Published in final edited form as:

Biotechnol Lett. 2014 September ; 36(9): 1901–1907. doi:10.1007/s10529-014-1551-0.

BMP2 cross-linked by transglutaminase 2 to collagen-plla scaffold promotes osteogenic differentiation in mesenchymal stem cells

Kelly E. Beazley and Maria Nurminskaya

Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD USA

Abstract

Transglutaminase 2 (TG2) was used to attach biologically-active BMP2 to collagen type I-coated poly-L-lactic acid (PLLA) nanofibrous scaffolds. Irreversibly cross-linked BMP2 retained its activity and induced Smad-dependent gene expression in cells seeded on PLLA-BMP2 scaffolds. These modified scaffolds promote osteogenic differentiation of human bone marrow-derived MSCs (hBMSCs) cultured in low-serum and growth factor-free medium and support deposition of the calcified matrix and induction of the molecular osteogenic markers Runx2, osteopontin, osteonectin and bone sialoprotein. Importantly, the PLLA-BMP2 scaffolds did not support chondrogenic differentiation in hBMSCs as there was no expression of chondrogenic markers aggrecan, Sox 9, and collagen type II, and no deposition of cartilaginous glycosaminoglycan-rich matrix. Thus, TG2-mediated cross-linking of BMP2 to a scaffold is a novel approach to induce osteoblast-specific programming of hBMSCs in a spatially controlled manner.

Keywords

Bone morphogenetic protein 2; Bone regeneration; Mesenchymal stem cells; Osteogenesis; Poly-L-lactic acid; Protein therapy; Transglutaminase 2

Introduction

Protein therapy using osteogenic factors is a promising technique for bone regeneration and implant placement. Bone morphogenetic proteins (BMPs) are probably the most important growth factors in bone formation and healing that cause mesenchymal stem cells to infiltrate into the fracture zone and to differentiate into bone- and cartilage-forming cells. Although at least 20 BMPs have been identified and studied, only BMPs 2, 4 and 7 stimulate in vitro the entire process of stem cell differentiation into mature osteoblastic cells (De Biase and Capanna, 2005). The addition of BMP2 to PLLA scaffolds enhances osteoprogenitor cell differentiation (Ciapetti et al. 2012); however, methods for retaining BMP2 on PLLA

Maria Nurminskaya (corresponding author) mnurminskaya@som.umaryland.edu Telephone: 410-706-7469 Fax: 410-706-8297.
Kelly E. Beazley kbeazley@som.umaryland.edu

Supporting information

Supplementary Table 1 – Primer sequences for human genes analyzed by real-time PCR.

scaffolds are scarce. Current research is focused on approaches to prolong local BMP effects by reducing their diffusion from the carriers and optimization of the latter (Zhang et al. 2012, Kisiel et al. 2013). Matrices mimicking the architecture of collagen type I fibers, such as electrospun nanofibrous poly-L-lactic Acid (PLLA) scaffolds, enhance osteogenic differentiation in stem cells (Wang et al. 2010) and are commonly used to mimic skeletal tissues (Prabhakaran et al. 2009; Nguyen et al. 2011).

Previous studies from our group and others have revealed the efficiency of transglutaminase 2 (TG2) in the covalent addition of growth factors to scaffolds. We have shown that TGF β 3 irreversibly cross-linked to collagen-coated nanofibrous PLLA scaffolds by TG2 preserved the biological activity, and modified PLLA-TGF β 3 scaffolds supported chondrogenic differentiation in human bone marrow-derived mesenchymal stem cells (hBMSCs) (Niger et al. 2013). Similarly, TG2-mediated cross-linking of the biologically active fragment of human parathyroid hormone [PTH(1-34)] emerges as an effective approach to functionalize fibrin matrices for improved bone formation in comparison to un-modified fibrin (Arrighi et al. 2009). In this study, we determined the pro-osteogenic potential of nanofibrous PLLA scaffolds modified with covalent cross-linking of BMP2. We show that BMP2 cross-linked to collagen-coated scaffolds by TG2 retains its biological activity and induces osteogenic differentiation and calcium deposition by hBMSCs. This proof-of-principal study identifies a new application for TG2 in tissue engineering to fabricate precast scaffolds that can drive local osteogenic differentiation in hBMSCs in vivo.

