frontiers in Materials

REVIEW
published: 15 July 2021
doi: 10.3389/fmats.2021.698915

Current Application of Beta-Tricalcium Phosphate in Bone Repair and Its Mechanism to Regulate Osteogenesis

Haiping Lu[1,2†], *Yinghong Zhou*[3†], *Yaping Ma*[1,2], *Lan Xiao*[3], *Wenjun Ji*[1], *Yi Zhang*[4,2*] and *Xin Wang*[1,3,2*†]

[1]Department of Orthopaedic Surgery, Affiliated Hospital of Zunyi Medical University, Zunyi, China, [2]Joint Orthopaedic Research Center of Zunyi Medical University & University of Rochester Medical Center (JCMR-ZMU & URMC), Zunyi Medical University, Zunyi, China, [3]Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, QLD, Australia, [4]Department of Hygiene Toxicology, School of Public Health, Zunyi Medical University, Zunyi, China

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***Correspondence:**
Yi Zhang
zhangyizmr@163.com
Xin Wang
lchwx@aliyun.com

[†]These authors have contributed equally to this work and share first authorship

Specialty section:
This article was submitted to Biomaterials, a section of the journal Frontiers in Materials

Received: 24 April 2021
Accepted: 05 July 2021
Published: 15 July 2021

Citation:
Lu H, Zhou Y, Ma Y, Xiao L, Ji W, Zhang Y and Wang X (2021) Current Application of Beta-Tricalcium Phosphate in Bone Repair and Its Mechanism to Regulate Osteogenesis. Front. Mater. 8:698915. doi: 10.3389/fmats.2021.698915

Large segmental bone loss and bone resection due to trauma and/or the presence of tumors and cysts often results in a delay in healing or non-union. Currently, the bone autograft is the most frequently used strategy to manage large bone loss. Nevertheless, autograft harvesting has limitations, namely sourcing of autograft material, the requirement of an invasive procedure, and susceptibility to infection. These disadvantages can result in complications and the development of a bone substitute materials offers a potential alternative to overcome these shortcomings. Among the biomaterials under consideration to date, beta-tricalcium phosphate (β-TCP) has emerged as a promising material for bone regeneration applications due to its osteoconductivity and osteoinductivity properties as well as its superior degradation *in vivo.* However, current evidence suggests the use β-TCP can in fact delay bone healing and mechanisms for this observation are yet to be comprehensively investigated. In this review, we introduce the broad application of β-TCP in tissue engineering and discuss the different approaches that β-TCP scaffolds are customized, including physical modification (e.g., pore size, porosity and roughness) and the incorporation of metal ions, other materials (e.g., bioactive glass) and stem cells (e.g., mesenchymal stem cells). 3D and 4D printed β-TCP-based scaffolds have also been reviewed. We subsequently discuss how β-TCP can regulate osteogenic processes to aid bone repair/healing, namely osteogenic differentiation of mesenchymal stem cells, formation of blood vessels, release of angiogenic growth factors, and blood clot formation. By way of this review, a deeper understanding of the basic mechanisms of β-TCP for bone repair will be achieved which will aid in the optimization of strategies to promote bone repair and regeneration.

Keywords: beta-tricalcium phosphate, bone fractures, early bone healing, fracture hematoma, osteogenesis

INTRODUCTION

Large bone loss as a result of trauma, tumor removal, infection, and developmental congenital disorders, often leads to delayed healing or non-union, and remains a critical challenge for orthopedic surgeons. Until recently, the standard clinical management to facilitate bone healing was the use of a bone autograft (Zheng et al., 2018). Still, the drawbacks of this technique, such as limited autogenous bone source and potential complications necessitates other bone substitutes for clinical use to be evaluated (Robering et al., 2020). The main challenge for large bone defect repair and regeneration remains the inadequate recruitment of mesenchymal stem cells (MSCs), reduced vascularization, and decreased growth factors stimulation within the scaffold construct to support cell viability and tissue growth. Consequently, enhancing the adhesion of MSCs, augmenting the release of growth factors, and promoting angiogenic potential of biomaterial scaffolds after implantation are pivotal for successful bone regeneration.

Beta-tricalcium phosphate (β-TCP) is a bioceramic material favored by orthopedic surgeons for bone repair due to its exceptional biocompatibility and bioactivity (Kang et al., 2020). Application of β-TCP alone can significantly increase bone regeneration in bone defect animal models compared with nanostructured carbon implants and porous titanium (Gilev et al., 2019) and studies suggest β-TCP can significantly enhance bone regeneration compared with bone autografts (Pereira et al., 2017). To create a favorable osteogenic environment, β-TCP scaffolds have been modified in a number of ways to boost bone healing, including modulating physical features (e.g., pore sizes, porosity and surface roughness), combining with ionic components, and the addition/delivery of growth factors.

However, conflicting results indicate the clinical use of β-TCP for bone repair remains questionable in several specific experiment models. For example, implantation of β-TCP into critical size circumferential defects of sheep ilium demonstrated significantly reduced new bone formation (1.1% ± 0.5) compared with control (11% ± 2.9), suggesting β-TCP may contribute to delayed healing (Choo et al., 2013). Furthermore, in a rat mandibular defect model, a delay in early bone healing compared to control was observed when β-TCP particles were applied locally (Wang et al., 2018a). One possible explanation for a β-TCP-mediated delay in bone healing could be the accelerated formation of fine fibers within blood clots as a consequence of Ca^{2+} and PO_4^{3-} ions released by β-TCP. Consequently, this may hinder early recruitment of MSCs for bone repair and regeneration.

These conflicting results surrounding β-TCP-mediated bone healing therefore warrant further investigation and evaluation of the clinical application of β-TCP for bone defect repair. It has been demonstrated that Ca^{2+} released from β-TCP plays a crucial role in the proliferation and differentiation of MSCs and osteoblasts (Lei et al., 2018). Since the release of Ca^{2+} is closely linked to the process of neovascularization in the fracture site, and blood clot structure at the fracture site is critical for bone healing, it is possible that Ca^{2+} released from β-TCP affects blood clot structure thus delaying bone regeneration. This review therefore focuses on the latest developments in β-TCP materials, the impact of its features on bone regeneration, and the role of β-TCP in blood clot formation and the initial stage of bone healing.

