

RESEARCH ARTICLE

Injectable thermosensitive chitosan/β-glycerophosphate/collagen hydrogel maintains the plasticity of skeletal muscle satellite cells and supports their *in vivo* viability

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Abstract

A cell carrier plays an important role in the maintenance, growth and engraftment of specific cells aimed for defined therapeutic uses in many tissue engineering strategies. A suitable microenvironment for the cells allows for the maximum efficacy of the hybrid device. We have prepared an injectable thermosensitive chitosan/β-glycerophosphate/collagen (C/GP/Co) gel and investigated its potential application as a support for the culture of skeletal muscle satellite cells (SMSCs). A cell viability assay was used to evaluate the *in vitro* cytocompatibility of the gel. Cell growth was assessed by scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM) and histological analysis. The influence of the C/GP/Co gel on the plasticity of SMSCs seeded at the surface of the gel was assessed by induction of the myogenic, osteogenic and adipogenic differentiation. C/GP/Co gel provided the appropriate environment for the culture of SMSCs *in vitro*. In addition, the C/GP/Co gel supported SMSC plasticity. *In vivo* testing of the SMSC-seeded gel was investigated by subcutaneous injection into the dorsum of nude mice. Cell viability was assessed both by *in vivo* imaging and histological examination of the explants. In conclusion, C/GP/Co hydrogel is a cytocompatible carrier for the *in vivo* delivery of SMSCs and supportive for SMSC plasticity. Thus, this gel has potential applications in tissue engineering and regenerative medicine.

Keywords: cytocompatibility; injectable hydrogel; plasticity of adult stem cells; skeletal muscle satellite cells; tissue engineering

Introduction

Skeletal muscle satellite cells (SMSCs), located between the basal lamina and the sarcolemma of muscle fibres are one of the most plentiful and accessible sources of adult stem cells. As the main component of skeletal muscle stem cells, SMSCs are primarily responsible for muscle growth and repair following injuries (Kuang and Rudnicki, 2008; Tedesco et al., 2010). SMSCs differentiate not only towards the myogenic lineage, but into some mesodermal lineages, such as osteogenic, chondrogenic or adipogenic, when exposed to specific stimulation (Asakura et al., 2001; Sarig et al., 2006; Hashimoto et al., 2008). Therefore, the prospect of applying SMSCs in tissue engineering, as well as in regenerative

medicine strategies, is capturing the interest of the scientific community. One major limitation in the application of SMSCs is the relatively poor survival and engraftment after transplantation, which is frequently attributed to the loss of “stemness” and viability of SMSCs, especially after subculture expansion *in vitro* (Peault et al., 2007; Gilbert et al., 2010; Borselli et al., 2011). Plasticity is one of the most important characteristics of stem cells, indicating its multi-lineage potential of differentiation. The importance of matrix elasticity in the efficiency of SMSCs cultures is well known, such that soft hydrogel substrates mimic the physiological tissue rigidity (Gilbert et al., 2010). These substrates promote growth and preserve the characteristics of SMSCs. Injectable hydrogels may, at least partially, recapitulate the main

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Abbreviations: C/GP/Co, chitosan/β-glycerophosphate/collagen; SMSCs, skeletal muscle satellite cells; SEM, scanning electron microscopy; CLSM, confocal laser scanning microscopy

biophysical and biochemical features of the niche. Besides being applicable in minimally invasive procedures, hydrogels are viable supports for tissue engineering. Furthermore, hydrogel substrates offer the competitive advantage of easily filling irregular defects when compared with rigid scaffolds. A pullulan/collagen hydrogel carrier could enhance the angiogenic activity of implanted mesenchymal stem cells by accelerating wound healing in a murine model (Rustad et al., 2012). Thus, we hypothesise that our hydrogel system may facilitate the cell growth and the delivery of SMSCs, overcoming the main limitations of this important cell source.

Chitosan/β-glycerophosphate (C/GP) in situ forming hydrogel, first reported by Chenite et al. (2000), is one of the most currently investigated thermosensitive injectable hydrogels for cell delivery. Introduction of collagen type I in combination with C/GP contributed to enhancing the cytocompatibility of this biomaterial system (Wang and Stegemann, 2010; Sun et al., 2011). Collagen type I is one of the most routinely used substrates in cultures of SMSCs, due to its activity in promoting cell adhesion and proliferation. The chitosan/β-glycerophosphate/collagen (C/GP/Co) hydrogel may be a preferable hydrogel for carrying SMSCs in tissue engineering by allowing to fine-tune the composition to obtain specific properties. In fact, the application of C/GP/Co hydrogel in cultures of human bone marrow-derived stem cells (BMSCs) and adipose tissue-derived stem cells (ADSCs) (Song et al., 2010; Wang and Stegemann, 2010).

To our knowledge, no reports have been published on the growth and differentiation of SMSCs in C/GP/Co hydrogels. We demonstrate cytocompatibility and efficacy in maintaining the plasticity of SMSCs cells with C/GP/Co hydrogels. C/GP/Co hydrogels are promising carriers for the delivery of SMSCs in specific tissue engineering applications not involving demanding stress environments.

Materials and methods

SMSCs isolation and identification

Under ether anaesthesia, the limb muscles of GFP transgenic mice (C57BL/Ka-βactin-EGFP) were removed. After cutting the limb muscles into small pieces, SMSCs were enzymatically dissociated by incubating the minced tissue with 2% dispase collagenase (Hyclone) solution for 1 h at 37°C. The enzyme digestion was neutralised with growth media containing Ham's F10 (Hyclone) and 20% foetal bovine serum (FBS, Gibco). Cells were filtered through a cell strainer (70–100 μm) and plated on a non-coated dish for 1 h at 37°C. The supernatant was gently removed and plate on a collagen-coated dish. This step was repeated 1 h later to purify the SMSCs. The media was changed and the cells were

plated. The purified SMSCs were cultured in medium containing Ham's F10, 20% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (growth medium) at 37°C with 5% CO₂ and 95% air. Primary SMSCs were cultured and expanded in growth medium with the addition of 10 ng/mL basic FGF. When 50% confluence had been reached, the cells were detached with 0.25% trypsin (Hyclone), and passaged using standard methods.