Materials and methods

PLLA scaffold preparation

Biodegradable nanofibrous scaffolds were prepared by electrospinning of poly-L-lactic acid (PLLA) (Sigma) in a 12% (w/v) hexafluoroisopropanol (HFIP) (Sigma) as described in our previous studies (Shanmugasundaram et al. 2012). In brief, electrospinning was performed at room temperature at 3 ml/h at 20 kV. The average fiber diam. was $\sim 0.3 \mu\text{m}$ and the nanofibrous scaffolds were approx. 150 μm thick. Prior to use, PLLA scaffolds were cut into disks, 6 mm diam. for 96-well plates and 11 mm diam. for 24-well plates. Scaffolds were washed in 70% (v/v) ethanol, then in PBS, and coated overnight with collagen type I (Chondrex, Inc, WA) at 0.1 mg/cm² according to Shanmugasundaram et al. (2012). To cross-link BMP2 to collagen-coated PLLA nanofibers, 0.01 U/ml purified liver TG2 enzyme (Sigma-Aldrich) was incubated with scaffolds and BMP2 (ProSpec, NJ) for 16 h. Cross-linked scaffolds were rinsed in PBS to remove unbound BMP2 and TG2 and then used for cell cultures.

BMSC Expansion and Culture

Human bone marrow-derived mesenchymal stem cells (hBMSCs) (Lonza Walkersville Inc., MD) were expanded in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen, CA) at 37°C, 5% CO₂. Cells were used on passage 2-3.

BMSC

Differentiation

For the induction of hBMSC osteogenesis, cells were seeded at $7.5 \times 10^4/\text{cm}^2$ and maintained in 0.1% FBS DMEM with high glucose and supplemented with 0.1% fetal bovine serum, 10^{-7} mM dexamethasone, 0.1 mM ascorbic acid, 5 mM β -glycerophosphate, 1% ITS premix (BD Biosciences, NJ), 1 mM sodium pyruvate, 0.35 mM L-proline, 4 mM L-glutamine and 1% penicillin/streptomycin. Purified BMP2 (ProSpec, NJ) was either added to the medium at 5-50 ng/ml or cross-linked to the scaffolds by TG2 as described above. Medium was changed twice weekly. Quantitative analysis of the deposited calcium was performed by *o*-cresthophthalein method following an overnight extraction in 0.1 M HCl. Cell numbers were estimated with Crystal Violet staining (Sigma-Aldrich).

RNA isolation and real-time RT-PCR analysis were performed as described previously (Beazley et al. 2013) using the primers in Supplementary Table 1.

Luciferase assay

A10 rat VSMC lines containing a SMAD-dependent luciferase reporter sensitive to TGF β /BMP activation or a control “promoterless” reporter were established by stable transfection with Cignal Reporter lentiviruses (Invitrogen). Cell lysates were prepared using Promega reagents (passive cell lysis buffer) and freezing at -20°C . Luciferase activity was measured in lysates using a luciferase assay system (Promega), and normalized to total LDH activity in the lysate determined with BioVision Cytotoxicity Assay reagents.

Data Analysis and Statistics

Western blots were analyzed by densitometry using Scion Image (Scion Corp). Real-time PCR and luciferase activity data were analyzed using Excel (Microsoft Corp). Data are expressed as means $\pm$ S.E. from at least three independent experiments ($n = 3$). Student's *t* test was used for comparison between two groups. For more than two groups, mean values were compared using one-way analysis of variance with comparison between groups by Tukey's honest significant difference test. A value of $p < 0.05$ was considered statistically significant.

Results and discussion

BMP2 cross-linked to scaffold by TG2 retains its biological activity

Crosslinking of BMP2 to collagen type I-coated PLLA scaffolds was performed by an overnight incubation of the scaffolds with 50 ng BMP2/ml and 0.01 U TG2/ml. To assay for self-absorption of BMP2 to the scaffolds, a control reaction included only BMP2 but no TG2. We employed an ELISA assay to monitor depletion of BMP2 from the incubation medium that would occur if BMP2 was cross-linked to the scaffold. In the control reaction lacking TG2, BMP2 is readily detected in the medium collected after an overnight incubation with scaffold at levels similar to a control aliquot of BMP2 that was not exposed to a scaffold (50 ng soluble BMP2/ml) (Fig. 1A, compare dark grey to hatched bar). This result shows that BMP2 does not self-absorb onto the collagen-coated PLLA scaffolds. In

contrast, in the presence of TG2, almost all BMP2 protein is cross-linked the scaffold, and is barely detected in the medium by ELISA after the overnight reaction (Fig. 1A)