THE INFLUENCE OF PHYSICAL PROPERTIES OF BETA-TRICALCIUM PHOSPHATE ON OSTEOGENESIS

The physical characteristics of implanted biomaterials can affect the proliferation rate of osteoblasts and has important effects on cytokine gene expression (Barbeck et al., 2021). It has been established that the porous structure of calcium phosphate material can significantly influence bone regeneration (Xie et al., 2016; Bastami et al., 2017; Seong et al., 2020; Zamani et al., 2021). The porous surface of bone materials enhances the mechanical interlocking between the bone substitute and the surrounding bone tissues, providing mechanical stability to the bone-material interface (Meka et al., 2019; Roopavath et al., 2019; Cao et al., 2019; Dos Santos Trento et al., 2020). Two different range of pore sizes have generally been studied: micropore (<5 μm in diameter) and macropore (>100 μm in diameter) (Blokhuis et al., 2000). The presence of micropores increase in the surface area of biomaterials while micropore structures have been demonstrated to enhance material resorption and boost osteoinduction (Davison et al., 2014; Rustom et al., 2019). Open and interconnected macropores are not only useful for cell migration and blood vessel formation, but also essential for the diffusion of nutrients, waste and pro-osteogenic factors (Loh and Choong, 2013; Lim et al., 2020). Therefore, porosity not only allows biomaterials to absorb growth factors, but also controls the complex interaction between bone materials and pro-osteogenic factors (e.g., bone morphogenetic proteins). In a femur defect model of rabbit, the honeycomb β-TCP scaffolds with interconnected pore structure (ihTCP) presented higher new bone formation volume compared to the unidirectional pore structure (uhTCP) (Lu et al., 2020a). Studies have demonstrated that materials with high porosity have a positive impact on bone formation (Hannink and Arts, 2011; Raeisdasteh Hokmabad et al., 2017; Ishikawa et al., 2018). Similarly, porous β-TCP has the ability to augment the dissolution and absorption of the material and boost the infiltration of MSCs, thus enhancing new bone formation (Karageorgiou and Kaplan, 2005; Lopez-Heredia et al., 2012). An *in vitro* study showed that murine osteoblast precursors (MC3T3-E1) and human adipose stem cells (hADSCs) exhibited excellent proliferation and osteogenic differentiation when seeded into porous β-TCP blocks (Kim and Kim et al., 2019). Furthermore, when β-TCP blocks with 58.1 ± 1.7% porosity were implanted into rabbit distal femoral bone defects for 4 weeks, the amount of newly formed bone in porous β-TCP blocks group was approximately 200-fold higher compared with the dense β-TCP blocks group (with 10.9 ± 2.3 porosity) (Putri et al., 2020). In a study operating Opening-Wedge High Tibial Osteotomy in 25 patients, β-TCP blocks with 60% porosity showed a superior bone formation compared to the β-TCP blocks with 75% porosity (Tanaka et al., 2008). To sum up, the suitable porosity of β-TCP block to boost bone regeneration is approximately 60%.

Surface roughness is a factor that influences cellular attachment and subsequent bone repair (Albrektsson and

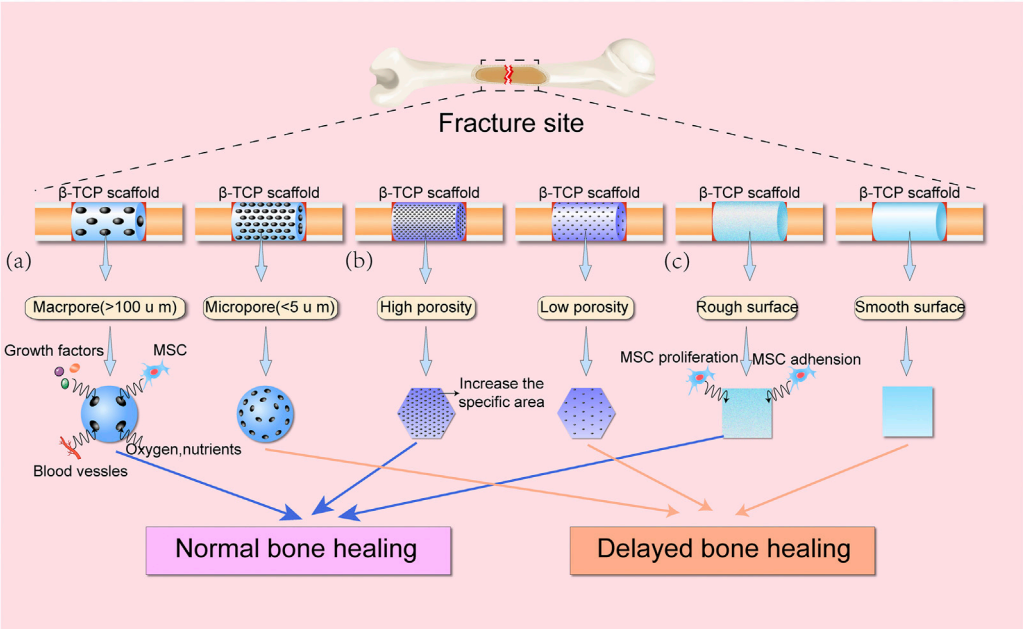

FIGURE 1 | Schematic diagram showing the effects of various physical features of β-TCP scaffolds on bone healing. **(A)** Macropores (>100 μm) provide greater space for access of growth factors, MSCs, blood vessels, oxygen, and nutrients into β-TCP scaffolds compared with micropores (<5 μm). **(B)** High porosity can significantly increase the specific areas of β-TCP scaffolds, as compared to β-TCP scaffolds with low porosity. **(C)** Rough surface of β-TCP scaffolds can improve MSC adhesion and proliferation, thereby promoting bone regeneration.

Johansson, 2001; Faia-Torres et al., 2015; Dantas et al., 2017; Dong et al., 2020). Materials with a high surface roughness can enhance the attachment of bone-forming cells, thereby promoting osteogenic differentiation and osteointegration, while low surface roughness may result in integration failure due to inferior cell attachment. It has been demonstrated that 8–10 arithmetical roughness average (Ra) calcium phosphate-coated titanium alloy showed a superior adhesion capacity of type I collagen compared with a 2-3 Ra calcium phosphate-coated surface (Ozerdem 2002). Additionally, using argon glow discharge plasma (GDP) to modify the surface of β-TCP can remove macro and micro particles of < 7 μm in size from β-TCP bigger particles surface thus promoting human mesenchymal stem cells (hMSCs) proliferation, osteoblastic differentiation, and increasing more new bone formation compared to the untreated β-TCP block (Choy et al., 2021). These results suggest that the porous structure and surface roughness of β-TCP-based materials can be optimized to enhance new bone formation. Furthermore, pores of different sizes can be adjusted to facilitate protein adsorption, cell infiltration and neovascularization (**Figure 1**).

METAL ION/ESSENTIAL ELEMENT - INCORPORATED BETA-TRICALCIUM PHOSPHATE COMPLEX

In addition to modulating the physical features of β-TCP, the incorporation of metal ions can improve the osteoinductive capacity of β-TCP scaffolds. The release of metal ions, such as cobalt (Co^{2+}), copper (Cu^{2+}), and magnesium (Mg^{2+}), and essential elements, such as zinc (Zn^{2+}) and silicon (Si^{4+}) can enhance the biological performance of bone regenerative scaffolds, which can induce angiogenesis and osteogenesis (Wang, et al. 2017a). Previous studies have demonstrated that metal ions can be easily incorporated into β-TCP (Matsunaga et al., 2015). Mg^{2+}, Zn^{2+} and other divalent cations smaller than Ca^{2+} can preferentially substitute the Ca (4) and Ca (5) cation sites in β-TCP. However, larger divalent cations such as strontium (Sr^{2+}) preferentially reside in sites Ca (1)-Ca (4), but not Ca (5) (Enderle et al., 2005; Ji et al., 2014). Mono- and trivalent cations tends to occupy Ca^{2+} on the partially occupied Ca (4) site. Si^{4+} is an alternative cation of P^{5+} on the tetrahedral sites in the PO_4 groups (Reid et al., 2005).

The addition of certain metal ions can lead to favorable effects on bone regeneration. For example, Co^{2+}-containing β-TCP composite scaffolds (CCP) can significantly induce the expression of osteogenic marker genes (*ALP, OCN* and *IBSP*) in human MSCs (hMSCs) compared with unmodified β-TCP scaffolds (Chen et al., 2015a). Similar findings have been demonstrated with the application of Cu^{2+}, which could enhance the osteogenic and angiogenic capacities of β-TCP powders (Zhang et al., 2019). This can be explained by the hypoxia-mimicking microenvironment induced by Co^{2+} and Cu^{2+}. The stabilization of hypoxia inducible factor 1-α (HIF-1α) can stimulate the endogenous release of angiogenic growth factors from the surrounding cells, which initiate the ingrowth of new blood vessels from host tissues, further facilitating

osteogenesis *via* the angiogenesis-osteogenesis coupling (Krock et al., 2011; Zhao et al., 2021).

