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Enhanced Proangiogenic Activity Of Endothelial Cells In Response To Polyglutamate Domain-Modified Qk Peptides Delivered On Bone Grafting Materials

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ENHANCED PROANGIOGENIC ACTIVITY OF ENDOTHELIAL CELLS IN RESPONSE TO POLYGLUTAMATE DOMAIN-MODIFIED QK PEPTIDES DELIVERED ON BONE GRAFTING MATERIALS

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ABSTRACT

Over 2 million bone grafting procedures are performed annually. To meet the high demand for these surgeries, commercial grafting materials sourced from allograft, xenograft, and synthetic calcium phosphate have become widely popular clinically. One limitation of these grafts, however, is their lack of growth factors that drive angiogenesis during bone tissue regeneration. Insufficient revascularization of the injured bone tissue leads to poor fracture healing. However, reincorporating angiogenic factors on grafting materials has proven challenging due to limited surface interactions between the protein and mineral graft surface. To address this challenge, we have synthesized a polyglutamate domain to the VEGF mimetic peptide, QK, in order to reliably deliver angiogenic factors on mineralized graft material. Polyglutamate domains anchor strongly through ionic interactions to the hydroxyapatite present within mineral graft materials. In this work, we have shown that a heptaglutamate (E7) domain synthesized to the QK peptide (E7-QK) greatly enriched the concentration (4-6 fold) of peptide coated onto graft materials. Moreover, enrichment of E7-QK on graft material led to enhanced activation of endothelial cells in direct contact with the scaffold over grafts coated with unmodified QK peptide. Furthermore, we have devised a method to deliver an angiogenic gradient of polyglutamate

modified QK peptides. The length of polyglutamate domain on the peptide directly correlates to the time of release of the peptide from the graft material. We combined a mixture of varied lengths of polyglutamate domains (E2, E4, E7) to the QK peptide (PGM-QK) to create a constant release gradient from mineral graft material. In this study, we characterized the release kinetics of each individual component of PGM-QK (E2-QK, E4-QK, and E7-QK) and found the longer the domain length, the longer the retention of the peptide on the graft material. Additionally, we showed that PGM-QK released from graft material provided an extended angiogenic stimulus. Endothelial cells treated with PGM-QK peptides released from graft material over the course of 6 days exhibited activation of angiogenic signaling pathways and enhanced migration. These results demonstrate the utility of polyglutamate domains to deliver a constant stimulus of bone healing factor from commercial graft material.

Keywords: Tissue engineering, Bone graft, angiogenesis, QK peptide, Polyglutamate domain, cell signaling

DEDICATION

I would like to dedicate this dissertation to my wife, Leslie Pensa. You have been my constant support and rock through this journey. Without you I could not have made it through graduate school. Thank you for being there with me through the highs and the lows, and always pushing me to be my best self. I love you, Les.

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DISCUSSION

INTRODUCTION

The human skeletal system

The human skeletal system has many important roles in support for the body such as structure, movement, protection, and metabolic function. Bones consist of a matrix that is approximately 70% inorganic mineral and 30% organic components [1]. Of these inorganic components, the network is primarily comprised of hydroxyapatite (HA), which is a calcium apatite crystal ($Ca_5(PO_4)_3(OH)$). Meanwhile, collagen type I fibers are the predominant organic component (95%) found within the bone matrix and serve in conjunction with mineral components to provide structure for the bone [1, 2]. These fibers are fortified with the hydroxyapatite crystals to form bone tissue that is rigid and able to withstand both compressive and tensile forces [3]. Other organic components of bone consist of proteoglycans and bone specific proteins that contribute to the homeostasis of bone, as well as guide the deposition of mineral and organic components of bone matrix during remodeling [1, 2].

The skeleton is developed through two distinct processes, endochondral or intramembranous ossification. Long bone formation occurs through endochondral ossification, where cartilaginous tissue is replaced with mineralized bone. During this progression, chondrocytes undergo hypertrophy and become ossified from at the primary (diaphysis) and secondary (epiphysis) ossification sites [4]. At these sites, blood vessels invade the developing bone tissue bringing in mesenchymal stem cells (MSCs) [4, 5]. MSCs are multipotent stem cells that reside within developed bone marrow and periosteum. During endochondral

ossification, the MSCs recruited differentiate into osteoblasts which replace the hyaline cartilage template with bone matrix [4]. The growth plate between these two ossification zones is comprised of chondrocytes that rapidly proliferate, driving elongation of the bone structures. Once fully developed, the growth plate “closes” and becomes ossified to complete the bone structure [4].

Intramembranous ossification is responsible for the flat bone development of skeletal structures such as the skull, clavicles, and mandible [4]. This process directly forms bone without a cartilaginous template structure. Developing tissues of mesenchyme and epithelia condense, while MSCs differentiate directly into osteoblasts. Osteoblasts initially excrete disorganized woven bone matrix known as the osteoid. The osteoid is then mineralized into a woven cancellous network that is characterized by its disorganized deposition of matrix. Over time, the matrix is remodeled through synergistic coordination between osteoblasts and osteoclasts into the organized rigid lamellar structure of cortical bone [4, 6]. Regeneration after a bone fracture follows closely to the process of intramembranous ossification to remodel the injured tissue [6].

