Biomedical Materials

**PAPER**

A thermoresponsive polydiolcitrate-gelatin scaffold and delivery system mediates effective bone formation from BMP9-transduced mesenchymal stem cells

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Abstract

Successful bone tissue engineering requires at the minimum sufficient osteoblast progenitors, efficient osteoinductive factors, and biocompatible scaffolding materials. We previously demonstrated that bone morphogenetic protein 9 (BMP9) is one of the most potent factors in inducing osteogenic differentiation of mesenchymal stem cells (MSCs). Here, we investigated the potential use of a biodegradable citrate-based thermosensitive macromolecule, poly(polyethyleneglycol citrate-co-N-isopropylacrylamide) (PPCN) mixed with gelatin (PPCNG) as a scaffold for the delivery of BMP9-stimulated MSCs to promote localized bone formation. The addition of gelatin to PPCN effectively enhanced the cell adhesion and survival properties of MSCs entrapped within the gel in 3D culture. Using the BMP9-transduced MSC line immortalized mouse embryonic fibroblasts (iMEFs), we found that PPCNG facilitated BMP9-induced osteogenic differentiation of iMEFs in vivo and promoted the formation of well-ossified and vascularized trabecular bone-like structures in a mouse model of ectopic bone formation. Histologic evaluation revealed that vascularization of the bony masses retrieved from the iMEFs + PPCNG group was significantly more pronounced than that of the direct cell injection group. Accordingly, vascular endothelial growth factor (VEGF) expression was shown to be significantly higher in the bony masses recovered from the iMEFs + PPCNG group. Taken together, our results suggest that PPCNG may serve as a novel biodegradable and injectable scaffold and carrier for gene and cell-based bone tissue engineering.
1. Introduction

Bone tissue engineering holds great promise as an effective approach to skeletal reconstruction [1–6]. Successful bone regeneration requires at least three essential components: osteogenic progenitor cells that can undergo effective osteogenic differentiation and produce extracellular matrix, biologically osteoinductive factors that are able to induce or enhance new bone ingrowth, and scaffolding materials that are easy to use, biocompatible and osteoconductive [4–6]. Mesenchymal stem cells (MSCs) are multipotent progenitors which can undergo self-renewal and differentiate into multiple lineages, including osteogenic, chondrogenic, and adipogenic lineages [7–12]. MSCs have been isolated from numerous tissues, and one of the major sources in adults is bone marrow stromal cells [3, 10–12].

Osteogenic differentiation from MSCs occurs via a sequential cascade that recapitulates most of the molecular events occurring during skeletal development [13]. While many signaling pathways, such as Wnt, insulin-like growth factors (IGFs), fibroblast growth factor (FGFs), and Notch, play important roles in regulating osteogenic differentiation [9, 14–22], bone morphogenetic proteins (BMPs) are considered as a group of the most potent osteoinductive factors [9, 23–25]. We have demonstrated that BMP9 is one of the most potent BMPs among the 14 types of BMPs in inducing osteogenic differentiation [2, 3, 26, 27]. BMP9 (also known as growth differentiation factor 2, or Gdf2) can effectively induce osteoblast differentiation by regulating several important downstream targets [28–32], as well as through cross-talk with other important signaling pathways [33–37]. We have recently found that BMP9 is resistant to Noggin inhibition, which may partially contribute to BMP9’s potent osteogenic activity [38]. Thus, it is conceivable that using BMP9-expressing progenitor cells may hold promise to promote bone regeneration in large bony defects and/or fracture non-union in clinical settings [1–3].

An ideal scaffold for cell-based bone tissue engineering should be able to deliver the cells easily and safely to the surgical intervention site; allow for the attachment of osteoblastic progenitor cells, provide a cell-friendly environment enabling progenitors to survive, propagate, and ultimately differentiate; enable the ingrowth of vascular tissue to ensure the survival of the transplanted cells; and undergo biodegradable process with a controllable rate of degradation into molecules that can be metabolized or excreted easily [39, 40]. Yang et al described a novel thermoresponsive and biodegradable polymer polyethylene glycol citrate-co-N-isopropylacrylamide (PPCN), which is synthesized by sequential polycondensation and radical polymerization of citric acid, polyethylene glycol, and poly-N-isopropylacrylamide [41]. The PPCN gel displays a hierarchical architecture of micropores that can potentially accommodate live cells and nanofibers that allow cell–material interactions at the nanoscale, similar to in vivo [41]. Furthermore, PPCN exhibits intrinsic antioxidant properties such as ability to scavenge free radicals, chelate metal ions, and inhibit lipid peroxidation that may protect cells from pro-inflammatory processes mediated by oxidative stress [41]. Finally, subcutaneous injection of PPCN in rats showed that the gel was resorbed over time and replaced by newly formed connective tissue without significant inflammation [41]. Although PPCN has beneficial properties for cell delivery, it lacks cell adhesion signals to promote cellular processes such as cell spreading and migration. Therefore, we hypothesized that an interpenetrating network created by adding gelatin to PPCN (PPCNG) will be a suitable material to deliver osteogenic progenitor cells to a site and locally form new bone.