Immunocytochemical staining for desmin was performed when SMSCs were in the logarithmic growth phase (Corbu et al., 2010). For myogenic differentiation, the medium was changed to myogenic induction medium containing DMEM (Hyclone), 2% horse serum (Hyclone), 1% penicillin/streptomycin (P/S) when the cells had reached 70% confluence. After 5 days, immunocytochemical staining for myosin heavy chain (MHC, Abcam) was carried out.

Preparation of C/GP/Co hydrogels

A 2.0 wt% chitosan (C; degree of deacetylation >90%, Sengong Technology, Inc., Shanghai, China) solution was prepared by stirring C powder in 0.1 M acetic acid for 4 h at room temperature. A 50 wt% β-glycerophosphate (β-GP, Sigma) solution was obtained by dissolving 1 g of β-GP in 1 mL of distilled water. The β-GP solution was added drop-wise into the C solution at a ratio of 1:5. The resulting C/GP solution was mixed with an equal volume of 2 mg/mL collagen type I (Co; BD Bioscience). Finally, the C/GP/Co solution was incubated at 37°C for gelation.

Cytocompatibility of the C/GP/Co hydrogel in vitro

Viability assay

The C/GP/Co group was prepared by loading liquid C/GP/Co (50 μL/well) in 96-well plates and maintaining the temperature at 37°C for 30 min. In the control group, plates were coated with collagen type I by standard methods. Briefly, collagen was diluted to 50 μg/mL in 0.02 M acetic acid, and used to coat plates (100 μL/cm²). After incubation at room temperature for 1 h, the remaining collagen solution was removed. Plates were rinsed three times with phosphate buffered saline (PBS) and air-dried for future use. An aliquot (100 μL) of the SMSC suspension (2×10^4 cells/mL) was pipetted into each well and incubated at 37°C in a humidified atmosphere containing 5% of CO₂. On Days 1, 3, 5 and 7, the CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each. After a 2 h incubation period, an aliquot from each well (100 μL) was transferred into new 96-well plates and the optical density at 450 nm was measured with a microplate reader (iMark, Bio-Rad). For comparison, an equal number of SMSCs were seeded for the control group and treated as described above. Each assay was replicated in six wells.

Analysis of cell growth in 3D cultivation

SMSCs were homogenously suspended in a C/GP/Co solution with 2×10^5 cells/mL. The cell suspension was pipetted into 24-well plates (500 μL /well) and allowed to gel at 37°C. Growth medium was added and renewed every 2 days. Immediately after gelation and on Day 7, SMSC-seeded hydrogels were washed with PBS, fixed with 4% paraformaldehyde and dehydrated in 30% sucrose solution. After being embedded in OCT (Optimal Cutting Temperature) medium, 10 μm thick sections were prepared and examined by fluorescence microscopy (BX53, Olympus).

After 3 days, random samples of SMSC-seeded hydrogels were obtained for scanning electron microscopy (SEM) and LCSM analysis. For the SEM observation, both seeded and unseeded scaffolds were washed and fixed with 3% glutaraldehyde for 2 h. The scaffolds were washed again, followed by a gradient-elution with ethanol and *tert*-butyl alcohol (TBA). Subsequently, the samples were vacuum dried, gold sputtered and analysed by SEM (S-3400N, Hitachi). For LCSM, SMSCs-seeded hydrogels were prepared, washed, fixed and dehydrated as described above for histological analysis. The hydrogels were sliced and finally analysed under a LCSM (FV1000, Olympus). Plain scanning (0.5 μm /section) and 3D reconstruction were conducted for the various sections.

In vivo testing of the cell encapsulated hydrogels

A total of six SPF male congenitally athymic nude mice, with a median age of ~10 weeks and weighing approximately 18–22 g, were acquired from the Animal Experiment Center of the Third Military Medical University. They were quarantined for 7 days. During the whole experiment, the nude mice were housed in sterilised cages supplied with filtered air, and sterile food was provided ad libitum. All surgery was done under sterilised conditions in a sterile room, using ether anaesthesia.

The implantation of the SMSC-encapsulated hydrogels was performed by two contralateral subcutaneous injections in the dorsum of each of the six mice. For each injection, 250 μL of C/GP/Co solution was gently suspended in a pellet containing 5×10^5 SMSCs. Before the injections, each mouse was anaesthetised with ethyl ether. A volume of 250 μL of SMSC-encapsulated solution was administered using a syringe equipped with a 26-gauge needle. The nude mice were killed 4 weeks after the injection by cervical dislocation in accordance with the Animal Care Guidelines from the Third Military Medical University and following international standards of care of animal experiments. The explants were individually dissected and used for histological analysis. Immediately after the injections, as well as before sacrificing the animals, an *in vivo* imaging system (Kodak In Vivo Fx) was used to track the state of the SMSCs.

Plasticity of SMSCs

SMSCs were seeded in plates coated with the C/GP/Co gel. An equal number of SMSCs were seeded in collagen coated plates as the control group. When the cell confluence reached a specified level, depending on the experiments described below, the growth culture medium was changed to differentiation-inducing medium for myogenic, osteogenic or adipogenic lineages.