Mg^{2+} is a known activator for phosphate-transferring enzymes (Gu et al., 2019) with Mg-doped bone substitute biomaterials able to regulate bone metabolism and facilitate bone regeneration by inducing a significant increase in osteogenic differentiation of MSCs (Wang and Yeung, 2017). An *in vitro* study demonstrated cell proliferation and viability of human bone marrow-derived MSCs (hBMSCs) and human umbilical vein endothelial cells (HUVECs) were significantly enhanced when treated with Mg-doped β-TCP scaffolds, compared with control β-TCP scaffolds (Gu et al., 2019). And Mg^{2+} in β-TCP/Mg-Zn composite scaffold was demonstrated to promote the differentiation of hBMSCs into osteoblasts *via* mitogen activated protein kinases (MAPKs)-regulated Runx2/Osterix (Osx) interaction (Wang et al., 2020a). However, in a murine dental alveolus grafting model, Mg-doped β-TCP granules scaffolds presented lower biosorption and less newly formed bone compared with the β-TCP group ($p < 0.05$) (Yassuda et al., 2013). The most plausible explanation for this is Mg^{2+} incorporation into β-TCP granules reduces resorption by downregulating parathormone (PTH) production *in vivo*, thereby inhibiting osteogenesis (Yassuda et al., 2013).

Zinc and silicon are two essential elements that have been widely studied to improve the osteoinductivity of β-TCP for osteogenesis. An *in vitro* study clearly demonstrated that both zinc oxide (ZnO)-doped β-TCP (Zn-TCP) and silicon oxide (SiO_2) doped β-TCP (Si-TCP) scaffolds could improve the expression of osteogenic marker genes, such as *BMP-2,* and *Runx2* compared with the unmodified β-TCP group (Fielding et al., 2019). Moreover, in a rabbit tibial defect model, a significant increase in new bone formation was observed in ZnO- and SiO_2-doped β-TCP scaffold groups compared with the β-TCP control group (Nandi et al., 2018). A plausible explanation for this effect is that Si^{4+} replaces P^{5+} in the β-TCP lattice, and Zn^{2+} replaces Ca^{2+}. The released Ca^{2+} ions can therefore enhance the expression of osteogenesis-related genes through the activation of intracellular calcium and Wnt signaling (Majidinia et al., 2018; Martineau et al., 2017).

The incorporation of 10 wt% manganese ion (Mn^{2+}) doped β-tricalcium phosphate (Mn-TCP) into calcium phosphate cement (CPC) has proper physicochemical properties and exhibits higher ability to promote osteogenic differentiation *in vitro* compared to the control group (0 wt% Mn-TCP-CPC), 20 wt% Mn-TCP-CPC group and 30 wt% Mn-TCP-CPC group (Wu et al., 2020). Nanosized-Ag-doped porous β-TCP scaffolds facilitate new bone formation and exhibits superior anti-infective properties as compared to pure β-TCP group, when tested in a rabbit femoral bone defect model (Yuan et al., 2020).

BETA-TRICALCIUM PHOSPHATE IN COMBINATION WITH OTHER MATERIALS

The degradation rate of β-TCP leaves insufficient time for osteoblast migration and colonization, impairing normal bone healing and limiting its clinical application (Pilliar et al., 2001). To address this problem, other materials have been combined with β-TCP to form composite scaffolds with superior biomechanical properties. In a recent study, β-TCP mixed with hydroxyapatite (HA) composite scaffolds (BCP) showed a remarkable increase in compressive strength (1.7 MPa) compared with normal β-TCP scaffolds (1.2 MPa) (Stastny et al., 2019). Moreover, the BCP scaffolds presented a steady and moderate degradation rate with a compressive strength of 0.5 MPa in the presence of an acidic buffer solution (pH = 5.5), which was demonstrated to be more beneficial for hMSCs adhesion, proliferation, and bone metabolism activity (Stastny et al., 2019). Furthermore, Cheng et al fabricated 25% collagen-15% thermosensitive hydrogel-60% β-TCP (CTC) composite scaffolds, which were showed to have better mechanical properties, osteoinductivity and weight-bearing capacity than 70% HA/30% β-TCP composite scaffolds when implanted into tibia defect in mice (Cheng et al., 2021).

An *in vitro* study found that a composite scaffold comprised of 30% β-TCP and 70% bioactive glass (BG) stimulated the proliferation of osteoblast-like MG-63 cells, compared with the β-TCP group (Seidenstuecker et al., 2017). Another study found that a nano-sized β-TCP and polylactic acid (PLA) mixture presented significantly higher disc height compared to the autograft group (Cao et al., 2017). Moreover, the β-TCP-PLA mixture membrane has been demonstrated to favor bone healing in the anterior esthetic area of the maxilla in a recent clinical study (Canullo et al., 2019). It has also been revealed that β-TCP-chitosan composite scaffolds can significantly enhance the proliferation and osteogenic differentiation of BMSCs *in vitro* (Wang et al., 2019a). Additionally, the addition of pulverized human bone into β-TCP-chitosan composite scaffolds presented excellent mechanical properties and induced significantly higher ALP activity than β-TCP-chitosan composite scaffolds when seeded with MG63 cells (Kowalczyk et al., 2021). Besides, Han et al found that the 50% PCL-50% β-TCP spinal fusion cage had optimal osteogenic capacity as compared to 60% PCL-40% β-TCP and 55% PCL-45% β-TCP spinal fusion cage *in vitro* (Han et al., 2021). Although large volume of β-TCP-based composites have been developed, further clinical trials are required before applying it to the clinic.

THE ADDITION OF STEM CELLS AND CELL-DERIVED ACTIVE SUBSTANCE TO BETA-TRICALCIUM PHOSPHATE

The addition of stem cells or cell-derived active substance, such as exosomes and extracellular matrix (ECM), to β-TCP can largely improve its osteogenic properties (Zhang et al., 2016). For example, in a monkey femur bone defect model, the ratio of successful bone union in composites containing β-TCP blocks and bone marrow mesenchymal stem cells (BMSCs) (5/7) was markedly higher compared to the β-TCP blocks alone (1/5) (Masaoka et al., 2016). In another study, β-TCP/gelatine scaffold combined with allogeneic adipose-derived stem cells (ASCs) showed a superior pro-osteogenic effect compared with β-TCP/gelatine scaffold and pure β-TCP scaffold in a

rabbit femoral bone defect model (Liu et al., 2021). Du et al. recently revealed that bone marrow mononuclear cells (BMMNCs)/β-TCP scaffold had a 1.5-fold more bone volume compared to the bone marrow-derived mesenchymal stem cells (BMSCs)/β-TCP scaffold after implanted into a tibia bone defect for 12 months (Du et al., 2021). Furthermore, in a recent clinical study, patients treated with MSC/β-TCP composite scaffolds presented significantly more new bone formation and improved functional recovery compared with the pure β-TCP (Chu et al., 2019).

The incorporation of exosomes derived from bone mesenchymal stem cells (BMSC) and hypoxia inducible factor-1α (HIF-1α) to β-TCP scaffolds significantly repaired rat critical-sized bone defects by facilitating new bone regeneration and neovascularization (Ying et al., 2020). Dental pulp stem cells (DPSCs)-derived extracellular vesicle (EVs)/β-TCP scaffolds was demonstrated to boost bone formation in the periphery of the defects (Imanishi et al., 2021). Moreover, in a pig mandibular bony defect model, MSCs and platelet-rich plasma (PRP)-impregnated polycaprolactone (PCL)-β-TCP bio-scaffold presented excellent ability to enhance bone regeneration around dental implants (Almansoori et al., 2021).