Matured bone structures are comprised of different layers specialized to carry out their specific role (Figure 1) [7, 8]. The outer layer of bone contains a periosteum comprised of a thin layer of tissue housing mesenchymal cells. This layer covers the cortical bone, a highly structured component of bone responsible for its mechanical properties. Cortical bone is a mineralized lamellar structure with osteon ring-like structures [7, 8]. This osteon structure is developed by osteoblasts that lay down matrix in concentric lamellar circles known as the

osteoid. Layer by layer of osteoid is mineralized and completed to become a rigid structure capable of withstanding heavy loads [4, 7, 8]. Once complete, these cells undergo apoptosis or terminally differentiate into osteocytes that localize to the central lacunae of the osteoid. Additionally, the lacunae of these concentric layers offer a channel for blood vessels and nerves to network in the bone. Internally, bones contain a cancellous bone network [7, 8]. This spongy bone network is porous and houses the marrow, vascular network, and cell reservoir. This area is largely responsible for the metabolic functions of bone [7].

Cells of the bone

Mesenchymal stem cells

Mesenchymal stem cells are multipotent cells derived from the mesoderm of developing tissue. These cells reside in both the bone marrow and periosteum tissue but can be found in adipose and developing tissues as well. Although primarily found in the marrow, these cells are in low density and only account for 0.001 to 0.01% of the resident cell population in bone marrow [9]. Mesenchymal stem cells differentiate to osteoblast, adipocyte, myocyte, and chondroblast cell lineages based upon exogenous stimuli and environmental factors [9, 10]. During a fracture injury, MSCs are recruited to the bone defect where they proliferate as well as differentiate into osteoblasts and chondrocytes. This differentiation is tightly regulated by endogenous factors such as bone morphogenic proteins (BMPs), fibroblast growth factors (FGFs), and transforming growth factors

(TGFs) [11]. Once differentiated, these cells are responsible for the deposition of new bone matrix.

Osteoblasts

Osteoblasts are derived from the differentiation of resident MSCs through the stimulation of pathways such as Wnt, BMP, FGF, and Hedgehog, and are mainly responsible for the deposition of both organic and inorganic matrix in bone [11, 12]. Osteoblasts are highly metabolic and rich in mitochondria [12]. One of their main functions is to deposit bone matrix proteins including collagen type I fibers that will produce a highly ordered structure known as the primary osteoid (Figure 2) [13]. Subsequently, the matrix mineralizes into fully reorganized bone tissue. In addition to their role as matrix depositors, osteoblasts also regulate bone tissue homeostasis. While undergoing differentiation and maturation, osteoblasts govern osteoclast activity through paracrine signaling of factors such as RANKL, osteoprotegerin, and macrophage colony stimulating factor (M-CSF) to maintain a controlled bone remodeling site. [14] Additionally osteoblasts sense the completion of the bone remodeling process and through autoregulation, signal the beginning of either apoptosis or maturation into osteocytes.

Osteocytes

Osteocytes are matured, terminally differentiated osteoblasts residing in the newly remodeling lacunae of the bone tissue [13]. Osteocytes are thought to participate in bone homeostasis by providing mechanosensing while also instigating remodeling and resorption of the surrounding bone tissue [15].

Osteoclasts

Osteoclasts are large multinucleated cells derived from monocyte cell type fusion [1]. These cells are largely responsible for removing the mineral and organic components of bone, thus maintaining a homeostatic relationship with the resident osteoblast. Osteoclasts remove old bone matrix by forming tight junctions within the bone tissue surface, then excreting high levels of protons to demineralize the bone, as well as Cathepsin K and MMPs to degrade the organic bone matrix (Figure 2) [16, 17]. The resorbing area of bone forms a pit known as the Howship's lacunae. Osteoclasts increase their efficiency in excreting these matrix degradative enzymes by forming a ruffled border within the sealed remodeling zone to maximize membrane surface area. The function of these cells is tightly regulated by stromal and preosteoblast which activate osteoclasts through their ligands, M-CSF and RANKL [1, 11, 18] . To signal the end of bone resorption, osteoblasts secrete the RANKL antagonist, osteoprotegerin (OPG), which induces inactivation and apoptosis of the osteoclasts [18].

Chondrocytes

Chondrocytes are responsible for the deposition of cartilaginous matrix consisting of collagen and proteoglycans [1]. They are the sole cell type native to the cartilage tissue. In bone formation, chondrocytes are involved with the process of endochondral ossification [1]. Chondrocytes produce type II and X collagens that act as a template for long bone formation. Additionally, chondrocytes play a role in the formation of the callus during bone fracture healing [19].

Other cells present in bone

The marrow cavity of bone is a hematopoietic reservoir for the body, containing the majority of hematopoietic stem cell populations [1]. The vasculature throughout bone contains endothelial cells and pericytes and serves as a conduit for transport of hematopoietic cells. Additionally, it has been shown that vascularization within bone plays a major role in the fracture healing process [20]. Other cell types present in bone marrow include adipocytes and lymphocytes [1].