Evaluation of the myogenic differentiation

After a 5-day induction, nuclei were stained with DAPI. The fusion index or the ability of cells to fuse into multinucleated cells was used to assess myogenic differentiation. The fusion index was calculated as follows: the ratio of nuclei present in multinucleated myotubes divided by the total number of DAPI fluorescent nuclei (Lafuste et al., 2005). A number ranging from 50 to 200 individual myotubes (cells with two or more nuclei) was counted in each fluorescence micrograph (Horsley et al., 2001). Myotubes were classified into two categories, those with two to four nuclei and those with five or more nuclei. The percentage of myotubes in each category was quantified. Six parallel replicates were performed for each specimen of the samples.

Osteogenic differentiation

When confluence had reached 80%, the growth medium was changed to osteogenic induction medium containing DMEM, 10% FBS, 1% P/S, 100 nM dexamethasone (Sigma), 10 mM β -GP (Sigma) and 50 $\mu\text{g}/\text{mL}$ ascorbic acid 2-phosphate (Sigma). Alkaline phosphatase (ALP) activity was assayed at Days 7, 14 and 21. Alizarin red staining was performed at Day 28 after osteogenic induction.

ALP activity was measured as described by Wang et al. (2011). The total amount of intracellular protein was measured using a bicinchoninic acid (BCA) protein assay kit (Pierce) after cultivation for 7, 14 and 21 days. ALP activity was determined by incubation with *p*-nitrophenyl phosphate (Sigma) and measuring the conversion to *p*-nitrophenol after 30 min. The absorbance of the solution was measured at a wavelength of 405 nm using a microplate reader. The value of absorbance was converted to a concentration using a calibration curve, obtained by measuring the absorbance of a series of dilutions of *p*-nitrophenol with a 1 U (active unit) equivalent to 1 g product of *p*-nitrophenol. Data is presented as units per g of protein. Six parallel replicates for each group were analysed.

Alizarin red staining was performed as described by Zhi et al. (2011). In brief, the specimens were washed three times with PBS and fixed in 75% ethanol at 4°C for 1 h. The specimens were washed three times with distilled water and stained with 1% Alizarin red S for 5 min. After rinsing three

times with distilled water, the specimens were photographed for visual analysis.

Adipogenic differentiation

When confluence had reached 100%, growth medium was changed to adipogenic induction medium containing DMEM, 10% FBS, 1% P/S, 10 µg/mL of insulin (Sigma), 0.5 mM dexamethasone (Sigma), 0.5 mM isobutylmethylxanthine (Sigma) and 0.1 mM indomethacin (Sigma).

After a 16-day induction, Oil Red O (Sigma) staining was done. Briefly, cells were fixed in 1% formaldehyde and stained for 20 min. The specimens were rinsed with 85% propylene glycol (Sigma) for 3 min, and washed with distilled water. The area of lipid droplets was measured (Benzer et al., 1994) with the software Image-pro plus 6.0.

Statistical analysis

Data are presented as the mean ± standard deviation (SD) and analysed with the Student's *t*-test. *P* < 0.05 was considered statistically significant.

Results

SMSCs identification

When analysed by fluorescence microscopy, SMSCs had a short spindle appearance with a round nucleus (Figure 1A). After staining for desmin, over 95% of the cells had positive

red staining in the cytoplasm (Figure 1B). Three days after myogenic induction, SMCS underwent a morphology change and fused into multinuclear small-sized myotubes (Figure 1C). A number of small-sized myotubes fused into larger-sized myotubes at Day 5 (Figure 1D). Following a 5-day myogenic induction, the cells were immunocytochemically assayed for MHC expression with DAPI counter-staining. A considerable amount of myotubes with dark red cytoplasm (Figure 1E) and blue nuclei (Figure 1F) were found. By merging the images, multiple nuclei could be identified in the myotubes (Figure 1G).

Macroscopic optical properties of C/GP/Co hydrogels

The gelation time of the C/GP/Co solution was 10 min and the pH was 7.15. C/GP/Co was a colourless transparent viscous liquid before gelation and transformed into a yellowish opaque hydrogel after gelation (Figure 1H).

Cytocompatibility in vitro

Cell viability assay

Cell viability in the C/GP/Co surface coating was measured over 7 days using a CCK-8 kit. Viability, measured by optical density, increased in the C/GP/Co group compared with the control group (Figure 2A). At Day 1, no significant difference in optical density was seen between the two groups. Longer culturing periods showed consistently and significantly higher cell viability in the C/GP/Co group compared to the control group.