3D AND 4D PRINTED BETA-TRICALCIUM PHOSPHATE-BASED SCAFFOLDS

It was only until recently that three-dimensional (3D) printed β-TCP-based materials were widely used in research and clinical applications due to the easily controllable size and shape. For example, the 3D printed β-TCP scaffolds with diverse pore size (500, 750, and 1,000 μm) not only possess excellent mechanical properties, but also boost proliferation of MG-63 cells (Seidenstuecker et al., 2019). Moreover, 3D-printed polycaprolactone (PCL)/β-TCP scaffolds showed promising potential for oromandibular reconstruction *in vivo* (Lee et al., 2020). However, the manufacturing process of traditional 3D printing is performed at high temperatures, which may lead to the loss of bio-efficacy of sensitive growth regulators, such as icariin (ICA) (Zhang et al., 2021). To incorporate various active growth regulators within the porous architectures of β-TCP materials, low-temperature based 3D printing technique is used. For example, the low-temperature extrusion-based 3D printing of composite scaffolds constructed by poly (ε-caprolactone) (PCL) and β-TCP encapsulated with ICA (ITP scaffolds) promoted differentiation of BMSCs *in vitro* (Zhang et al., 2021). Though 3D-printing allows for the manufacturing of complex shapes on demand, those shapes of printed products do not change over time. The emerging 4D printing technology is gaining great interest because of its changeable shapes. Wang et al. constructed β-TCP-based nanocomposite scaffolds by incorporating black phosphorus nanosheets and osteogenic peptide into β-TCP/poly (lactic acid-co-trimethylene carbonate) (TCP/P (DLLA-TMC)) by 4D printing. The shape of the 4D-printed scaffolds can be reconfigured to adjust precise fitting in irregular bone defects when the scaffold temperature rapidly increases to 45°C (Wang et al., 2020b). To date, the β-TCP-based 4D printed products have rarely been reported in bone tissue engineering, however, these materials have substantial potential as bone substitutes although further research is warranted.

BETA-TRICALCIUM PHOSPHATE-MEDIATED MECHANISMS FOR OSTEOGENESIS REGULATIES

Although the pro-osteogenic effects of β-TCP remain controversial, β-TCP affect bone repair by interacting with the surrounding tissues through the regulation of growth factors, cytokines and ions. A possible mechanism of β-TCP-regulated osteogenesis is that β-TCP can directly or indirectly (*via* the release of ionic components such as Ca^{2+} and $PO4^{3-}$) facilitate osteoblast differentiation, promote vascularization, regulate the release of growth factors, and alter blood clot formation thus boosting bone healing at defect sites.

The Effect of Ca^{2+} on Osteoblast Differentiation

Numerous studies have confirmed the crucial role of Ca^{2+} released from β-TCP plays in proliferation and differentiation of MSCs and osteoblasts. It has been suggested that 2–4 mmol/L Ca^{2+} *in vitro* is favorable to the proliferation and survival of mouse primary osteoblasts (mOBs), whereas slightly higher concentrations (6–8 mmol/L) can facilitate mOBs differentiation and matrix mineralization (Maeno et al., 2005; Wang et al., 2020c). Other studies have found that a higher concentration of Ca^{2+} (up to 14 mmol/L) can lead to a rounded shape osteoblast morphology while a lower concentration of Ca^{2+} (≤8 mmol/L) can promote the migration of human osteoblasts (Lei et al., 2018; Nakamura et al., 2010).

It has been suggested that Ca^{2+}-mediated osteoblast differentiation involves the activation of the Calmodulin and Calmodulin-dependent kinase II (CaM–CaMKII) pathway (Cary et al., 2013; Liu et al., 2020). The activation of α-CaMKII boosts autophosphorylation of threonine 286, resulting in increased affinity of CaMKII for Ca^{2+}/CaM by nearly 1000-fold while also maintaining the enzyme in a partially activated or autonomous state (Zayzafoon et al., 2005). The phosphorylated α-CaMKII subsequently modulates the activity of several transcription factors, such as CRE-binding protein (CREB) and extracellular signal-regulated kinase (ERK) (Zayzafoon et al., 2005; Lu et al., 2020b) which transactivate the serum-response element (SRE) and cAMP-response element (CRE) within the promoter of *c-fos* respectively (Kukushkin et al., 2002; Sen et al., 2015). This in turn enhances expression of *AP-1*, a transcription factor involved in differentiation of bone forming cells and ultimately results in the differentiation of osteoblasts (**Figure 2**) (Wagner 2002; Babu et al., 2013). Another study suggested L-type voltage-gated calcium channels (L-VGCCs) play a role in modulating the Ca^{2+} released from β-TCP. Blockade of L-VGCCs with nifedipine significantly inhibited the expression of

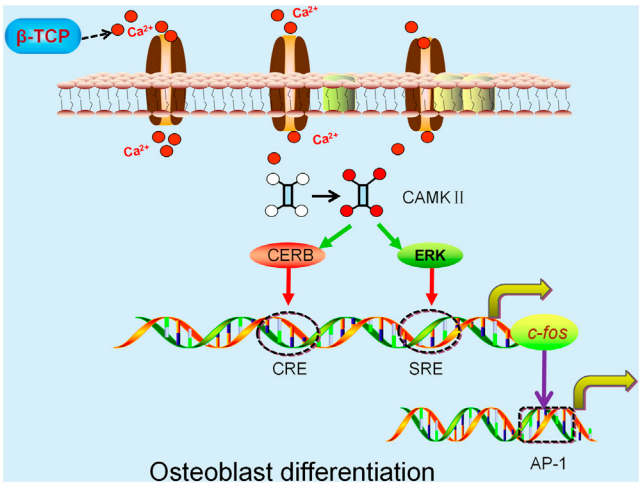

FIGURE 2 | β-TCP-released Ca^{2+} activates the α-CaMKII pathway and modulates CREB and extracellular ERK activity. The activation of CRE-binding protein (CREB) and extracellular signal-regulated kinase (ERK) increase the transactivation of SRE and CRE respectively, both of which regulate the *c-fos* promoter resulting in enhanced expression of AP-1 and ultimately osteoblast differentiation.

the osteoblast differentiation protein, bone morphogenetic protein 2 (BMP-2), indicating that osteogenic differentiation may be regulated by L-VGCCs (Barradas et al., 2012). Similarly, another study has demonstrated inhibition of L-VGCCs can suppress the osteogenic differentiation of rat BMSCs (Wen et al., 2012). Interestingly, conflicting results have been reported using mouse BMSCs. Ma et al. demonstrated L-VGCCs treated with the blocker, benidipine enhanced mouse BMSCs differentiation by upregulating the Wnt/β-catenin pathway in an ovariectomized (OVX) mouse model (Ma et al., 2015). This phenomenon is probably due to different L-VGCCs subtypes which exist in cells from different tissues and species (Tan et al., 2019).