In this study, we investigate whether an interpenetrating network of a citrate-based thermoresponsive macromolecule and gelatin is a suitable delivery vehicle for BMP9-stimulated MSC cells to improve bone formation. While PPCN alone is biocompatible but does not mix well with cells in vivo, we show that gelatin can effectively enhance the cell adhesion and survival properties of PPCN in 3D culture in vitro. Moreover, using the BMP9-transduced MSCs, we found that PPCNG facilitated BMP9-induced osteogenic differentiation of MSCs in vivo and promoted the formation of well-ossified and vascularized trabecular bone-like structures in a mouse model of ectopic bone formation.

2. Materials and methods

2.1. Cell culture and chemicals

Mouse melanoma line B16 was obtained from ATCC (American Type Culture Collection, Manassas, VA, USA). The immortalized mouse embryonic fibroblasts (iMEFs) were previously characterized [42, 43]. The 293pTP cells were the HEK-293 cells that were modified for efficient adenovirus packaging and amplification [44]. These cell lines were maintained in complete Dulbecco’s modified eagle medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin at 37 °C in 5% CO₂. Gelatin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Matrigel Growth Factor High Concentration Basement Membrane Matrix was purchased from Corning Inc. (Corning, NY, USA). Unless indicated otherwise, all chemicals were purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

2.2. Synthesis of PPCN

The polymer PPCN was synthesized by sequential polycondensation and radical polymerization of citric acid, poly (ethylene glycol) (PEG), and poly-N-isopropylacrylamide as described [41]. The chemical, biodegradable and thermoresponsive features were evaluated for each newly synthesized batch as reported [41]. Prior to mixing with cells, PPCN powder was...
dissolved in phosphate-buffered saline (PBS; 100 mg ml$^{-1}$ stock solution), sterilized by syringe filtration using 0.22 µm filters, and kept at either room temperature or 4 °C.

2.3. Construction and generation of recombinant adenoviruses AdBMP9, AdR-PBase, AdR-GLuc, AdGFP and AdGRFP

Recombinant adenoviruses expressing human BMP9, Gaussia luciferase (GLuc), green fluorescent protein (GFP) and red fluorescent protein (RFP) (AdGRFP, or AdGFP) were respectively constructed using the AdEasy system as described [26, 27, 45–47]. Briefly, the human BMP9, piggyBac transposase (PBase) or GLuc coding region was polymerase chain reaction (PCR) amplified and subcloned into an adenoviral shuttle vector, and used to generate and amplify recombinant adenovirus in 293pTP cells [44]. The shuttle vector, and used to generate and amplify (PCR) amplified and subcloned into an adenoviral or GLuc coding region was polymerase chain reaction (PCR) amplified and subcloned into an adenoviral vector, and used to generate and amplify recombinant adenovirus in 293pTP cells [44]. The resulting adenoviruses were designated as AdBMP9, AdR-PBase, and AdR-GLuc, which also express GFP or RFP, respectively [36, 38, 48–51]. An analogous adenovirus expressing GFP and/or RFP (AdGRFP, or AdGFP) was used as a control [33, 52, 53]. For all adenoviral infections, polybrene (4 µg ml$^{-1}$) was added to enhance infection efficiency as reported [54].