Bone regeneration after injury

Bone fracture healing can occur in discrete primary or secondary healing categories [21]. Primary bone healing involves the direct fusion of the bone fracture back together without the formation of callus [22]. The osteoblasts and osteoclasts produce a structured bone matrix rather than a woven bone to be remodeled later. This healing process is the rarer of the two subsets, as it requires direct abutment and rigid fixation to form correctly [21]. Secondary healing occurs through a multi-step process involving recruitment of MSCs, inflammatory cells, and new vasculature. During secondary healing, a hematoma is formed which is converted into granulation tissue composed of fibroblasts and new vasculature [21]. This new vasculature provides the injury site with the endogenous cells and growth factors required to remodel the injured tissue. This site is converted into a primary soft callus of loosely organized extracellular matrix excreted by recruited osteoblasts. The matrix is then mineralized to a

hardened bone callus [21, 23]. Once calcified, the callus is slowly reorganized through cooperation between osteoblasts and osteoclasts to form fully healed cortical bone. Secondary healing is tightly regulated by several factors such as BMPs, VEGF, FGFs, PDGFs, and RANKL to coordinate between cell populations that are responsible for the regeneration of the bone tissue [6, 21, 24]. Both primary and secondary healing processes are dependent on the size and level of trauma involved in the fracture. Larger defects can result in non-union fractures and require surgical interventions such as fixation or bone graft implantation to repair the bone defect [21].

Mineralized bone graft materials

Bone grafts are used by clinicians for a number of applications in the orthopedic, reconstructive, and dental fields. An estimated 2 million grafting procedures are performed annually [25]. Procedures range in a wide variety from trauma reconstruction to outpatient procedures. The main similarity between all these applications is that the graft material provides a conduit for new bone to grow and develop. The current gold standard for bone grafts is autologous bone harvested from the patient's iliac crest or other non-load bearing site [26]. These grafts are ideal as they contain the patient's own cells and factors that promote healthy graft integration with the surrounding bone tissue [27]. Although autogenous grafts are desirable for their osteoconductive and osteoinductive potential, these grafts are limited in their quantity. Harvesting requires secondary surgery sites and may not yield enough graft material for major traumas [28, 29]. In addition,

autogenous sources can be variable in their ability to regenerate based upon tissue quality. Factors such as age, gender, and disease can play a significant role in determining the use of autografts for procedures [25, 30]. One specific example is the use of autogenous grafts in elderly populations. Studies have shown poor patient prognoses for patients over 65 undergoing lumbar spinal fusion with autologous graft due to poor graft quality and co-morbidities from harvesting bone from secondary sites [31].

For these reasons many clinicians use non-autogenous graft sources such as allograft, xenograft, and synthetic mineralized materials (calcium phosphate and hydroxyapatite materials) [27]. These grafting materials fulfill the role as an osteoconductive substrate, are produced in large quantities, and eliminate the need for secondary surgical sites. Moreover, allograft and xenograft contain the microarchitecture of bone tissue optimal for osteoblast attachment and integration within the graft material [32, 33]. However, these grafting materials lack the osteoinductive cells and growth factors that autogenous grafts rely on for rapid and efficient bone recovery. Moreover, allograft and xenograft are chemically and/or physically treated to reduce the potential for an immunogenic response or introduction of pathogens; however, these processing steps typically eliminate cells and damage or remove organic factors that provide osteogenic potential [27]. Synthetic sources lack any organic factors completely and are purely mineral based.

Clinical applications of allograft bone

Currently, the bone allografts used in the clinic are produced and distributed as either fresh-frozen bone allograft (FFB) or freeze-dried bone allograft (FDBA) [34, 35]. Under each category there are several commercial suppliers of these bone grafting materials, offering a wide range in bone graft size (granules vs whole sections) and type (cortical vs. cancellous). FFB and FDBA both provide an osteoconductive matrix that mimics the graft recipient's own bone matrix most. However, their use within the clinic is limited due to controversy surrounding the risk of pathogen transmission as well as immune response [35]. FFA frozen allografts are harvested directly from donors, washed in an antibiotic sterilization and immediately stored at -70℃. This limited procedure allows retention of the mechanical properties of the grafts and also limits the loss of osteoinductive factors such as bone morphogenic proteins. However, studies have reported that patients receiving FFA have been infected with HIV, resulting in the growing concerns for when FFA should be used thus limiting its usage in clinic today [35]. FDBA are processed by harvesting the tissue, treating with antibiotic solutions, and sterilization followed by lyophilization. These grafts can be stored for long-term use at room temperature and have a far lower risk of pathogen transfer than FFB. However, the sterilization of FDBA has been shown to remove any osteoinductive factor within the graft as well as compromise the structural integrity of the graft, itself [36]. For example, sterilization techniques such as ethylene oxide or gamma irradiation compromise the quality of these bone grafts

leading to slower reintegration with host bone [36]. To mitigate issues surrounding the limited osteoinductivity of allograft, physicians have used alternative methods to replenish the osteoinductive potential of these grafts such as combining allograft with autograft or recombinant growth factors [37, 38].