The Effect of Beta-Tricalcium Phosphate on Neovascularization

To survive, osteoblasts (OBs) must reside within 150–200 μm of capillaries to obtain a sufficient supply of oxygen and nutrients (Nguyen et al., 2012). Conversely, inadequate angiogenesis can result in necrosis in the central region of bone tissues, and delay the bone regeneration process. Therefore, the extent and rate of vascular growth are thought to be important determinants in effective bone healing (Liu et al., 2015). Ca^{2+}, one of the major components of β-TCP, plays a critical role in blood vessel formation. Studies over the past decade has demonstrated that intracellular Ca^{2+} concentration is associated with the proliferation and movement of endothelial cells. The intracellular Ca^{2+} wave drives proliferation, homing and tubulogenesis of endothelial progenitor cells (EPC) (Moccia et al., 2014; Moccia et al., 2012). Ca^{2+} exhibits a unique regulatory role in cytoplasm, organelles, and nuclei by acting as an allosteric activator or inhibitor of various intracellular enzymes. It can also interact with other proteins to regulate calcium-dependent enzymes and ion channels. The most significant example is CaM, a Ca^{2+} decoder for cell proliferation that is involved in regulating the CaMKII family and several other membrane channels. Multiple studies have shown that CaMKII is directly involved in several transition points in the progress of the cell cycle (G1 to S) (Kahl and Means, 2003; Zeng et al., 2020; Koval et al., 2019). Moreover, calcium-dependent enzymes initiate the activation of several nuclear factors related to DNA division, such as cyclin, which is closely associated with the proliferation of endothelial cells (Li et al., 2016; Hui et al., 2011). Vascular endothelial growth factor (VEGF), a potent regulator of vascular formation, stimulates the proliferation, migration, differentiation and formation of capillary-like structures in endothelial cells *via* the Ras/mitogen-activated protein kinase (Ras/MAPK), phosphatidylinositol 3-kinase (PI3K/AKT) and phospholipase Cγ/inositol triphosphate (PLCγ/IP3) signaling pathways in the presence of Ca^{2+} after binding to vascular endothelial growth factor receptor 2 (VEGFR2) (Jin et al., 2017; Kim et al., 2018). Given the critical role of Ca^{2+} in angiogenesis, it is not surprising that β-TCP can effectively induce neovascularization. In a rabbit tibial defect model, significantly increased angiogenesis measured by average number of blood vessels, was observed in the β-TCP group (4.47 ± 0.2) compared with the bioactive glass group (1.58 ± 0.246) (Anghelescu et al., 2018). In clinical practice, similar results were observed when using β-TCP-based dental implants (Dautova et al., 2018). These results suggest β-TCP can augment angiogenesis, which could be the reason for its osteoinductivity (Kusumbe et al., 2014; Rather et al., 2019).

The Incorporation of Bone Regenerative Growth Factors in Beta-Tricalcium Phosphate for Osteogenesis

With a deeper understanding of bone repair and fracture union, studies have shown that the biological performance of scaffolds can be improved by incorporating certain growth factors (such as the BMP family). An *in vitro* study found that β-TCP particles exhibited excellent capacities to deliver growth factors from platelet concentrates such as BMP-2 and BMP-7, compared with the control group (non β-TCP particles) (Bonazza et al., 2018). Interestingly, in a rat femoral defect model, the residual β-TCP in the BMP-delivery treatment group was notably lower than the β-TCP control group, indicating that BMP may facilitate the degradation of β-TCP and subsequently enhance bone healing (Xie et al., 2019). Based on the aforementioned evidence, it is speculated that β-TCP could boost the process of bone regeneration *via* interacting with growth factors such as BMPs.

It has been demonstrated that growth factors play an important role in cell migration, cell proliferation, and tissue angiogenesis, further facilitating new bone formation at the defect

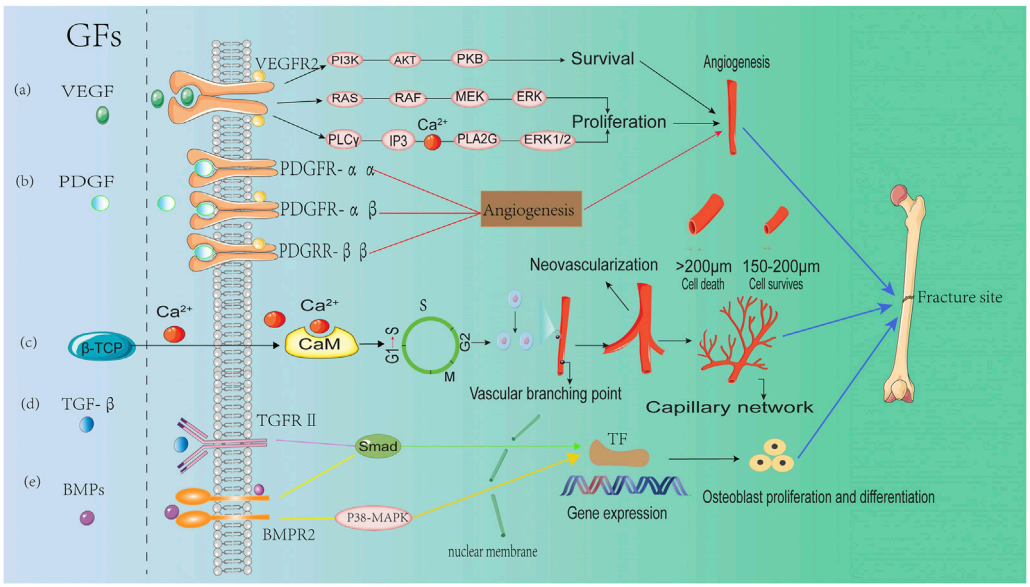

FIGURE 3 | The role of growth factors in regulating angiogenesis during bone formation **(A)** Vascular endothelial growth factor (VEGF) regulates osteogenesis through three major signaling pathways after binding to VEGFR2: the PI3K/AKT pathway, the Raf-MEK-ERK pathway and the PLCγ-IP3 pathway. **(B)** Platelet derived growth factor (PDGF) regulates angiogenesis by binding to and activating PDGFR-αα, PDGFR-αβ, and PDGFR-ββ receptors. **(C)** Ca^2 released from β-TCP, binds to calmodulin (CaM) to promote cell cycle progression (G1→S), enhancing endothelial cell proliferation and facilitating neovascularization and bone healing. **(D)** Transforming growth factor beta (TGF-β) induces up-regulation of osteogenic gene expression through the SMAD signaling pathway after binding to transforming growth factor beta receptor2 (TGFR2). **(E)** Bone morphogenetic proteins (BMPs) facilitate the expression of osteogenic genes *via* bone morphogenetic protein receptor type-2 (BMPR) and the activation of SMAD and p38-MAPK signaling pathways.

sites (Kim et al., 2014; Mochizuki et al., 2020). Osteogenesis is the process by which new bone is synthesized by graft cells (mainly cells that survive on the surface of the graft) or host cells. During material-mediated osteoinduction, the migration and proliferation of undifferentiated host MSCs will be recruited to the defect sites, driven by growth factors such as BMPs, transforming growth factor beta (TGF-β) and platelet derived growth factor (PDGF). These growth factors have been found to induce early bone healing by binding to fibrinogen or extracellular matrix (ECM) proteins (Martino et al., 2013; Hulsart-Billstrom et al., 2015).

In a dog dehiscence defect model, β-TCP with fibroblast growth factor-2 (FGF-2) was demonstrated to increase bone volume compared with the β-TCP control (Fukuba et al., 2021). Similarly, the effect of FGF-2 on bone regeneration has been demonstrated in a canine maxillary saddle defect model (Hoshi et al., 2016). Furthermore, when β-TCP scaffolds containing 0.3% FGF-2 was implanted in patients with periodontal defects, the percentage of bone growth in the FGF-2-β-TCP treatment group was significantly increased compared with the β-TCP treatment group over a period of 13 months (Cochran et al., 2016). BMPs, members of the TGF-β superfamily, can stimulate DNA recruitment and cell replication *via* SMAD and p38-MAPK signaling pathways, thereby promoting the directional differentiation of MSCs into osteoblasts. Accordingly, in an *in vitro* study, β-TCP granules were used to carry BMP-2, which led to a more pronounced osteogenic effect compared with the β-TCP control (Kuroiwa et al., 2019). Moreover, in a study using a rat calvarial bone defect model, BMP-2-loaded β-TCP granules significantly induced bone healing compared with the β-TCP group (**Figure 3**) (Lee et al., 2021).