2.4. Establishment of stable firefly luciferase expression lines B16-FLuc and iMEF-FLuc

The stable expression lines were established using our recently modified piggyBac transposon system [43, 51, 55]. Briefly, exponentially growing B16 and iMEF cells were transfected with the piggyBac transposon vector pMPB-FLuc using Lipofectamine Reagent by following manufacturer’s instructions (Invitrogen). At the end of transfection, cells were infected with AdR-PBase. At 36 h after transfection/infection, the cells were subjected to blasticidin B selection. Stable cell pools were usually obtained after 5–7 d, and confirmed with high levels of firefly luciferase activity using the firefly Luciferase Assay kit (Promega, Madison, WI, USA).

2.5. PPCN-based 3D culture in vitro

Subconfluent iMEF cells were infected with AdGFP or AdR-GLuc (multiplicity of infection, or MOI = 50) for 24 h. The infected cells were collected and kept on ice. The cell pellets were resuspended in cold PPCN (1:1 dilution with PBS; PPCN final concentration at 50 mg ml$^{-1}$) or PPCN-gelatin (i.e. PPCNG, 1:1 dilution with 0.2% gelatin/PBS; PPCN final concentration at 50 mg ml$^{-1}$). The cell-polymer mixture was seeded into 12-well plates that were pre-incubated at 4 °C. Each seeding contained 40 µl of cell-polymer mixed with 1 ~ 5 × 10^5 cells. Then, the plates were incubated at 37 °C 5% CO2 for 20 min, followed by adding 2 ml of complete DMEM medium. In order to avoid the interference of the cells leaking into the 2D surface, the gels were transferred to new plates the next day. Fluorescence signals were examined under a fluorescence microscope at indicated time points. Each assay condition was done at least in triplicate.

Similar cell-polymer mix experiments were set up for testing adenovirus-mediated transgene delivery, except that the cells were not pre-infected. Rather, the adenoviral infection was carried out after gelation by adding adenoviral vector directly to the medium. Fluorescence signals were examined under a fluorescence microscope at indicated time points. Each assay condition was done at least in triplicate.

2.6. GLuc assay

Subconfluent iMEF cells were infected with AdR-GLuc (MOI = 50) for 24 h. The infected cells were collected, resuspended in cold PPCN or PPCNG, and seeded into 12-well plates as described above. At the indicated time points, 50 µl medium was removed from each well for GLuc assay using the Biolux Gaussia Luciferase Assay Kit (New England Biolabs) as described [37, 50, 54, 56, 57]. Each assay condition was done in triplicate.

2.7. Subcutaneous implantation of cell-containing PPCN scaffold

The use and care of animals were approved by the Institutional Animal Care and Use Committee at The University of Chicago (protocol #71 108). All experimental procedures were carried out in accordance with the approved guidelines. For the experiment to test the PPCN-cell mixing features, subconfluent B16-FLuc cells were collected and resuspended in 80 µl of PPCN only (40 µl PPCN + 40 µl PBS), PPCN-matrigel (40 µl PPCN + 40 µl matrigel), or cells only (80 µl PBS). The cell-polymer mixtures were kept on ice and injected subcutaneously into athymic nude mice (Envigo/ Harlan Research Laboratories, Indianapolis, IN, USA; n = 5, female, 5–6 week old; 2 × 10^6 cells per injection site).

Subcutaneous heterotopic bone formation was carried out as reported [27, 37, 46, 58]. To evaluate the mixing features of PPCN with cells, subconfluent iMEF-FLuc cells were infected with AdBMP9 or AdGRFP for 36 h, then collected and resuspended in 80 µl PPCN-matrigel mix (40 µl PPCN + 40 µl 0.2% gelatin), or cells only (80 µl PBS). The iMEFs + PPCN mix- ture was kept on ice and injected subcutaneously into athymic nude mice (Envigo/Harlan Research Laboratories; n = 5, female, 5–6 week old; 2 × 10^6 cells per injection site). The animals were maintained ad lib in the biosafety barrier facility.

2.8. Xenogen bioluminescence imaging

Xenogen bioluminescence imaging of small animals was carried out as described [53, 59–63]. Briefly, at the indicated time points, the athymic nude mice were anesthetized with isoflurane and were injected intraperitoneally with D-luciferin sodium salt (Gold Biotechnology Inc., St. Louis, MO, USA) at 100 mg kg$^{-1}$ in 0.1 ml PBS. The animals were subjected to imaging with Xenogen IVIS 200 system (Xenogen Corporation, Alameda, CA, USA). The images were obtained by superimposing the emitted light over the gray-scale
Data were expressed as mean ± SD. All of the quantitative experiments were performed in triplicate or repeated three times. Data were expressed as mean ± SD. Statistical significance between treatment groups and/or controls was determined by one-way analysis of variance and the Student’s t-test. A p-value less than 0.05 was considered statistically significant.