Clinical applications of xenograft material

Xenografts are commercially available bone grafting materials that have been widely successful in the clinic. The most popular xenograft materials are derived from bovine, porcine and coralline organisms [25, 39, 40]. These grafting materials contain the microarchitecture found within native bone that has been shown to improve graft integration. Xenografts are processed even further compared to alloplastic materials and are devoid of any organic components, thus removing the risk of an immune response [40]. However, the lack of organic components eliminates the osteoinductive potential of the grafts. Additionally, it has been shown that the processing techniques used to ablate organic components results in a brittle graft material that requires external fixation for load bearing applications [41]. However, their abundant availability and lowered risk of pathogen transfer has made xenograft materials highly popular within the clinic, particularly for dental applications [25, 41].

Clinical applications of synthetic calcium phosphate (CaP) materials

Several studies have shown the success of calcium phosphate grafts in resolving bone defects in patients [25, 42]. The most widely used calcium phosphate

materials are comprised of either hydroxyapatite (HA) or β-tricalcium phosphate (β-TCP). These synthetic materials differ in chemical composition and crystallinity making it possible to amend their structural properties as well as rates of degradation within the body [43]. These grafts have shown great promise in the clinic as excellent substrates for new bone formation as they are highly osteoconductive and biocompatible. Additionally, some have speculated that CaP mineral possesses some osteoinductive influence over resident MSCs perpetuating their usefulness in the clinic, although the exact mechanism by which they contribute to osteoblastic differentiation is still unclear [44]. It has been shown that MSCs seeded onto stiffer matrices such as mineral HA are prone to osteoblastic differentiation [45]. Meanwhile others have speculated that CaP-containing materials serve as a mineral ion reservoir for calcium(Ca^{2+}) and phosphate ions (PO_4^{3-}) which facilitate osteoblastic differentiation [46]. While the exact mechanism is not yet known, CaP materials have been very successful in the clinic.

Calcium phosphate graft materials are oftentimes available as a porous scaffold or ceramic material. One drawback of these materials is that they are pure mineral and therefore are brittle, and not well-suited for load bearing applications [47]. However, products such as OSTEON™ II, Osteogen™, and SyOss® have been popular in the dental field where there are several non-load bearing scenarios. Another feature of these grafts is that they resorb more slowly than other sources such as allograft or xenograft due to their denser mineral content, allowing them to be used for procedures requiring long-term stability [42,

48]. Additionally, synthetic CaP materials are ideal for applications where the patient's culture or religious beliefs do not permit the use of xenograft or allograft material. CaP materials are also devoid of organic components, making the immunogenic and pathogenic risk minimal compared to the xenograft and allografts. Moreover, as a synthetic material CaP can be manufactured with high reproducibility to meet the tight regulatory and manufacturing standards across the globe.

Protein therapy to improve bone graft materials

To improve the bone healing response from non-autologous materials, several groups have attempted to deliver these grafts in conjunction with recombinant proteins [34]. These proteins are targeted to address the main components of bone graft healing including osteogenicity, angiogenesis, and cell attachment. The delivery of osteogenic factors has been shown to have clinical success [42]. Some of the most potent osteoinductive factors identified are BMPs. Specifically, rBMP-2 and rBMP-7 are clinically approved to be used in conjunction with mineral scaffolds in bone grafting procedures [49-51]. While these proteins enhance bone graft reintegration through stimulating direct differentiation of MSCs to osteoblasts, the use of BMPs has been associated with some serious off-target effects [52]. Another avenue to invigorate a rapid bone regeneration is to target the angiogenic response of bone healing. Studies have shown that tissue revascularization plays a major role in healthy bone regeneration, especially in early stages during initial bone callus formation. Delivery of factors

such as VEGF and FGF has been reported to enhance tissue revascularization and improve bone healing [24, 49]. These factors, however, function through a chemotactic gradient. There is currently a lack of methods that enable gradient delivery of angiogenic factors from the surface of commercial bone graft materials, which has limited the development of angiogenic therapies for bone applications. In addition to osteogenic and angiogenic proteins, grafts have been modified with factors that promote cell attachment to the graft surface. Generally, these factors are based on integrin-binding sequences derived from native extracellular matrix proteins such as collagen and fibronectin. Peptide sequences such as DGEA or RGD facilitate cell attachment and interaction with the implanted scaffold [53, 54].

Functionalizing graft materials with proteins/peptides

At present, the most widely used method to deliver growth factors on graft materials in the clinic is through passive absorption. The broad appeal of this method lies in the simplicity of use for the clinician. In preparation for this method, grafts are soaked in a solution of reconstituted protein and then implanted directly into the body. However, passive absorption of these proteins and peptides has been shown to be very inefficient in the delivery of these factors *in vivo* [55]. To coat these grafting materials with a therapeutic dosage of growth factors, supraphysiological doses are required, and once implanted, the efficacy of such factors is diminished due to dissemination outside the bone healing site. As an example, rBMP2 has been shown to elicit serious deleterious

side effects such as inflammation, ectopic calcification, and edema due to the dissemination of rBMP2 away from its graft carrier [56]. Due to the poor interactions between mineralized graft surfaces and protein/peptide therapeutics, a great effort has been placed on functionalizing these proteins/peptides with graft binding domains enhancing their delivery on grafts, ameliorating some off-target effects.