Modifying Beta-Tricalcium Phosphate in the Regulation of Blood Clots to Enhance Bone Regeneration

The Effect of Blood Clots on Bone Healing

After implantation into the fracture site, the surface of substitute materials comes into contact with peripheral blood, which results in the formation of a blood clot around the graft (Milleret et al., 2015). In the case of a bone fracture, the blood clot acts as a 'natural scaffold' when the extrinsic coagulation pathway is activated (Einhorn and Gerstenfeld, 2015). Following a fracture, a cross-linked fibrin network is formed *via* the generation of thrombin, which strengthens clot formation by converting fibrinogen into fibrin. Coarse and loose fibrin clots have an increased rougher surface structure and a higher porosity which means it is more prone to undergo fibrinolysis, an enzymatic process by which fibrin is degraded and which has a beneficial process to the repair and regeneration of bone tissues (Yermolenko et al., 2011; Varin et al., 2013). Conversely, dense blood clots composed of thin and dense fibrin are highly resistant to fibrinolysis and result in a significant delay in bone regeneration (Wang et al., 2018b). As a consequence, the change in fibrin structure directly affects

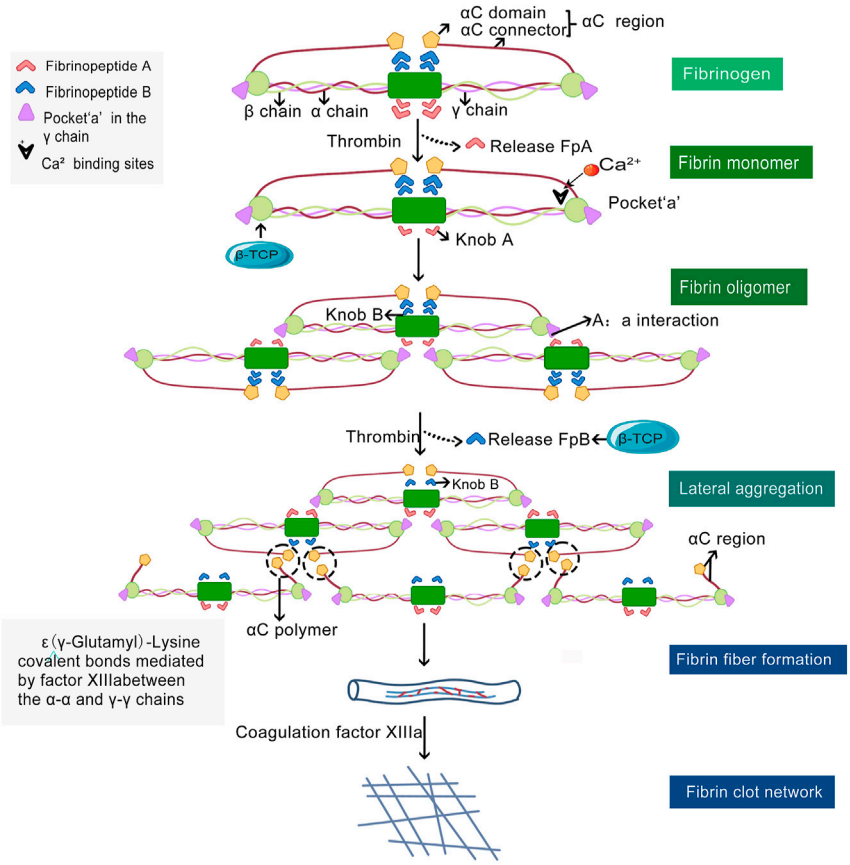

FIGURE 4 | Polymeric fibrin network formation and β-TCP regulatory sites. Thrombin mediates the of monomeric fibrinogen to polymeric fibrin by catalyzing the cleavage and release of fibrinopeptides (Fp)A (light red dovetail arrows) and FpB (blue dovetail arrows) from fibrinogen. Fibrin monomers originating from FpA aggregate longitudinally and form fibrin oligomers, which in turn polymerize to form protofibrils. The protofibrils undergo lateral polymerization *via* intermolecular interaction at αC regions to form αC polymers (black dashed circles) and results in the formation of thick fibers. Finally, the fibrin clot, a cross-linked gel-like meshwork forms and plays an important role in normal hemostasis. β-TCP may regulate fibrin polymerization at the cleavage sites when FpB is released and at the primary binding sites (black arrow heads) of the fibrinogen γ chain (orange color).

the bone healing process, as fibrinolysis of the thrombus is responsible for cell growth and the release of growth factors (Laurens et al., 2006; Wang et al., 2016). Blood clots with a tight fibrin network delay bone healing due to the low porosity and delayed degradation (Wang et al., 2017b). On the contrary, thick fibrin blood clots with higher porosity facilitate osteoblast migration and adhesion, thereby promoting bone regeneration (Collen et al., 1998). Studies have demonstrated that the structure of blood clots at defects is closely related to thrombin concentration. Low concentrations of thrombin produce turbid, coarse and loose fibrin, leading to highly permeable fibrin clots. While high concentrations of thrombin result in non-turbid, thin and dense fibrin network chain and results in low permeability blood clots (Campbell et al., 2009; Rahmany et al., 2013).

Blood clots can also regulate bone regeneration *via* secreting growth factors. For example, platelet concentrate (PC) contains high levels of crucial growth factors for bone healing, including PDGF, TGF-β, insulin-like growth factor (IGF), epithelial growth factor (EGF) and VEGF (Qiao et al., 2017; Nagaraja et al., 2020; Mijiritsky et al., 2021; Blatt et al., 2021). Moreover, PC also contains many soluble cytokines (CKs), such as pro-inflammatory response factors and anti-inflammatory response factors (Fang et al., 2020). These soluble CKs are important mediators of tissue healing process (Ma et al., 2019; Pennati et al., 2020). Studies have shown that PC can promote not only migration, proliferation, differentiation, and angiogenesis of adipose mesenchymal stem cells (ADSCs), but also ectopic osteogenesis (Chen et al., 2015b; Wang et al., 2015; Liou et al., 2018; Wang et al., 2019b; Ni et al., 2021).

The Effect of Beta-Tricalcium Phosphate on Blood Clots

Since blood clot formation is the first stage in the generation of new bone after material implantation, it is necessary to understand how β-TCP can regulate the formation and structure of new blood clots. Wang X et al. reported β-TCP solutions supplemented with increasing concentrations of fibrinogen resulted in a concomitant decrease in fibrin diameters (Wang et al., 2018a). Furthermore, the density of

blood clots in the fibrinogen-200 mg/ml group was markedly higher than the control group (Wang et al., 2018a) β-TCP is able to modulate the density of blood clots by regulating the formation of the fibrin network. This process involves the sequential cleavage of fibrin peptides (Fp)A and FpB from the central region of the fibrinogen molecule with subsequent longitudinal and lateral polymerization (Weisel and Litvinov, 2017). The fibrin monomers are gathered longitudinally through a knob-pocket interaction to form a fibrin oligomer, which then polymerizes to form fibrils (Weisel and Litvinov, 2013). Lateral polymerization occurs when fibrils form αC polymers through intermolecular interactions mediated by activated factor XIII resulting in the formation of thick fibers. Together, longitudinal and lateral polymerization results in fibrin forming a crosslinked gel-like network (Weisel and Litvinov, 2013). During the process of fibrin polymerization, β-TCP may target this pathway by binding to the fibrinogen γ chain of fibrinogen and result in the release of FpB (**Figure 4**).