3. Results

3.1. Gelatin effectively enhances the cell adhesion and survival properties of PPCN in 3D culture in vitro

Here, we sought to explore the potential use of PPCN as an injectable thermoresponsive scaffold for MSC-based bone tissue engineering. We first tested if the PPCN polymer could mix with cells and support cell proliferation in vivo. Through a subcutaneous injection of firefly luciferase-tagged melanoma line B16-FLuc cells, we found that when directly mixed with PPCN, mixed with PPCN + matrigel, or directly injected subcutaneously the cells exhibited significant luciferase activity at 2 days after injection, while at 15 days after injection the luciferase activity decreased in the cells mixed PPCN only group, compared to cells mixed with PPCN + matrigel group and even the cells only group (figure 1(A)). Histologic evaluation of the retrieved injection sites indicates that, in the cells + PPCN group, the cells and PPCN polymer were not mixed well and relatively segregated from each other (figure 1(B), panel a), while the cells mixed rather well with the PPCN + matrigel carrier (figure 1(B), panel b), which was significantly different from the cellular masses formed in the cells only group (figure 1(B), panel c).

These in vivo results indicate that while biocompatible, the PPCN polymer may not allow efficient cell adhesion and hence fail to populate the cells uniformly, rendering PPCN alone less than ideal as a scaffold material.

While the above results suggest that matrigel may improve the cell adhesion feature of PPCN polymer, matrigel contains many growth factors and extracellular matrix proteins that may interfere with specific biofactor-stimulated cell-based tissue engineering [70, 71]. With the knowledge that gelatin is commonly used as a coalescing agent in several industries (e.g., foods, pharmaceuticals) [72, 73], as well as a natural biomaterial that can promote cell adhesion, migration and tissue engraftment [74–79], we sought to evaluate whether gelatin could be used to enhance the cell adhesion properties of PPCN. To easily monitor the cell proliferation and survival status in PPCN only or PPCN + gelatin (PPCN) based 3D culture, we labeled our previously characterized MSC line iMEFs with an AdGFP (figure 2(A), panel a). The infected iMEFs cells were shown to form hemisphere-like 3D gels in 37 °C culture medium (figure 2(A), panel b), which were firm enough to be transferred to new wells (figure 2(A), panel c). When GFP expression was examined, we found that while both PPCN and PPCNG groups had high levels of GFP expression at 4 days after plating, GFP expression significantly decreased in the PPCNG group, as compared with that in the PPCNG group at day 10 (figure 2(B), panels a versus b). These results suggest that gelatin may enhance cell adhesion and survival characteristics of PPCN-based 3D culture.

We previously demonstrated that adenovirus can effectively deliver transgenes into matrigel-based intestinal organoid culture [80]. Here, we sought to test if the adenoviral vector can mediate effective transgene delivery into the PPCN-based 3D culture. When iMEF cells were mixed with PPCN or PPCNG, and infected with AdGFP, GFP expression was readily detected in both PPCN or PPCNG based 3D culture at days 2 and 5 after infection, although the infected cells were more evenly distributed in PPCNG culture than that in PPCN only culture (figure 2(C), panels a versus b).
Based on the above results, we further evaluated whether the addition of gelatin can enhance the functional activity of the cells cultured within PPCN. In order to monitor cell proliferation, cell survival, as well as the transgene function, we took advantage of an adenovirus AdR-GLuc, which expresses GLuc, a secreted form of luciferase enzymes [50, 53, 54, 64]. The iMEF cells were effectively infected with AdR-GLuc and mixed with PPCN or PPCNG to form 3D culture (figure 3(A), panels a–c) and when quantitative GLuc activity assays were carried out at 2, 4, 5, and 7 d after 3D culture, we found that the GLuc activity was significantly higher in PPCNG culture than those in the PPCN’s cultures at all tested time points ($p < 0.01$) (figure 3(B)). Thus, these results indicate that that the addition of gelatin can significantly enhance the expression and functional activity of the transgene’s in PPCN-based 3D culture. The above in vitro results demonstrate that gelatin can significantly improve several important functional aspects of PPCN as a scaffolding biomaterial, including cell proliferation, cell survival, cell-scaffold interactions, and transgene expression in 3D culture.