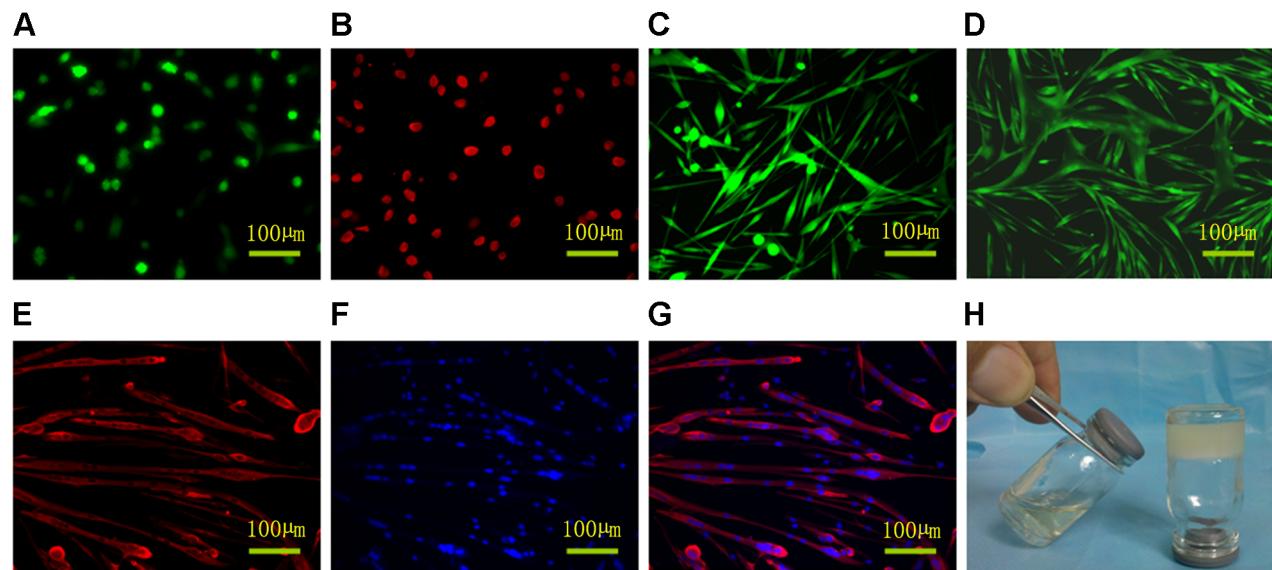


Figure 1 SMSCs identification (A–G) and the appearance of the C/GP/Co hydrogel (H). (A) Typical short spindle-shaped morphology of SMSCs. (B) Red staining of the SMSC cytoplasm with desmin. (C) Small-sized myotubes after myogenic induction for 3 days. (D) Large-sized myotubes after myogenic induction for 5 days. (E) Dark-red myotubes with MHC staining. (F) Counter staining with DAPI. (G) Merged image of (E) and (F). (H) Appearance of the C/GP/Co hydrogel before and after thermal gelation.

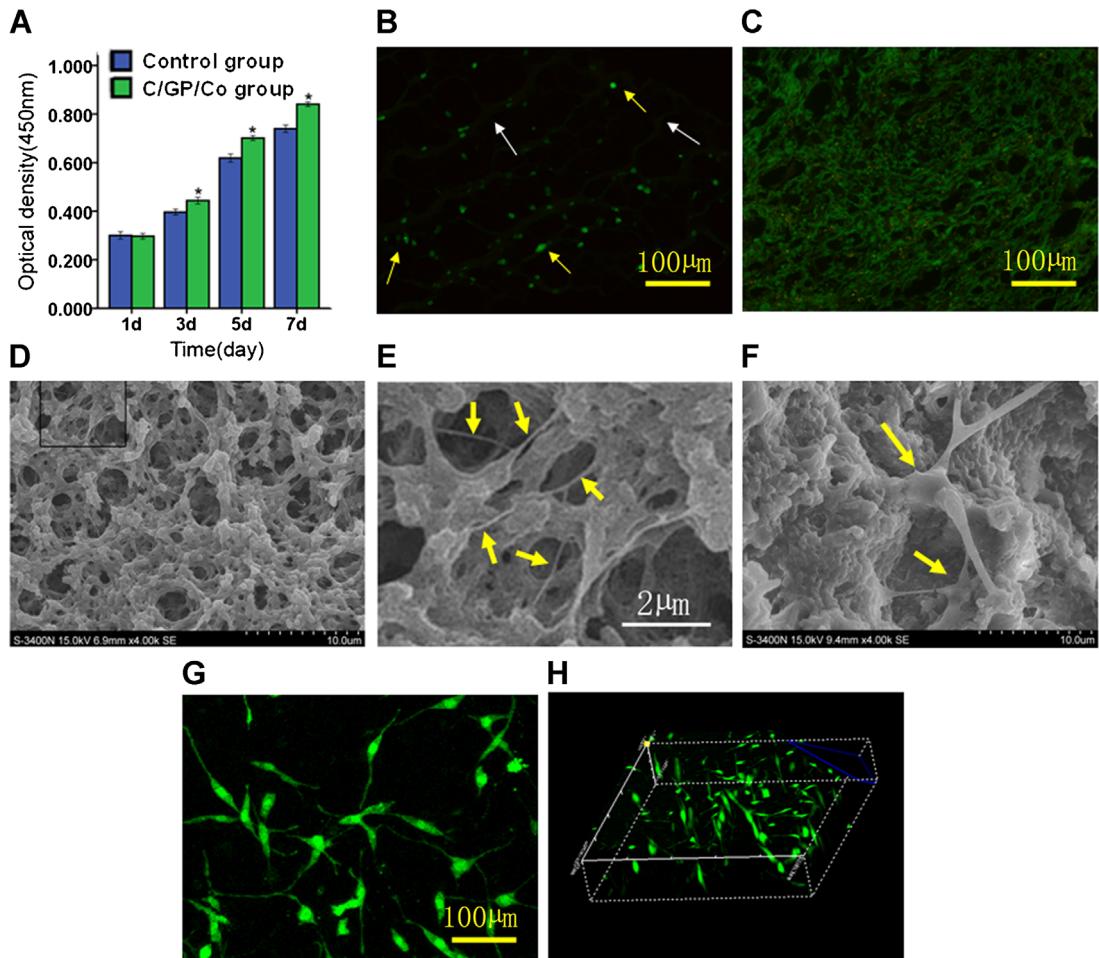


Figure 2 In vitro cytocompatibility of the C/GP/Co hydrogel. (A) Cell viability assay: optical density was significantly higher in the C/GP/Co group compared to the control group, except at Day 1. (B) Morphology and distribution of SMSCs immediately after gelation. The white and yellow arrows indicate scaffold and GFP-labelled SMSCs, respectively. (C) SMSCs exhibit robust proliferation and migration into the bulk of the hydrogel after 7 days. (D) SEM image of the unseeded hydrogel: it exhibits a porous cobweb-like network. (E) Amplified image of a selected area in (D) to show collagen fibrils. (F) SEM image of a SMSCs-seeded hydrogel after 3 days: the arrows indicate the location of SMSCs. (G) Top image of LCSM scanning after 3 days (H) 3D construction obtained from the sectioning performed by LCSM. * $P < 0.05$, bar = 100 μm .