One method explored in the bone grafting field to improve the delivery of proteins/peptides on mineralized grafting surfaces is to incorporate bone graft binding sequences onto growth factors. Many of the bone-binding sequences engineered onto these factors are inspired from native proteins such as bone sialoprotein (BSP), osteocalcin (OCN), and satherin. BSP and other native proteins contain negatively-charged glutamate or aspartate-rich domains that ionically interact with the bone matrix hydroxyapatite [57-60]. This ionic interaction strongly localizes these proteins to the bone matrix. This finding has motivated our group and others to design factors with acidic polyglutamate or polyaspartate domains to localize mimetic peptides such as RGD, DGEA, FHRRIKA, KRSR, and BMP2pep (BMP-2 mimetic) on HA-containing material [53, 61-68]. In seminal work by the Kuboki group, a heptaglutamate domain (E7) was first shown to localize the RGD sequence with high affinity to HA material [69]. Additionally, their work confirmed that the interaction between the HA material and E7-RGD was due to the ionic interactions between the E7 domain and HA, not the RGD sequence [69]. Our lab has demonstrated that the enrichment of these factors on implanted materials improves overall bone

regeneration. In a prior study, we showed that E7-BMP2pep-coated grafts placed into rat mandibular defects induced a more sustained activation of resident osteoblasts than implanted rBMP2 treated grafts, as observed through ^{18}F-NaF labeled imaging [61]. Moreover, greater overall bone formation was also noted as a result of this prolonged activity. These results demonstrated that the enrichment of peptide on bone graft, as well as increased peptide retention *in vivo* due to the polyglutamate domain, played a significant role in the efficacy of the peptide treatment on bone healing [61]. Additionally, our group has shown that modulating the length of the polyglutamate domain can alter the peptide's affinity for HA, thereby modulating the peptide release from graft material over time [70, 71]. In Culpepper et al., diglutamate, tetraglutamate, or heptaglutamate sequences were synthesized to DGEA (E2-DGEA, E4-DGEA, E7-DGEA) [71]. It was shown that extending the number of glutamates within the polyglutamate domain led to increased retention of the peptide on the graft (i.e. E2-DGEA released first, and E7-DGEA lastly) [71]. These results demonstrate that the release kinetics of polyglutamate-modified peptides can be empirically modulated. It was further theorized that a combination of peptides containing these different domain lengths could be utilized to produce a constant release of peptide from graft material. This dissertation explores that theory to deliver a sustained gradient of a proangiogenic factor using a mixture of variable-length polyglutamate domains.

Other groups have sought to permanently couple bioactive factors onto mineralized materials through covalent binding. Covalent binding is a common

approach for protein/peptide immobilization within the polymer chemistry field, but few strategies have proven successful in linking these entities to mineral material [54, 55, 72-74]. One successful approach has been performed by Moon et al. where biphasic calcium phosphate (a mixture of HA and β-TCP) was subjected to aminosilanization of the surface, followed by chemically conjugation of FGF-2 [73]. This method highly enriched the concentration of protein on the graft surface. Methods such as this are appealing as they reduce the initial protein concentration needed at delivery and reduce the off-target effects caused by dissemination. However, covalently coupling factors to graft materials has potential limitations, as well. Namely, the chemical conjugation process can denature protein structure and disrupt the function of the coupled growth factor [75-77]. Additionally, once coupled, these factors are tightly bound, making this technique impractical for some growth factors that require release for their primary function.

Another method to localize bone healing factors to graft materials involves the use of bisphosphonate groups [78-84]. Bisphosphonates were found to preferentially bind to calcium found within native bone matrix and directly inhibit bone resorption by disrupting the formation of the ruffled border of osteoclasts [85]. Bisphosphonates have been highly effective in patients suffering from osteoporosis, Paget's disease, and several types of cancers infiltrating the bone [85]. However, bisphosphonates have been shown to have serious long-term side effects such as osteonecrosis of the jaw [85-87]. Researchers have sought to chemically conjugate growth factors to bisphosphonates to enrich their

concentration on CaP grafting materials. Similar to polyaspartate or polyglutamate-modified proteins, the bisphosphonate-conjugated proteins were able to enrich the concentration of factors such as BMP-2 or bFGF on graft material [79, 88]. However, conjugation of these factors to bisphosphonate groups requires complicated chemical coupling between the growth factor and bisphosphonate group, which could impede the signaling function of the growth factor.

Human vascular system

The vascular system derived from the mesoderm is the key supplier of nutrients, oxygen, and metabolites throughout the body [89, 90] . This intricate system is comprised of several specialized vessels that circulate blood such as arteries, veins, and capillaries [91]. The arteries are responsible for delivering oxygenated blood. They are composed of an endothelial intima, smooth muscle media, and fibrous adventitia. These vessels maintain and regulate blood pressure through their characteristic thick smooth muscle layer, which dilates and contracts depending on external stimuli and chemokines delivered from the baroreceptors of the brain [89]. Each arterial branch intersects with a capillary network to deliver oxygenated blood to specific tissues. These capillaries are thin layers of endothelial cells surrounded by basement membrane. The narrow diameter and thin walls of the vessels allow for nutrients and gases to readily permeate to the surrounding tissue. In addition, the capillaries reuptake the deoxygenated blood from the tissue to be returned through the venous system.