The Effect of Ca^{2+} on Blood Clots

The release of Ca^{2+} from β-TCP plays a prominent role in the polymerization of fibrinogen. Fibrinogen contains both high and low Ca^{2+}-binding sites; three high- and 8–10 low-affinity calcium binding sites (Weisel and Litvinov, 2017). Of the high-affinity Ca^{2+} binding sites in the γ chains, γ1, is associated with amino acid residues γAsp318, γAsp320, γGly324, and γPhe322 and coordinated with two strongly bound water molecules (Spraggon et al., 1997; Kamijo et al., 2019). The remaining high-affinity Ca^{2+} binding sites (named β1) are located at the β-nodules within loop β381–385 and both have a coordinating water molecule (Everse et al., 1998). Of the low-affinity Ca^{2+} binding sites, γ2 and β2 have been well characterized. Formed as a consequence of molecular rearrangements as a result of crystal packing, γ2 sites are located in loops γ294–301 and exert only a moderate influence on the crystal structure and functional properties of fibrinogen (Kostelansky et al., 2004a; Kostelansky et al., 2004b; Kostelansky et al., 2007). The β2 sites are formed by residues γGlu132, βAsp261, βAsp398, and the backbone carbonyl oxygen of γGlu132 (Kostelansky et al., 2004a; Kostelansky et al., 2004b). The β-nodules are anchored to the coiled-coil connector for functioning *via* β2 sites, which are involved in the process of lateral aggregation of protofibrils (Kostelansky et al., 2004a).

The binding of Ca^{2+} to high-affinity sites protects the γ chains from enzymatic degradation (Odrljin et al., 1996). and modulates fibrin polymerization by augmenting lateral aggregation to form thicker and denser fibers (Weisel and Litvinov, 2013). Consequently, functionality can be seriously damaged by mutations influencing these Ca^{2+} binding sites (Brennan et al., 2007). Furthermore, the conformational changes which occur when FpB is cleaved is Ca^{2+} dependent with the affinity of the Ca^{2+} binding sites changing upon FpB release (Dyr et al., 1989; Mihalyi 1988). The functional relevance of the β2 site was elucidated when the binding of a knob 'B' mimetic peptide (Gly His-Arg-Pro) in the presence of Ca^{2+} resulted in a 10-fold increased binding to fibrinogen compared with in the absence of Ca^{2+}. This suggests the β2 site may be involved in the conformational change associated with binding of Gly His-Arg-Pro (Everse et al., 1999). The interval formed by dense fibers is inadequate to permit the entrance of osteogenic factors to the fracture site, resulting in delayed bone healing.

Ca^{2+} Binding to Factor XIII Regulates the Crosslinking of Fibrin Monomers

Under normal physiological conditions, Ca^{2+} is an essential factor involved in the extrinsic coagulation pathway (**Figure 5**). Plasma factor XIII, a 325.8 kDa heterotetrameric proenzyme which stabilizes fibrin, is composed of two catalytic A subunits (FXIII-A) and two inhibitory B subunits (FXIII-B) (Ashcroft et al., 2000; Muszbek et al., 2011; Singh et al., 2019). It is the last factor in the coagulation cascade and facilitates crosslinking between fibrin monomers, protects fibrin from shear stress, and guards clots from premature degradation (Duval et al., 2014; Vasilyeva et al., 2018; Alshehri et al., 2021). For factor XIII to react with fibrin monomers, it is converted to an activated transglutaminase and functions in the presence of thrombin and Ca^{2+}. The activation of plasma factor XIII under physiological conditions comprises two steps, firstly, the cleavage of a peptide from the N-terminal end of FXIII-A at Arg37-Gly38 by thrombin and secondly, the separation of FXIII-A from FXIII-B (in the presence of Ca^{2+}) to form the active configuration. Thrombin can also inactivate FXIII-A by cleaving at Lys513-Ser514. Ca^{2+} is a key regulator of the proteolytic degradation of plasma factor XIII and can prevent thrombin-mediated inactivation of FXIII-A even at a very high concentrations of thrombin (Mary et al., 1988). After FXIII-A and FXIII-B subunits have been dissociated, Ca^{2+} binds to the high-affinity binding sites on FXIII-A to convert the free FXIII-A into an active transglutaminase, which subsequently facilitates the cross-linking and stabilization of fibrin monomers (Hornyak and Shafer, 1991; Hethershaw et al., 2014; Protopopova et al., 2019; Mangla et al., 2021). The formation of crosslinks, including α–α, γ–γ, and α–γ crosslinking, have a variety of functions (Shainoff et al., 1991; Piechocka et al., 2017). The α–α and to a certain extent, γ–γ crosslinking play a vital role in regulating the physical properties of clots (e.g. stiffness, elasticity and tautness) compared with α–γ crosslinking (which affects the rigidity of clots less) (Duval et al., 2014; Standeven et al., 2007). In addition, the rate of crosslinking varies. For example, γ–γ crosslinking is significantly quicker than α–α crosslinking which is likely due to the complexity of αC polymer formation (Guthold et al., 2007; Helms et al., 2012).

The Effect of Polyphosphate Ions on the Polymerization of Fibrin

Inorganic polyphosphate (polyP) is a linear polymer composed of 60–100 orthophosphate residues linked by high-energy phosphoanhydride bonds (Muller et al., 2009; Ruiz et al., 2004). and functions to regulate the physical properties of fibrin clots and regulate fibrin polymerization (Whyte et al., 2016). It has been suggested that incorporation of the negatively charged polyP into fibrin is through the net positive charge on the αC regions of fibrinogen (Smith and Morrissey, 2008). This interaction increases the length of fibrin monomers, possibly as a result of the αC region reaching beyond the D-region of the fibrin monomer. Furthermore, polyP may function to enhance

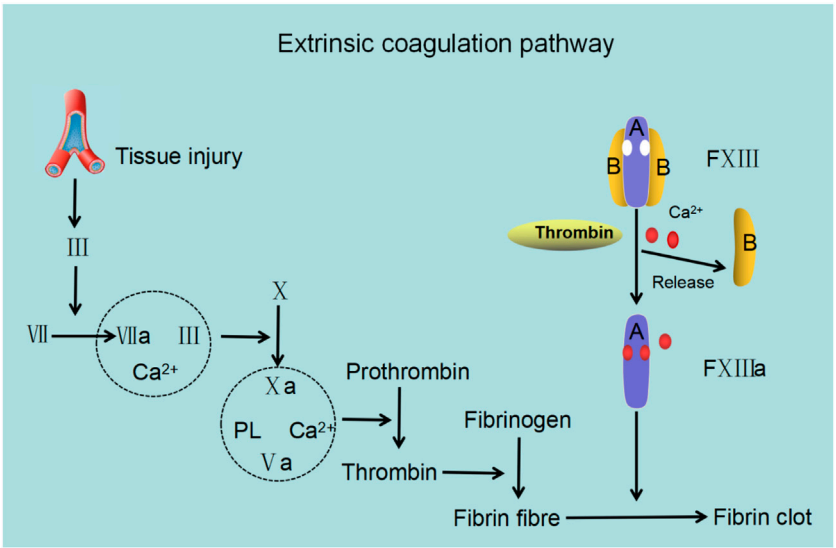

FIGURE 5 | The role of factor XIII in the extrinsic coagulation cascade. Following trauma, factor **III** (FIII; tissue factor) stimulates factor VII (FVII) to form an activated complex (TF–FVIIa) wherein TF–FVIIa activates factor X (FX) to form FXa and converges into the common coagulation cascade. In the presence of Ca^{2+} and phospholipid (PL), FXa and its cofactor FVa form the prothrombinase complex (FXa–FVa-PL-Ca^{2+}), which boosts the conversion of prothrombin to thrombin. Lastly, thrombin catalyzes fibrinogen to produce fibrin, leading to fibrin clot formation and achievement of hemostasis. Factor **XIII** (FXIII) contains two A subunits and two B subunits. The B subunit dissociates from the A subunit with the assistance of Ca^{2+} and thrombin and exposes Ca^{2+} binding sites resulting in the binding of Ca^{2+} and FXIII facilitating the stabilization and cross-linking of fibrin to form fibrin clots.