3D growth in the seeded hydrogel

Immediately after gelation, the round SMSCs were evenly distributed in the C/GP/Co hydrogel (Figure 2B). After culturing for 7 days, the SMSCs exhibited robust proliferation and migration into the bulk of the hydrogel (Figure 2C).

The hydrogel had a porous cobweb-like network (Figure 2D), in which tiny collagen fibrils were connected to each other (Figure 2E). The pore size of the hydrogel was $3.51 \pm 0.97 \mu\text{m}$, as measured by Image-pro Plus 6.0 software. Spindle-shaped MSCs in the hydrogel migrated along the pores of the hydrogel, extending processes that seemed to connect cells in the hydrogel porous structure (Figure 2F).

In a top view image compounding the various slices obtained by LCSM, scattered SMSCs emitting green fluores-

cence could be seen in all the sections and had extended protrusions (Figure 2G). In the 3D compounded image, a large number of cells displayed homogeneous dispersion and intercellular extended protrusions (Figure 2H).

In vivo testing of cell encapsulated hydrogels

Immediately after SMSC-encapsulated hydrogel injections into nude mice, two elliptical subcutaneous masses could be identified in each mouse (Figure 3A). A strong fluorescence signal was detected with the in vivo imaging system at the injection sites at this time (Figure 3B). Neither skin necrosis nor an inflammatory response was found during the follow-up periods. After 4 weeks, the subcutaneous implant did not change volume (Figure 3D), and the emitted fluorescence

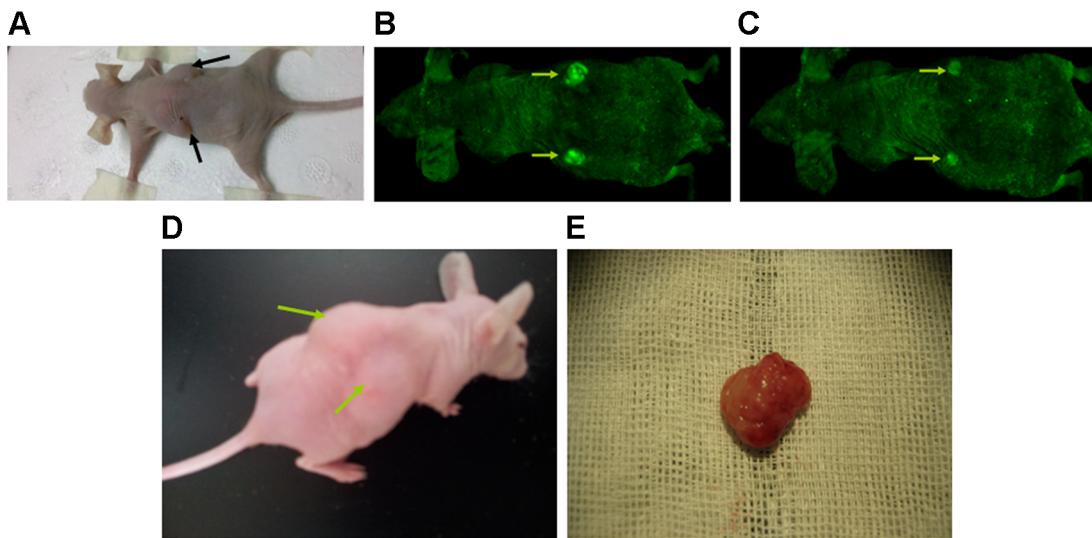


Figure 3 In vivo biocompatibility of the C/GP/Co hydrogel. (A) Photograph of a nude mouse after injection. (B) Fluorescent photograph of a nude mouse immediately after injection. (C) Fluorescent photograph of a nude mouse before sacrifice. (D) Photograph of a nude mouse before sacrifice. (E) Dissected hydrogel tissue. The arrows in (A–D) indicate the locations of subcutaneous implants.

remained visible, but had a lower intensity, as compared to early time-points (Figure 3C). Fibrous encapsulations were not found in the dissected hydrogel, which remained elastic, but stiffer than before the injections (Figure 3E). The histological analysis revealed that the SMSCs co-transplanted in the hydrogel maintained viability and remained distributed in the visual field (Figure 4A). HE staining showed extensive ingrowth of blood vessels (Figure 4B).

Influence of the hydrogel on SMSC plasticity

In the myogenic induction assay, the C/GP/Co coated surface in tissue culture polystyrene (TCP) showed improved differentiation efficiency compared with the control group (collagen coated surface). The fusion index and the percentage of myotubes with five or more nuclei in the

C/GP/Co group were significantly higher compared to the control group (Figure 5A–D).