The venous system is comprised of vessels containing an endothelial intima, thin smooth muscle media, and thick fibrous adventitia, and is responsible for delivering deoxygenated blood back to the heart [89]. The vessels also contain specialized one-way valves to ensure that returning blood maintains its venous return during the pulsatile flow caused by the beating heart [89] .

Cells present in the vascular system

Endothelial cells

Endothelial cells comprise the thin inner lumen of the vascular network [89]. These cells form tight junctions to ensure proper transport of nutrients throughout the body. Endothelial cells are derived from the mesoderm and oriented as a single-layer of squamous cells known as the endothelium. These cells serve important functions in fluid transport of the body acting as sensors for vasoconstriction and vasodilation [92]. In addition, the endothelium acts as a semi-selective barrier allowing the free movement of select cells, factors and metabolites necessary for targeted tissues [93]. Endothelial cells also are responsible for the early stages of angiogenesis [94, 95]. When stimulated by factors such as VEGF or FGF, these cells reorganize to create sprouts, thus initiating the growth of new vasculature towards the tissue source of these factors [94-97].

Smooth muscle cells

Smooth muscle layers comprise the medial layer of blood vessels. These cells are elongated spindle-like cells containing abundant amounts of actin and

myosin filaments which are important for contraction [89, 92]. These cells form cell-cell junctions to coordinate with surrounding smooth muscle cells to provide a structure to vessels known as the smooth muscle layer. The smooth muscle layer is largely responsible for maintaining the blood pressure by contracting or relaxing based upon stimuli from circulating factors [89].

Pericytes

Pericytes are rounded nuclei cells that are embedded on the outer basement membrane of blood vessels. These cells excrete a matrix to surround and fortify the blood vessels [89]. Additionally, these cells have been shown to secrete paracrine factors to nearby endothelial cells aiding in the stimulation and proliferation of endothelial cells during angiogenesis [89, 92].

Angiogenesis

Angiogenesis is the process of developing new vasculature from existing blood vessels. This process can occur as intussusceptive or sprouting angiogenesis [94]. Intussusceptive occurs within the vessel creating a bifurcation in the center and thus producing two individual vessels. Sprouting occurs when endothelial cells branch out from the vessel wall, creating new vessels that invade into the periphery [94]. Sprouting angiogenesis is the most common type of angiogenesis that occurs, whereas intussusceptive angiogenesis is more heavily involved in the developing tissues. Intussusceptive angiogenesis occurs quickly and is thought to be predominant in developing embryos where rapid neovascularization is required [94]. Meanwhile, sprouting angiogenesis

predominates in the healing tissue due to its ability to branch out far from existing vasculature. During injury response, sprouting angiogenesis is signaled by secreted factors such as VEGF, bFGF, Ang-1, and Ang-2. Endothelial cells that detect a high concentration of these angiogenic factors become tip cells, and upregulate expression of tyrosine kinase receptors, VEGFR-2 and Tie-2 [95]. Tip cells are characterized by their filopodial extensions and are positive for both CD-31 and CD-34 expression [98]. Surrounding endothelial cells elongate to become stalk cells and divide to follow the tip cell towards the chemokine gradient of angiogenic factors. MMP-2 and MMP-9 weaken the cell-cell junctions allowing for the lengthening of the new vessels [94]. Once vessel growth is complete, Ang-1 binds to Tie-2 to promote establishment of the endothelial layer and recruit pericytes to stabilize the structure [94]. Pericyte attachment is one of the final stages in vessel maturation and this event signals the endothelial cells to quiesce. Once complete, there is a new, fully functioning vessel that connects the original vasculature to the nearby healing tissue.

Vascular endothelial growth factor's role in angiogenesis

Vascular endothelial growth factor (VEGF) is one of the most potent angiogenic factors. While there are five VEGF proteins in the VEGF family (VEGFA, VEGFB, VEGFC, VEGFD, and VEGFE), the most widely studied for its angiogenic potential is VEGFA [5, 99, 100]. VEGFA contains three primary isoforms, VEGF-189, VEGF-165, and VEGF-121, that range in receptor affinity and role. VEGF-165 has been shown to be a potent inducer of angiogenesis. This protein can

interact with VEGFR-1(Flt-1) and VEGFR-2 (Flk-1/KDR), both of which are receptor tyrosine kinases expressed on the endothelial cell surface [101] . While the exact role of VEGFR-1 has yet to be determined, VEGFR-2, when stimulated by VEGF proteins, is largely responsible for the angiogenic response in endothelial cells [101]. The activation of VEGFR-2 induces endothelial cell migration, proliferation, and survival through downstream signaling pathways such as MAPK, PI3K, and Ras [101]. VEGF protein is regulated in the body through HIF-1α and other hypoxic-driven transcription factors in response to tissue injury [5]. Upon injury, VEGF expression is upregulated in the damaged tissue, and the protein is also cleaved from heparin-sulfate-containing extracellular matrix proteins; together these processes enable the establishment of a chemokine gradient [5]. The gradient established from injured tissue to the surrounding vascular has been shown to play a vital role in VEGF function [101, 102]. Bolus release of high concentrations of VEGF has been shown to produce immature, leaky vasculature in many cancer types [100]. In contrast, lack of VEGF in the wound response leads to insufficient revascularization of tissues. Administration of recombinant VEGFA has been a primary focus in the field of tissue engineering for its ability to improve tissue regeneration and graft integration through enhanced vascularization [97, 103-105]. However, one challenge associated with VEGFA delivery is the inherently short half-life (4 hours) and dependency on a chemokine release gradient [102, 106]. To address these issues, VEGFA has been delivered on carriers to increase its half-life and also control the release of rVEGFA when implanted into tissue. One prevalent