Figure 1. PPCN polymer is biocompatible but does not allow efficient cell adhesion in vivo. (A) The B16-FLuc were mixed PPCN, PPCN + matrigel, or cells only, and injected subcutaneously into athymic nude mice. The animals were subjected to Xenogen IVIS 200 bioluminescence imaging at D2 and D15. Representative images are shown. (B) The animals were sacrificed at D15 of injection, and the injection sites were retrieved for H and E staining. Representative staining results from PPCN + B16-FLuc (a), PPCN + matrigel + B16-FLuc (b) and B16-FLuc cells only (c) are shown. B, B16-FLuc cells; M, matrigel; and P, PPCN polymer.
3.2. PPCNG scaffold facilitates BMP9-induced osteogenic differentiation of MSCs *in vivo*

We next evaluated whether PPCNG can be used as a scaffold material for effective progenitor cell-based bone tissue engineering *in vivo*. Subcutaneous ectopic ossification is a commonly utilized model for bone formation [32, 34–36, 40, 81, 82]. However, most studies including ours, are conducted by directly injecting large amounts of osteoblast progenitor cells subcutaneously without the use of scaffold materials [37, 38, 49, 65, 83, 84], which often leads to the incomplete ossification of injected cell masses. Here, we sought to determine whether PPCNG can serve as an effective cell delivery vehicle and scaffold for the formation of ectopic bone by BMP9-transduced iMEF cells. We previously demonstrated that BMP9 is one of the most potent BMPs
in promoting osteogenic differentiation of mesenchymal progenitors [3,24–27,46].

Using the firefly luciferase tagged-iMEF cells, we were able to monitor cell proliferation and mass formation simultaneously. We found that although the bioluminescence signal was readily detectable at 7 d after subcutaneous injection in all four groups, signal intensity decreased among BMP9-transduced iMEFs injections (both PPNCG and cells only groups) at 14 and 21 d after injection. However, the bioluminescence signal increased in intensity among iMEF cells transduced with the control virus AdGRFP (figure 4(A), a and c versus b and d). These results indicate that BMP9 was able to promote osteogenic differentiation and hence decrease cell proliferation of the iMEFs. Quantitatively, the iMEF cells transduced with AdGRFP exhibited significantly higher proliferative activity in PPCNG group than that in the direct iMEFs injection group within the first two weeks (figure 4(A), panel a), which is consistent with findings in vitro that demonstrated the superior cell proliferation and survival within the PPCNG scaffold. On the other hand, the iMEF cells transduced with AdBMP9 and mixed with PPCNG exhibited significantly lower bioluminescence signal than that of the directly injected BMP9-transduced iMEFs group (p < 0.001) (figure 4(B), panel b). Nonetheless, no significant bioluminescence signal was detected in all groups at 32 d after subcutaneous injection, while only BMP9 treatment groups exhibited retrievable masses at the injection sites (data not shown). These results are consistent with our previous reports, in which direct subcutaneous injection of BMP9-transduced iMEFs exhibits an early proliferation stage, followed by a stage of osteogenic differentiation [27,46], while iMEFs themselves rarely form any masses in subcutaneous injection after 4 weeks [42,43].

3.3. PPCN–Gelatin scaffold promotes the formation of more mature trabecular bone-like structures in a mouse model of ectopic bone formation

The tissue masses formed at the sites of subcutaneous injections were retrieved and subjected to μCT analysis. We found that there were no significant volumetric differences of the bony masses formed between the iMEFs + PPCNG and the directly injected cells groups (figure 5(A), panels a and b). However, BMP9-transduced iMEF cells in PPCNG scaffold formed a uniformly distributed network of trabecular bone-like structures, accounting for approximately 94.5% (average area per cross-section) of the bony mass (figure 5(B), panels a and b), while the directly injected BMP9-transduced iMEFs formed unevenly mineralized bony masses with more mature bone at the periphery of the mass (approximately 31.4% of average area per cross-section) and cartilage and undifferentiated/necrotic cells at the interior region of the masses (figure 5(B), panels c and d).