ALP is one of the most widely recognised functional biomarker of osteoblastic differentiation and its activity is closed related to matrix formation (Gerstenfeld et al., 1987; Zou et al., 2008; Serigano et al., 2010). In the osteogenic induction assay, ALP activity in both groups increased up to Day 14, and decreased thereafter. On Day 7, 14 and 21, ALP activity (U/g protein) in the control group was 6.88 ± 0.80 , 13.38 ± 0.67 and 3.70 ± 0.48 , respectively, compared to 12.58 ± 1.00 , 22.33 ± 1.61 and 7.05 ± 0.94 in the C/GP/Co group correspondingly. ALP activity in the C/GP/Co group was significantly higher than that observed in the control group at all time-points (Figure 6). At Day 28, Alizarin red staining for osteogenic differentiation showed a significantly larger area of red staining in the C/GP/Co group than in the

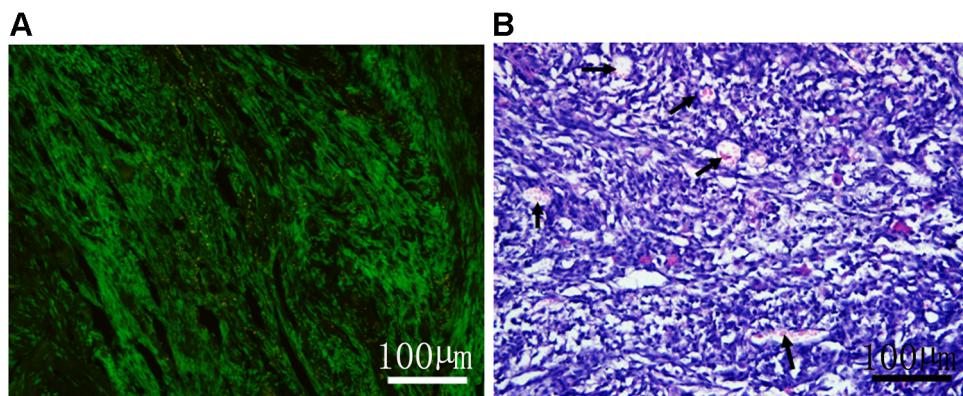


Figure 4 Histological analysis of the SMSC-seeded hydrogel after the 4-week transplantation: (A) green fluorescence. (B) HE staining. The arrows indicate the newly formed blood vessels. Bar = 100 μm.

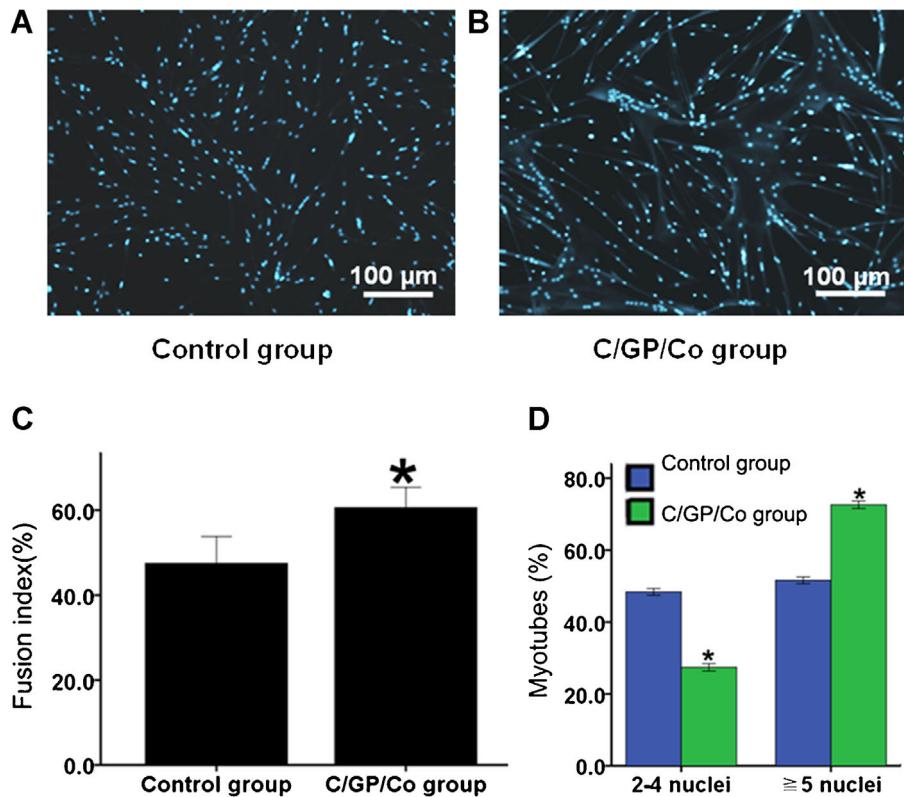


Figure 5 Influence of the C/GP/Co gel surface on myogenic differentiation of SMSCs ($n = 6$). After a 5-day induction, DAPI staining was performed to show the nuclei of myotubes in the control group (A) and the C/GP/Co group (B). (C) Fusion index in the C/GP/Co group was significantly higher compared to the control group. (D) The percentage of myotubes with five or more nuclei was significantly higher compared to the control group. * $P < 0.05$, bar = 100 μm .

control group (Figure 7A–C). Enhanced staining is a positive indication of the efficacy of the C/GP/Co hydrogel in supporting the differentiation of stem cells.

In the adipogenic induction assay, red staining droplets were visible in both the C/GP/Co group and in the control.

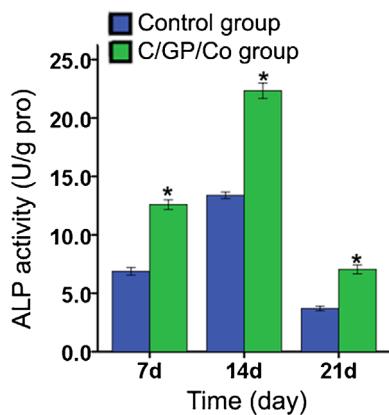


Figure 6 Dynamic monitoring for alkaline phosphatase (ALP) activity during a period of 21-days of osteogenic induction. The ALP activity in the C/GP/Co group was significantly higher at all time-points, compared to the control group. * $P < 0.05$.