TG2-mediated cross-linking of certain proteins attenuates their activity (Cooper et al. 1997). Therefore, we examined whether BMP2 retains its biological activity after cross-linking to collagen type I-coated PLLA nanofibers. For this, A10 cells stably infected with a SMAD-dependent luciferase reporter (A10-Smad cells) were incubated for 48 h on the modified scaffolds (Fig. 1B, light grey bars). In cells cultured on control scaffolds pre-incubated overnight with either BMP2 or TG2 alone, we did not detect SMAD-dependent luciferase activity. In contrast, scaffolds modified with BMP2 by TG2-mediated cross-linking induced SMAD-dependent gene expression. Activity of the luciferase reporter in these cells was similar to that induced by soluble 50 ng BMP2/ml retained in the medium from scaffolds treated with BMP2 alone in the absence of TG2 (Fig. 1 B, dark grey bars). Next, we analyzed the dose-dependent response of A10-Smad cells to cross-linked versus soluble BMP2, and demonstrate a good correlation between the activities of the immobilized and soluble BMP2 in the range of 5 to 50 ng/ml (Fig. 1C).

Together, these results confirm the biological activity of BMP2 cross-linked to collagen type I-coated PLLA nanofibrous scaffolds up to 150 ng/cm² and present a new method for local delivery of immobilized active BMP2.

Stability of cross-linked BMP2

Stability of the modified scaffolds is an important factor to consider in tissue engineering, and therefore we tested whether the biological activity of BMP2 in the TG2 cross-linked scaffolds was maintained over time. First, we analyzed whether BMP2 was covalently and irreversibly cross-linked to the scaffolds by measuring BMP release into the medium during 7 days incubation of the cross-linked scaffolds. Medium collected at days 1, 3, 5 and 7 was applied to A10-Smad cells and luciferase activity was measured 48 h later. Medium collected from non-modified control scaffolds not exposed to either BMP2 or TG2 was used to set the baseline level of luciferase activity in the reporter cells (Fig. 2A, black bars). In agreement with minimal self-absorbance of BMP2 into the scaffolds, medium from the scaffolds incubated with BMP2 in the absence of TG2 contained high levels of active morphogen on day 1 after treatment (Fig. 2A, light grey bars). Importantly, incubation of the scaffolds with a combination of BMP2 and TG2 completely removed active morphogen from the medium on the first day after treatment indicating that all BMP2 protein was cross-linked to the scaffold. This cross-linking is covalent and irreversible as no BMP2 was released into the medium over 7 days (Fig. 2A, dark grey bars). To determine whether cross-linked BMP2 retained its activity after 7 days, scaffolds were seeded with A10-Smad cells after the last collection of medium and activity of luciferase was analyzed 48 h later (Fig. 2B). This analysis revealed that BMP2 cross-linked to PLLA scaffolds induced expression of the reporter transgene as effectively as did a fresh aliquote of 50 ng soluble BMP2/ml demonstrating that activity of BMP2 is maintained for at least 7 days after the cross-linking reaction.

BMP2 cross-linked by TG2 promotes osteogenic differentiation of hBMSCs

Next, we analyzed whether immobilized BMP2 promotes osteogenic differentiation in hBMSCs. Primary hBMSCs (P2-4) were cultured on 3D collagen-coated PLLA nanofibrous scaffolds in the presence of either cross-linked or soluble BMP2 for 12 days. Deposition of calcified matrix was analyzed as a marker of osteoblast maturation. Cells seeded on non-modified scaffolds served as negative controls. Only scaffolds modified with cross-linked BMP2 supported calcium accrual in hBMSCs to levels similar to that induced by soluble BMP2 freshly added every other day to culture medium (3.5 ± 0.3 -fold increase compared to 4.3 ± 0.2 increase in calcium accumulation) (Fig. 3A). Of note, induction of calcium accumulation in hBMSCs was specific for BMP2 as TGF β 3, another protein of the TGF-beta superfamily, was insufficient to induce deposition of the calcified matrix (Fig. 3A, light grey bar).

To confirm osteogenic differentiation at the molecular level, we analyzed expression of the markers of stem cells and osteogenesis by real-time RT-PCR in hBMSCs on either noncrosslinked PLLA or PLLA-BMP2 scaffolds. Stem cell markers were down-regulated (Fig. 3B) while osteogenic markers were induced (Fig. 3C) including a significant, greater than 10-fold increase of Runx2 and osteopontin gene expression, an 8-fold increase of osteocalcin expression and a 2-fold increase in BSP expression. These results demonstrate that cross-linked BMP2 retains its pro-osteogenic activity.

Cross-linked BMP2 does not induce chondrogenic differentiation in hBMSCs

BMP2 induces chondrogenic differentiation in mesenchymal cells in vitro (Denker). To determine whether cross-linked BMP2 stimulates dual osteochondrogenic differentiation in our scaffold culture, we analyzed the deposition of glycosaminoglycan-rich (GAG) cartilaginous matrix with Alcian Blue dye (Fig. 4A, black bars) and expression of the early chondrogenic markers by RT-PCR (Fig. 4B) on BMP2-crosslinked PLLA scaffolds (PLLA-BMP2) compared to unmodified scaffolds. These analyses did not detect any sign of chondrogenic differentiation on the PLLA-BMP2 scaffolds that did support the deposition of calcified matrix (Fig. 4A, grey bars).