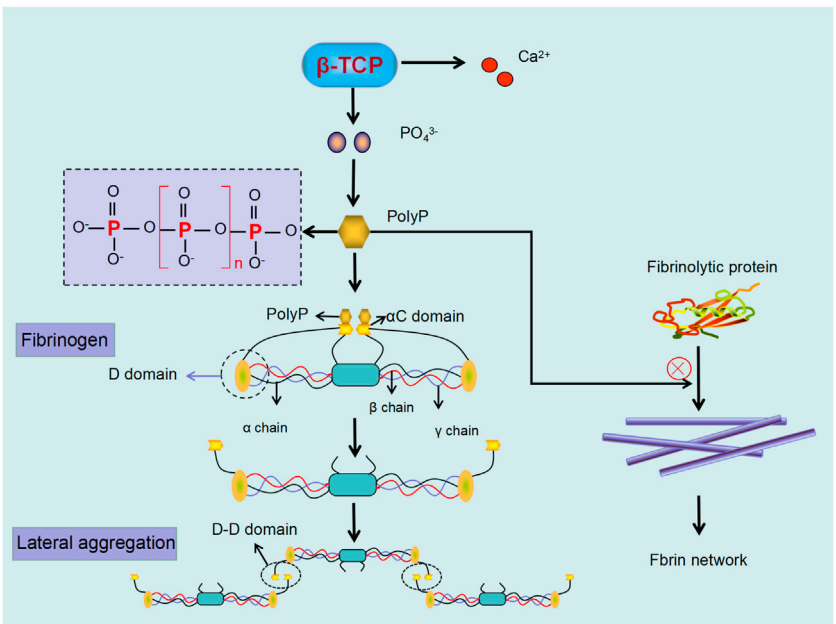

FIGURE 6 | Phosphate ions (PO_4^{3-}) released from β-TCP can polymerize to form PolyP, which regulate fibrin polymerization and enhance resistance to fibrinolysis. PolyP binding to αC region in fibrinogen results in extension beyond the D-region, with subsequent D-D interaction, resulting in lateral aggregation of fibrin. Moreover, the formed PolyP can further defend fibrinolytic protein from linking to the fibrin, leading to dense and undissolved fibrin.

D-D interactions between fibrin monomers as a result of lateral extension of αC region instead of longitudinal. This may account for shortened fibrin oligomers (Whyte et al., 2016). It is likely that polyP functions as a nucleus that attracts fibrinogen resulting in denser areas of fibrin (Whyte et al., 2016; Mutch 2016). Moreover, the presence of polyP can lead to more turbid fibrin clots and thicker fibrin fibrils compared with clots without polyp (Smith and Morrissey, 2014). PolyP

seems to be directly integrated into fibrin clots, by currently an unknown mechanism.

In addition to altering the structure of the fibrin network, polyP also regulates fibrinolysis. The process of fibrinolysis, is regulated by tissue plasminogen activator (tPA) and urokinase (uPA). Both tPA and plasminogen exert their functions by attaching avidly to C-terminal lysine residues on the fibrin surface (Foley et al., 2011). PolyP can inhibit fibrinolysis through modulating the binding of tPA and plasminogen to fibrin, although the detailed mechanism remains unclear (Morrissey and Smith, 2015; Mutch 2016). From the above considerations, we speculate that phosphate ions (PO_4^{3-}) released from β-TCP form polyP which can lead to fibrin clots that are dense and more resistant to fibrinolysis. Moreover, as dense clots can hinder the migration of osteogenesis factors (Wang et al., 2016). and MSCs into the fracture site, polyP formation may be one of the possible mechanisms by which β-TCP delays bone regeneration (**Figure 6**).

SUMMARY

In this review, we have described recent advances in the application of β-TCP for bone regenerative medicine. β-TCP with its customizable pore size, porosity, and roughness, exhibits substantial advantages for the repair and regeneration of damaged bone tissues. Furthermore, the incorporation of other materials to modulate its hardness and ability to degrade can improve the mechanical performance of β-TCP. The porous structure of β-TCP can act as cargo carriers to deliver pro-osteogenic growth factors with favorable releasing kinetics and results in reduced side effects due to its inherent bioactivity. Compared to other biological materials, β-TCP has a more appropriate degradation rate, which is beneficial to the delivery of therapeutic ionic dissolution products.

Through the phenomenon β-TCP delays bone healing are observed just in several specific experimental models, the role of hematoma formation in osteogenesis is highlighted. The formation of a blood clot as a 'natural scaffold' is fundamental to early bone healing. Coarse and loose fibrin clots have a rougher surface structure, resulting in higher porosity and is more prone to undergo fibrinolysis. Consequently, this is favorable to the ingrowth of blood vessels and the adhesion of growth factors and MSCs, leading to successful bone regeneration. On the contrary, dense blood clots composed of thin and dense fibrin are highly resistant to fibrinolysis, resulting in a significant delay in the growth of new blood vessels, entry of growth factors and MSCs, leading to delayed bone regeneration. β-TCP, directly or indirectly (through its release of Ca^{2+} and PO_4^{3-} ions), affect blood clot structure by regulating fibrin polymerization. The release of Ca^{2+} and subsequent binding to FXIII-A can accelerate fibrin polymerization and lead to fibrin that is highly resistant to fibrinolysis. The release of PO_4^{3-} ions may initiate the formation of dense fibrin which is not susceptible to degradation. From this perspective, β-TCP may delay early bone regeneration and therefore further investigation is warranted to explore the effects of modified β-TCP materials on blood clot/vessel formation to accelerate bone healing.

As yet, several methods have been discovered to control the structure of hematoma to facilitate bone regeneration, such as applying the copper (Cu) dose gradient to realize the well-controlled nitric oxide (NO) releasing, which results in hematoma that is porous and susceptible to degradation. However, no perfect parameter or technique has been attained to endow a designable biomaterial with the desirable properties to completely control hematoma configuration. For this, more researches are required to gain knowledge of the key factors that have influence on the hematoma performance, thus finding suitable methods to manipulate hematoma formation to boost bone healing.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

FUNDING

This work was financially supported by the National Natural Science Foundation of China (Grant No. 86060620, 31960209, and 31760266), Guizhou Science and Technology Fund Project (Grant No. (2020)1Y093), the Scientific Innovation Foundation for Returned Overseas Chinese Scholars of Guizhou Province (Grant No. 2018-07), the 2018 Zunyi "15851 Talent Elite Project" (Grant No. National Natural Science Foundation of China 31760266), Doctoral Science Research Startup Funding of Zunyi Medical University (Grant No.F-934 and No.2017-01). Qian Wei Ji Ban Han (Grant No. 2017-24).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.