The area of lipid droplets was significantly higher in the C/GP/Co group than in the control group (Figure 8A–C).

Discussion

An effective and functional carrier to deliver selected cell populations is an important prerequisite for the success of any tissue engineering strategy. Indeed, such a carrier should provide adequate cues and a microenvironment for seeded cells to maximise the therapeutic efficacy of the device.

Injectable hydrogels can be administered by minimally invasive procedures. Hydrogel carriers are soft substrates that easily interact with the flexibility of the muscle extracellular matrix, and are thus strong candidates to develop carriers for the *in vivo* delivery of SMSCs (Gilbert et al., 2010). C/GP hydrogel is one of the most frequently investigated injectable hydrogels and is a promising cell carrier (Cho et al., 2008; Kim et al., 2008). GP fraction, as a key component of this hydrogel, is responsible to bring chitosan pH and osmolality to a cytocompatible level. However, at high concentrations GP is cytotoxic because it leads to a high-osmolar environment which is detrimental to cell survival (Song et al., 2010). Some previous studies have reported that

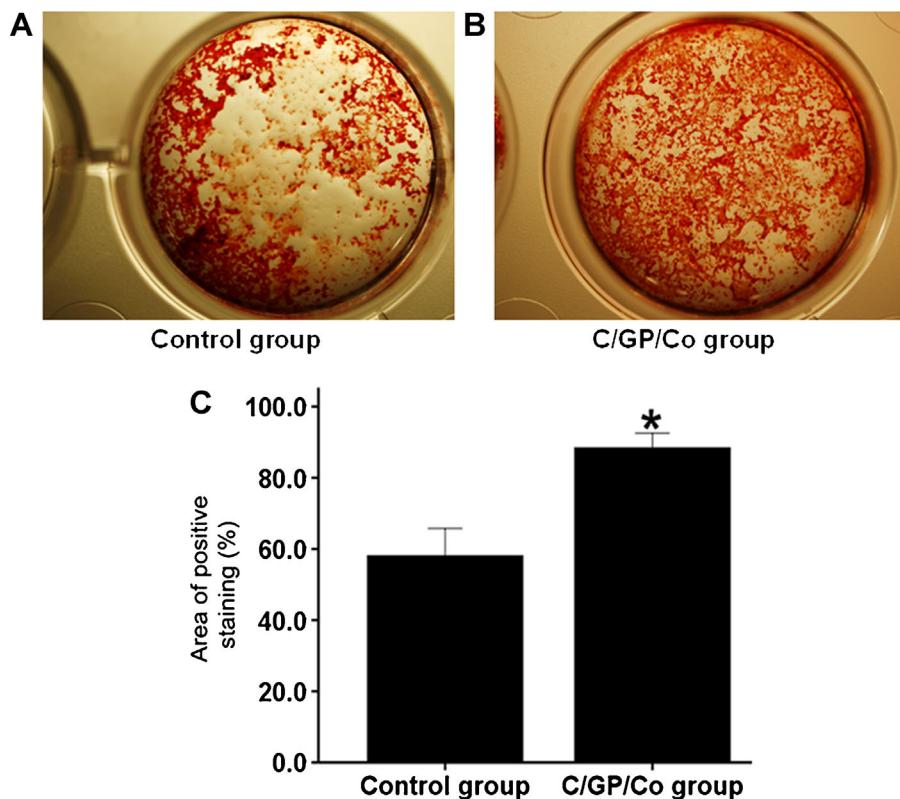


Figure 7 Alizarin red staining for osteogenic differentiation performed at Day 28 ($n = 6$). (A) Alizarin red staining in control group. (B) Alizarin red staining in C/GP/Co group. (C) The area of positive staining with Alizarin red was significantly larger in the C/GP/Co group compared to the control group. $*P < 0.05$.

chitosan solutions with GP can undergo thermal gelation (Chenite et al., 2000; Cho et al., 2008; Richardson et al., 2008). Ideally, the osmotic pressure of a hydrogel should be isotonic to the extracellular fluid. Chitosan hydrogels with high salt (GP) concentration produce ionic strengths unsuitable for cells and inhibited cell growth (Ahmadi and de Bruijn, 2008; Cho et al., 2008). In order to reduce the cytotoxicity of chitosan-based injectable hydrogels and improve cell viability, several researches have used low concentration of inorganic phosphate salts (Kim et al., 2010, 2011; Nair et al., 2007), or added cross-linkers, such as hydroxyethyl cellulose, genipin and glyoxal, responsible for the gelation of chitosan based hydrogels with low concentrations of GP (Li and Xu, 2002; Hoemann et al., 2007; Yan et al., 2010; Moura et al., 2011; Wang and Stegemann, 2011), while the gelation temperature is maintained at 37°C. Collagen type I is frequently used as a substrate for the culture of different stem cells, including in the clinical practice (Shih et al., 2006); thus we selected this component as the additive to our hydrogel. pH and gelation time and temperature of the C/GP/Co hydrogel were all compatible in our work with the requirements of an injectable hydrogel. In addition, the porous structure is interconnected and suitable for nutrient diffusion, such that the hydrogel surfaces are adequate for the

adhesion of cells and cell proliferation. The pore size was $3.51 \pm 0.97 \mu\text{m}$, consistent with some previous studies (Wang and Stegemann, 2010), but inconsistent with others (Crompton et al., 2005; Sun et al., 2011). The difference in pore size may be partly due to the different pretreatment protocols used in the SEM analyses.