Conclusions

Several clinical trials have proven that effects of BMPs are more potent and specific than that of other members of the TGF-beta superfamily. Here, we demonstrate the specific action of cross-linked BMP2 as an osteo-inductive factor on collagen-coated PLLA scaffolds. The nanofibrous structure of the scaffolds may contribute to restricted cell lineage differentiation towards an osteoblastic phenotype in hBMSCs despite the demonstrated ability of soluble BMPs to stimulate both chondrogenic and osteogenic differentiation in vitro.

Efficient bone repair in higher mammals may require a prolonged period of time for BMP-stimulated osteogenic differentiation of the local and circulating progenitor cells (Gautschi et al. 2007). Current methods of delivering these morphogens as loosely-incorporated into matrices during synthesis by absorption or in lipid-based vesicles do not retain the

instructive proteins in place for appreciable time due to diffusion and new methods to retain active morphogens at the site of implantation are clinically needed. A new approach to immobilize biologically active BMP2 on PLLA scaffolds is described that supports a rapid translation of our findings into clinical models as PLLA nanofibrous scaffolds are resorbable, FDA-approved, mechanically suitable for generating bone scaffolds, and their degradation product (lactic acid) is nonimmunogenic. In addition, we envision that TG2-mediated cross-linking of BMPs will be effective on other scaffolds as well. Further, we recently showed that TG2 can be also employed to modify PLLA scaffolds with biologically active TGF β 3 which efficiently directs chondrogenic differentiation of hBMSCs (Niger et al. 2013), suggesting that TG2 can be used to generate more complex biphasic osteochondral scaffolds capable of delivering multiple biologically active growth factors in a spatially-regulated manner to direct hBMSCs to multiple lineages within the same scaffold.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Ms. S. Das, Dr. Shanmugasundaram and Dr. Egtestad for technical help with PLLA scaffolds. This study was partially supported by NIH grants DK071920 and AR057126 (to M.N.) and T32AR007592 fellowship (to K.B.)

References

- Arrighi I, Mark S, Alvisi M, von RB, Hubbell JA, Schense JC. Bone healing induced by local delivery of an engineered parathyroid hormone prodrug. *Biomaterials*. 2009; 30:1763–1771. [PubMed: 19124152]
- Beazley KE, Lima F, Borrás T, Nurminskaya M. Attenuation of Chondrogenic Transformation in Vascular Smooth Muscle by Dietary Quercetin in the MGP-Deficient Mouse Model. *PLoS One*. 2013; 8:e76210. [PubMed: 24098781]
- Ciapetti G, Granchi D, Devescovi V, Baglio SR, Leonardi E, Martini D, Jurado MJ, Olalde B, Armentano I, Kenny JM, Walboomers FX, Alava JI, Baldini N. Enhancing osteoconduction of PLLA-based nanocomposite scaffolds for bone regeneration using different biomimetic signals to MSCs. *Int J Mol Sci*. 2012; 13:2439–2458. [PubMed: 22408463]
- Cooper AJ, Sheu KR, Burke JR, Onodera O, Strittmatter WJ, Roses AD, Blass JP. Transglutaminase-catalyzed inactivation of glyceraldehyde 3-phosphate dehydrogenase and alpha-ketoglutarate dehydrogenase complex by polyglutamine domains of pathological length. *Proc Natl Acad Sci U S A*. 1997; 94:12604–12609. [PubMed: 9356496]
- De Biase P, Capanna R. Clinical applications of BMPs. *Injury* 36 Suppl. 2005; 3:S43–6. S43–S46.
- Gautschi OP, Frey SP, Zellweger R. Bone morphogenetic proteins in clinical applications. *ANZ J Surg*. 2007; 77:626–631. [PubMed: 17635273]
- Kisiel M, Martino MM, Ventura M, Hubbell JA, Hilborn J, Ossipov DA. Improving the osteogenic potential of BMP-2 with hyaluronic acid hydrogel modified with integrin-specific fibronectin fragment. *Biomaterials*. 2013; 34:704–712. [PubMed: 23103154]
- Nguyen LT, Liao S, Chan CK, Ramakrishna S. Electrospun Poly(L-Lactic Acid) Nanofibres Loaded with Dexamethasone to Induce Osteogenic Differentiation of Human Mesenchymal Stem Cells. *J Biomater Sci Polym Ed*. 2011
- Niger C, Beazley KE, Nurminskaya M. Induction of chondrogenic differentiation in mesenchymal stem cells by TGF-beta cross-linked to collagen-PLLA [poly(L-lactic acid)] scaffold by transglutaminase 2. *Biotechnol Lett*. 2013; 35:2193–2199. [PubMed: 23892982]