strategy for VEGF delivery is to incorporate the protein into hydrogel carriers [107-112]. Once implanted, the hydrogels degrade over time to release the VEGF slowly into the surrounding tissues. Hydrogels are well suited for protein delivery but lack the mechanical rigidity and strength often needed for bone graft applications. However, there have been several studies demonstrating the vast improvement in bone regeneration when rVEGF is delivered into a bone defect site. For example, rVEGF-containing polylactic-co-glycolic acid (VEGF-PLGA) scaffolds implanted into irradiated osseous defects showed improvement in bone vascularization and overall bone volume, as measured through Laser doppler perfusion and μCT imaging [113]. Although these results are promising, few studies have focused on how to incorporate rVEGF on mineralized grafting materials, which have their own inherent characteristics benefitting a healthy bone graft healing process [103, 114].

QK peptide induction of angiogenesis

While rVEGF is a very effective angiogenic agent, there are technical limitations associated with producing recombinant therapeutic proteins. These include high production costs, low-purity, variability in protein bioactivity, as well as the requirement for host-cell systems which can introduce pathogens or immunogenic antigens [115]. Additionally, recombinant proteins may have diminished signaling capacity when compared to the endogenous proteins [116]. One alternative to address these limitations is the use of peptide-mimetics of growth factors. Several studies have shown that these bioactive sequences of

amino acids, modeled after the binding sequences of native proteins, are capable of activating target receptors in a manner comparable to the full-length protein. Mimetic peptides do not require a host-cell system as they are produced by a commercial peptide synthesizer, yielding a more pure and cost-efficient product than recombinant proteins. The mimetic peptide for the VEGF protein, referred to as the QK peptide, was identified by D'Andrea et al. [117]. The QK domain is comprised of the sequence, KLTWQELYQLKYKGI, which was modeled after the helical binding region of VEGF-165 (amino acids 17-25) to the VEGFR-2 receptor [117]. Studies have shown that the QK peptide activates the VEGFR-2 receptor and key downstream targets such as the MAPK, Ras, and PI3K/Akt signaling pathways (Figure 3) [117]. QK peptides have also been shown to have a greater resistance to degradation, evidenced by their longer half-life of 24 hours over rVEGF in serum-stability analyses [118]. QK peptide treatments have been shown within a hindlimb ischemia model to induce vascularization comparable to full-length rVEGF, as measured by capillary density and hindlimb perfusion [119]. Moreover, the QK peptide can be readily modulated with other sequences to enhance binding and delivery on scaffold materials. In Chan et al., a collagen-binding sequence was coupled to QK peptide to enhance integration with a hydrogel scaffold [120]. These peptides were shown to be bioactive even with the collagen binding domain modification giving promise that the QK domain may be modified with other scaffold binding domains. In this dissertation, we explore utilizing such a technique to localize and deliver the angiogenic QK peptide on bone graft material.

Research Objectives

Bone grafts are used for a wide variety of clinical applications in the dental and orthopedic fields. Autologous grafts are highly successful at promoting bone regeneration due to their retention of the patient's own cells and growth factors. However, clinical use of this graft material is compromised by the limited amount of donor bone available, as well as the risk of secondary surgical site complications. Commercial grafts derived from allograft, xenograft, and synthetic CaP materials have been developed to meet the high demand for bone grafting surgeries. These grafts are successful within the clinic but lack the cells and growth factors necessary to achieve the same levels of bone regeneration found in autograft procedures. The tissue engineering field has identified several growth factors key to the regeneration of bone but has yet to find a method to reliably deliver said factors with these commercial grafting materials.

One major healing cascade necessary for healthy bone graft integration is angiogenesis. Revascularization of the tissue following a bone grafting procedure is crucial during the early stages of bone regeneration as it supplies the necessary endogenous cells and growth factors to the injured bone tissue. Studies have shown that the lack of vascularization within the bone graft site can lead to unresolved non-union bone fractures [121-123]. Angiogenic factors such as the QK peptide, derived from the active signaling domain of VEGFA, have been shown to induce an angiogenic response in healing tissues. However, the

delivery of growth factors and peptides on mineralized material has proven challenging due to limited interactions between the factors/peptides and the graft surface. Currently, these factors/peptides are passively coated onto grafting materials. This method leads to high variability in what is delivered to the patient due to poor protein/peptide coating efficiency and supraphysiological doses administered to mitigate the poor coating method. Moreover, once implanted, there are no measures to control the release of factors from the bone graft. It has been shown that the signaling function of angiogenic factors is dependent upon release of the factors as a chemotactic gradient. However, current delivery methods of bioactive factors on bone graft material yield a bolus release of the factors, which in the case of angiogenic molecules such VEGF, has been shown to result in leaky immature vasculature [102]. In this dissertation a novel method has been developed to functionalize the QK peptide so that it can be reliably delivered on HA containing graft material.