We have evaluated the application potential of the C/GP/Co hydrogel by characterizing its cytocompatibility in vitro, as well as in vivo. The cell viability assay showed that the hydrogel was not cytotoxic. The SMSCs exhibited good growth when encapsulated in the hydrogel and cultured in vitro, as demonstrated by SEM, LCSM and histological analysis. Our results strongly suggest that the C/GP/Co hydrogel provides an environment that is supportive for SMSC biological activity by inducing its multilineage potential of differentiation. To investigate its efficacy as an injectable cell carrier in vivo, SMSC-populated hydrogels were transplanted into the subcutaneous dorsum of nude mice for 4 weeks. In vivo fluorescence imaging and histological analyses confirmed that SMSCs implanted in the hydrogel were viable and that the hydrogel maintained its structural integrity over the course of the experiment.

Enhanced cytocompatibility due to the introduction of collagen type I in the formulation may be related to three

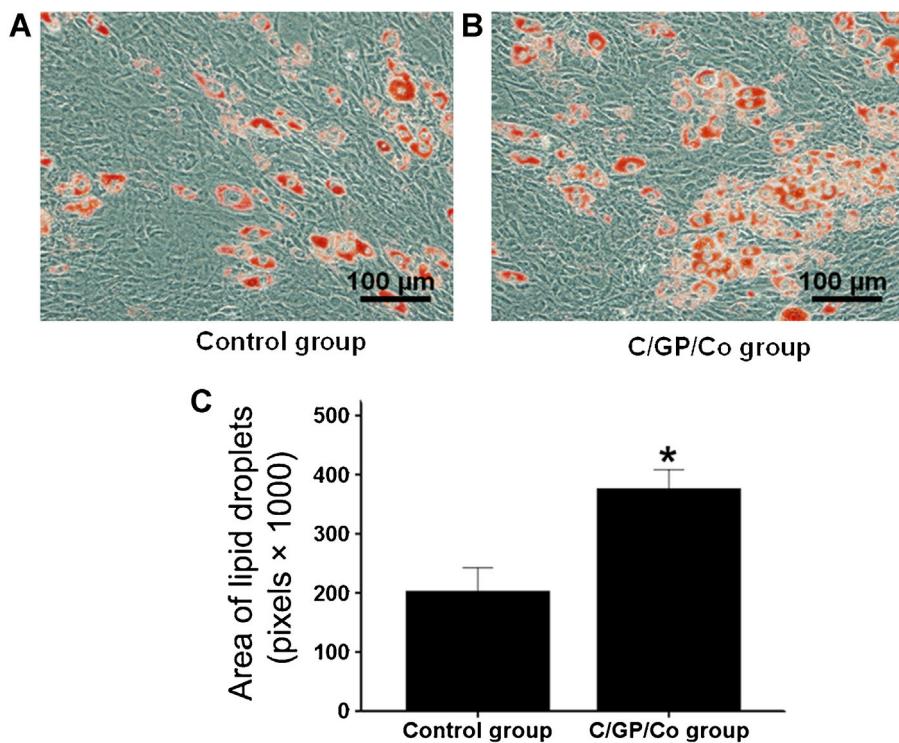


Figure 8 Oil red O staining for adipogenic differentiation performed at Day 16. Oil red O staining was performed to show the lipid droplets (the area of red staining) in cytoplasm of cells in control group (A) and C/GP/Co group (B). (C) The area of lipid droplets was significantly larger in the C/GP/Co group compared to the control group. * $P < 0.05$, bar = 100 μm .

positive properties of collagen. First, collagen type I has multiple cell binding sites (Xu et al., 2000) and facilitates cell adhesion and proliferation (Eslaminejad et al., 2009; Hesse et al., 2010; Mathews et al., 2011). Second, collagen type I is routinely stored in an acidic solutions. When it is neutralised by β -GP, which is a weak base, collagen type I may reconstitute into its usual fibril morphology (Kadler et al., 1996; Sun and Ma, 2011). Although collagen is not implicated in the polymerisation of chitosan, it modifies the interspaces left by the gradual cross-linking of the chitosan molecules. These additional fibrils will enhance the surface area and provide ECM-mimetic biochemical signals that are favourable for cell attachment and growth. Third, the introduction of collagen could reduce the opportunities of contact between cells and the β -GP phase in the hydrogel, reducing its eventual cytotoxic effect.

The plasticity or potential of multi-lineage differentiation is a key property defining the stemness of cells and is critical for the use of stem cells in tissue engineering. An ideal support for the culture and delivery of SMSCs is expected not only to ensure cell viability, but also to maintain its plasticity. In a preliminary investigation to determine the effect of the C/GP/Co hydrogel on the plasticity of SMSC, the results of myogenic, osteogenic and adipogenic differentiation all suggested that the C/GP/Co surface provided a superior

environment to maintain the plasticity of SMSCs when compared to control surfaces.

In tissue engineering, different growth factors are incorporated into the cell supporting structures to promote growth or induce the differentiation of the seeded cells (Lee et al., 2011). To analyse the biocompatibility of the C/GP/Co scaffold objectively, we did not use growth or differentiation inducing factors in the hydrogel. SMSC viability, both *in vitro* and *in vivo*, indicates that they could be induced to differentiate after being loaded into the hydrogel. Although we did not evaluate the committed differentiation of SMSCs *in vivo*, preliminary results indicate that upon induction the cells should promote tissue formation. Further experiments investigating the differentiation of SMSCs in *in situ* forming hydrogels are currently under study. Recent studies have already reported the adipogenic and osteogenic differentiation of mesenchymal stem cells in a hydrogel of similar composition (Wang and Stegemann, 2010; Sun and Ma, 2011).

Conclusions

Injectable thermosensitive C/GP/Co hydrogel is cytocompatible and maintains the plasticity of SMSCs. This novel composite hydrogel combined with SMSCs may serve as a

promising device for tissue engineering and regenerative medicine strategies.

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