- Prabhakaran MP, Venugopal J, Ramakrishna S. Electrospun nanostructured scaffolds for bone tissue engineering. *Acta Biomater.* 2009; 5:2884–2893. [PubMed: 19447211]
- Shanmugasundaram S, Logan-Mauney S, Burgos K, Nurminskaya M. Tissue transglutaminase regulates chondrogenesis in mesenchymal stem cells on collagen type XI matrices. *Amino Acids.* 2012; 42:1045–1053. [PubMed: 21830118]
- Wang J, Liu X, Jin X, Ma H, Hu J, Ni L, Ma PX. The odontogenic differentiation of human dental pulp stem cells on nanofibrous poly(L-lactic acid) scaffolds in vitro and in vivo. *Acta Biomater.* 2010; 6:3856–3863. [PubMed: 20406702]
- Zhang Q, He QF, Zhang TH, Yu XL, Liu Q, Deng FL. Improvement in the delivery system of bone morphogenetic protein-2: a new approach to promote bone formation. *Biomed Mater.* 2012; 7:045002–6041/7. [PubMed: 22556155]

Figure 1. BMP2 cross-linked to collagen-coated PLLA nanofiber scaffolds retains its biological activity

A – ELISA for BMP2 protein present in medium after overnight incubation on scaffolds.

The bar graph presents quantitative analysis of replicate experiments (n=3; *, p<0.05). Soluble BMP2 (50 ng/ml) served as a positive control.

B – expression of SMAD-dependent luciferase reporter in A10 cells cultured for 48 h on scaffolds pre-treated for 16 h with TG2 (0.01 U/ml), BMP2 (50 ng/ml) or their combination to allow for BMP2 cross-linking to the scaffolds (dark grey bars) and in cells cultured for 48 h in residual medium removed from scaffolds after pre-treatment (light grey bars). (n=4, *, p<0.05).

C – dose-dependent activation of SMAD-dependent luciferase expression in A10 cells by both soluble and cross-linked BMP2; n=3.

Figure 2. Irreversible TG2-mediated cross-linking of active BMP2 to scaffolds

A – expression of SMAD-dependent luciferase reporter in A10 cells treated with medium conditioned on scaffolds that were pre-treated for 16 h with TG2 (0.01 U/ml), BMP2 (50 ng/ml) or TG2+BMP2 combination. **B** – PLLA-BMP2 modified scaffolds incubated in serum-free medium for 7 days were seeded with A10-Smad reporter cells and luciferase expression was measured 48 h later. Cells treated for 48 h with soluble BMP2 (50 ng/ml) served as positive control. (n=4, *, p<0.05; **, p<0.01; ***, p<0.001).

Figure 3. Induction of osteogenic differentiation in hBMSCs by cross-linked BMP2

A – deposition of calcified matrix in hBMSCs cultured on modified collagen-coated PLLA scaffolds. Prior to cell seeding, scaffolds were incubated overnight with TG2 (0.01 U/ml), BMP2 (50 ng/ml) or their combination to allow for BMP2 cross-linking to the scaffolds. Cell-seeded scaffolds treated with soluble BMP2 or TGF β 3 served as positive and negative controls, respectively. Three independent experiments in duplicate were performed (***, $p < 0.001$). **B,C** – real-time PCR analysis for expression of stem cell markers (**B**) and osteogenic genes (**C**) in hBMSCs cultured on BMP2-modified PLLA scaffolds compared to unmodified scaffolds. ($n=3$; *, $p < 0.05$; ***, $p < 0.001$).

Figure 4. Cross-linked BMP2 does not induce chondrogenic differentiation in hBMSCs

A – deposition of glycosaminoglycan (GAG)-rich and calcified matrix in hBMSCs cultured on modified collagen-coated PLLA scaffolds. Prior to cell seeding, scaffolds were incubated overnight with BMP2 (50 ng/ml) alone or in the presence of TG2 (0.01 U/ml to allow for BMP2 cross-linking to the scaffolds. (n=3; **, p<0.01). **B** – expression of chondrogenic gene markers in hBMSCs cultured on BMP2-modified PLLA scaffolds compared to unmodified scaffolds. (n=3; *, p<0.05; **, p<0.01).