By using the trichrome staining, we found that BMP9-transduced iMEF cells formed relatively mature woven bone-like structures in PPCNG injection group (figure 6(A), panels a and b). However, most of the bone masses retrieved from the directly injected

Figure 3. Gelatin significantly enhances transgene expression in PPCN-based 3D culture in vitro. (A) The iMEFs were infected with AdR-GLuc for 24 h (a) and collected to mix well with PPCN or PPCNG on ice, and seeded into 12-well cell culture plates. The plates were then gently moved to 37 °C CO2 incubator for gelation (b). At 4 h, the gels were transferred to a new set of 12-well plates (c). (B) At the indicated time points, culture medium were taken for GLuc assay. Each assay was done in triplicate. Each experimental condition was set up in triplicate. ‘**’, p < 0.01 when the mean GLuc activities between PPCNG and PPCN groups were compared.
BMP9-transduced iMEFs group only exhibited a few mature bone structures (figure 6(A), panels c and d). Furthermore, histologic evaluations indicated that the bony masses retrieved from PPCNG group were more vascularized than that of the direct cell injection group. We subsequently examined the expression level of VEGF in the bone masses recovered from both groups and found a strong positive staining of VEGF in the bony masses from the PPCNG group (figure 6(B), panel a), while the direct cell injection group only displayed a few sparsely distributed positively stained cells (figure 6(B), panel b). Taken together, these in vivo results strongly suggest that PPCNG may provide a superior scaffolding environment for biological factors, such as BMP9, to stimulate the terminal osteogenic differentiation of MSCs in vivo.

4. Discussion

4.1. PPCNG can be used as a flexible and versatile scaffolding vehicle for stem cell-based tissue engineering and regenerative medicine

Effective bone tissue engineering requires a combination of potent bone formation stimulating factors, osteoblastic progenitors, and biocompatible scaffold materials. We previously demonstrated that BMP9 is the most potent osteogenic factor among the 14 types of human BMPs in inducing bone formation from MSCs [3, 24–27, 46]. Numerous efforts have been devoted to the development of scaffolding materials for bone tissue engineering [85]. Potential bone scaffold materials include inorganic ceramics (e.g. hydroxypatite, coralline-derived hydroxyapatite,
tricalcium phosphate, calcium sulphates, glass ceramics, calcium phosphate-based cements, and bioglass), metals, and synthetic biodegradable polymer composites [39, 86, 87]. Furthermore, there is a growing interest in developing artificial bone-mimetic nanomaterials with controllable mineral content, nanostructure, chemistry for bone tissue engineering and substitutes [88, 89]. Nonetheless, there is a scarcity of biocompatible scaffold materials for progenitor cell-based gene therapies for the regeneration of bone.

In this study, we demonstrated that a PPCN-gelatin interpenetrating polymer network may be used as biocompatible scaffold and cell delivery vehicle for cell-based bone tissue engineering. PPCN is a novel nanoscale polymer, and was shown to display a hierarchical architecture of micropores and nanofibers with a lower critical solution temperature of 26 °C and no significant volume reduction upon gelation [41]. We found that PPCN can gel robustly at 30 °C at a final concentration as low as 50 mg ml⁻¹. Our in vivo studies showed that a significant biodegradation of PPCN gel started approximately 3 weeks after subcutaneous injection while a complete resorption of PPCN gel usually occurred at 4–5 weeks after injection.

4.2. Gelatin promotes cell entrapment and possesses potential neoangiogenic activity

We have demonstrated that the addition of gelatin effectively enhanced cell entrapment in PPCN and promoted the formation of mature and vascularized bony masses from BMP9-transduced MSCs. Gelatin is a commonly used natural biopolymer in tissue engineering [74–79], and has several exceptional advantages, such as commercially available at low cost and readily available, biodegradability and biocompatibility, low antigenicity, no harmful byproducts upon enzymatic degradation, containing motifs such as arginine-glycine-aspartic acid sequences that can mediate cell adhesion, and a large number of accessible functional groups for modification (e.g. crosslinking) and targeting ligands (e.g. drug delivery vehicles) [72, 90]. Gelatin-containing hydrogel has been recently shown to enhance the engraftment of transplanted cardiomyocytes and angiogenesis to

Figure 5. PPCNG scaffold facilitates the formation of woven and trabecular bone-like structure for BMP9-induced heterotopic bone formation. (A) The injection sites were retrieved at 32 d after implantation. No bony masses were retrieved from AdGRFP-transduced iMEFs either in PPCNG or cells only group (data not shown). Representative images of the 3D reconstruction of the retrieved masses are shown (a). The 3D volumetric data were quantitatively analyzed using Amira 5.3 software (b). (B) After μCT imaging, the retrieved masses from PPCNG (a and b) and iMEF cells only (c and d) groups were decalcified and subjected to paraffin-embedding, sectioning and H and E staining. TB, trabecular-like bone; MB, mature bone; Ca, cartilage. Representative images are shown.
ameliorate cardiac function after myocardial infarction [74]. It was reported that human cardiospheres derived from hyaluronan-gelatin hydrogel or gelatin/collagen scaffolds can be used in cardiogenic engineered tissues [77, 78].

