**}p \leqslant 0.005$; $n \geqslant 3$. Results are shown as mean ± SD.

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use of photosynthetic scaffolds for controlling the stem cell differentiation rate in 3-D cultures.

Previously, scaffolds have been loaded with calcium-peroxide-based oxygen generating particles that release oxygen through its chemical degradation [20] or by incorporating fluorinated porous zeolite particles in the scaffold [21]. Both approaches have been shown to support hypoxic cells seeded in the scaffold by increasing the oxygen levels in ~30%. Because of its simplicity, the approach described here may represent several advantages compared to other technologies. Among others, it is easy and inexpensive to implement, the local oxygen tension can be spatiotemporally modulated and could be potentially used for nearly any kind of materials combined with any kind of cells. Some drawbacks of this approach are the need to use light-transmitting biomaterials and potential problems related with immune rejection in vivo. The combined use of scaffolds and light-emitting-diode technologies and the encapsulation of microalgae in immune-compatible materials [22] could be investigated in future studies to minimize the possible problems mentioned above.

Taken together, this work provides the first evidence for the development of photosynthetic tissues and proposes the use of photosynthesis to homogeneously supply engineered tissues with oxygen in vitro. Future approaches may also consider the use of photosynthetic scaffolds in vivo, thus providing an alternative source of oxygen delivery to blood vessels.

5. Conclusion

The combined use of biomaterials seeded with photosynthetic cells generates scaffolds that constantly deliver oxygen in response to light, creating normoxic microenvironments, even in the absence of an external oxygen supply. The results presented here represent the first scientific evidence that photosynthesis can overcome the need of oxygenation in tissue engineering approaches. This work suggests the use of photosynthetic cell transplantation to modulate a broad spectrum of hypoxic conditions in vitro and in vivo.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1, 2, 4, 5, and 6 are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2013.12.055.

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