In our study, high vascularization was observed in the bony masses formed in PPCNG, suggesting that the addition of gelatin may not only enhance cell engraftment but also promote neovascularization. This observation is supported by several other recent studies. The implantation of human blood-derived endothelial colony-forming cells and white adipose tissue-derived MSC-laden gelatin-phenolic hydroxyl hydrogels into immunodeficient mice led to the rapid formation of functional anastomoses between the bioengineered human vascular network and the mouse vasculature [91]. Heparin-modified gelatin hydrogel was also shown to have the capability to induce angiogenesis to a certain degree [92]. Furthermore, a porous gelatin-alginate scaffold was shown to exhibit excellent biocompatibility with neovascularization on the surfaces in vitro [93]. Interestingly, although MSCs were able to attach and proliferate in the porous gelatin-alginate scaffold similarly during both in vitro culture and in vivo implantation, the subcutaneous microenvironment was found to suppress MSC differentiation along the osteogenic lineage compared to in vitro conditions [93], highlighting the importance of using potent osteogenic factors, such as BMP9 reported in our study, to ensure effective bone tissue engineering in vivo.

Based on our in vitro and in vivo results, we believe that PPCN possesses several unique and advantageous features for bone tissue engineering. First, PPCN is biocompatible and highly suitable for stem cell-based tissue engineering as cells can proliferate and survive well in the polymer. Second, the thermo-responsive feature of PPCN allows easy manipulations of stem cell-based tissue regeneration as the manipulated/engineered cells...
can be easily mixed with PPCN at lower temperature and entrapped within the PPCN during gelation at body temperature without significant shrinkage of the resulting scaffold. Third, the injectable feature of PPCN enables one to deliver prefabricated biofactor-progenitor cells-scaffolds directly to repair sites with ease, and to contour the gelled material regardless of the complexity of the 3D shape of repair sites. Of note, unlike other thermosensitive injectable hydrogels such as PNIPAAm [94], PPCN gel retains its original volume upon gelation. Fourth, PPCN gel is resorbed in vivo within the period of time (e.g. 4–5 weeks) that should be sufficient for most cell-based tissue engineering and regeneration endeavors. Nonetheless, PPCN can be further modified to suit a particular application if necessary. Our results indicate that the addition of gelatin not only significantly enhances the cell proliferation, cell survival and cell distribution properties of the PPCN, but also provides an angiogenic component to the scaffold, potentially optimizing cell survival and differentiation into the desired tissue type. This suggests that PPCN may be further optimized as a novel, biodegradable and injectable biomaterial for tissue engineering and regenerative medicine.

5. Conclusions

In this study, we investigated the potential use of a biodegradable citrate-based thermosensitive macromolecule PPCN mixed with gelatin (PPCNG) as a scaffold and delivery vehicle for BMP 9-stimulated MSCs to promote ectopic bone formation. We found the addition of gelatin to PPCN effectively enhanced the cell adhesion and survival properties of cells entrapped within the PPCN gel in vitro. PPCNG facilitated BMP9-induced osteogenic differentiation of MSCs in vitro and promoted the formation of well-ossified masses recovered from the iMEFs within the PPCNG gel in vivo and promoted the formation of well-ossified and vascularized trabecular bone-like structures in a murine model of ectopic bone formation. Histologic evaluation revealed that vascularization of the bony masses recovered from the iMEFs + PPCNG group was significantly more pronounced than that of the direct iMEFs injection group. Accordingly, VEGF expression was shown significantly higher in the bony masses recovered from the iMEFs + PPCNG group. Thus, our results strongly suggest that the PPCN-gelatin interpenetrating network may serve as a novel biodegradable and injectable scaffold for gene/cell-based bone tissue engineering.

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