Previous work in our lab has shown that a heptaglutamate domain (E7) synthesized to mimetic peptides serves as an anchoring moiety for these peptides on HA graft surfaces [53, 61-65, 70]. The conceptual foundation underlying this approach is derived from knowledge of mechanisms used by native bone proteins to localize to mineralized tissues. Specifically, native bone-specific proteins contain polyglutamate or polyaspartate-rich domains that anchor tightly to HA present in bone through ionic interactions. Our lab has used this principle to successfully deliver a number of peptides aimed at enhancing bone regeneration on bone grafting material, including BMP2 mimetics, DGEA, and

RGD sequences [53, 61-65, 70, 124]. In light of these studies, we hypothesized that the polyglutamate domain would be an effective tool to deliver the VEGF-derived QK peptide on bone grafting material (Figure 4). In the first manuscript, we synthesized an E7 domain to the angiogenic QK peptide (E7-QK) to enrich the concentration of the peptide on bone grafting material. From our studies, we reported a 4-6 fold increase in peptide concentration on manufactured HA disks and ABB graft after coating. Additionally, we reported that the E7-QK peptide retained full bioactivity with the addition of the polyglutamate domain, as shown through comparable cell migration, tubule formation, and activation of angiogenic signaling molecules, p-ERK1/2 and p-Akt, in HUVEC cells when treated with solutions of E7-QK or unmodified QK peptide. Moreover, we demonstrated that the increase in peptide concentration on graft surface led to a greater proangiogenic response from endothelial cells in contact with the graft. Specifically, HA grafts were coated with either E7-QK or unmodified QK. After coating, disks were washed and HUVEC cells were directly seeded onto the graft surface. Cells seeded onto the E7-QK coated disks were shown to have greater activation of proangiogenic cell signaling markers (ERK1/2 and Akt) over cells seeded onto grafts coated with unmodified QK peptide, highlighting the functional import of enriching the concentration of QK peptide on graft surface through the use of the polyglutamate domain. These findings address a major gap in the field of bone graft tissue engineering. Namely, we have developed a method to mitigate the issues of poor delivery kinetics and need for supraphysiological dosages surrounding current clinical practices of passively coating graft materials

with growth factors. In this manuscript, we have devised a method to efficiently and reliably coat bone graft with an angiogenic factor, thereby reducing the dosage variability delivered to patients. Moreover, we demonstrated that E7-QK delivered in either a free solution or coated on a graft bound elicited a proangiogenic response in endothelial cells indicating it would be a successful therapeutic once delivered by bone graft.

While the E7-QK peptide was demonstrated as an effective tool to enrich the concentration of angiogenic peptide on graft material, angiogenic factors such as the QK peptide are optimally delivered as a chemotactic gradient to promote healthy tissue vascularization. Several studies have shown that gradient release of angiogenic factors provides directionality for the developing vasculature and aids in the healthy maturation of the blood vessels [102]. Previous work in our lab has shown that modulating the length of the polyglutamate domain alters the binding affinity of the polyglutamate-modified peptide to bone grafting material [70, 71]. In detail, E2-DGEA, E4-DGEA, and E7-DGEA were shown to release from graft material based upon their polyglutamate domain, demonstrating that the longer the domain, the longer the peptide is retained on the graft surface [70, 71]. We postulated that a mixture of QK peptides with different lengths of polyglutamate domains (PGM-QK) would be able to maintain a constant release gradient of angiogenic peptide from grafting material (Figure 5). Specifically, E2-QK, E4-QK, and E7-QK were combined to create PGM-QK. In the second manuscript included within this dissertation, we demonstrated that all three polyglutamate modified peptides were shown to

activate p-ERK1/2 and p-Akt in endothelial cells, individually and as a combined PGM-QK mixture. Each individual component of PGM-QK (E2-QK, E4-QK, and E7-QK) was shown to have its own respective binding and release profile from HA graft based upon the length of the polyglutamate domain. Ultimately, we demonstrated that peptides released from PGM-QK coated graft materials maintained a proangiogenic stimulus for up to 6 days, as evidenced by migration and cell signaling (p-ERK1/2 and p-Akt) in HUVECs treated with solutions containing peptides released from graft materials. The work performed within this study is the first demonstration of the capability of polyglutamate domains to deliver a gradient of peptide from bone grafting material. We determined the individual release kinetics of each PGM-QK were dependent on their polyglutamate domain length. As a combined mixture of PGM-QK, we hypothesized that graft material coated in PGM-QK would deliver a functional therapeutic gradient of QK. We validated this hypothesis by showing in this study that the gradient of PGM-QK delivered on bone graft is functionally active as evidenced by the activation of endoethelial cells by peptide collected after 6 days. These findings suggest that the use of PGM-QK coated grafting materials would be an effective method to deliver an angiogenic factor to improve bone regeneration following a bone graft procedure. Overall the work performed in this dissertation demonstrates that polyglutamate domains improve the method of delivery of bone healing factors on grafting materials as compared with standard passive coating techniques, giving promise to new therapies for bone regeneration.

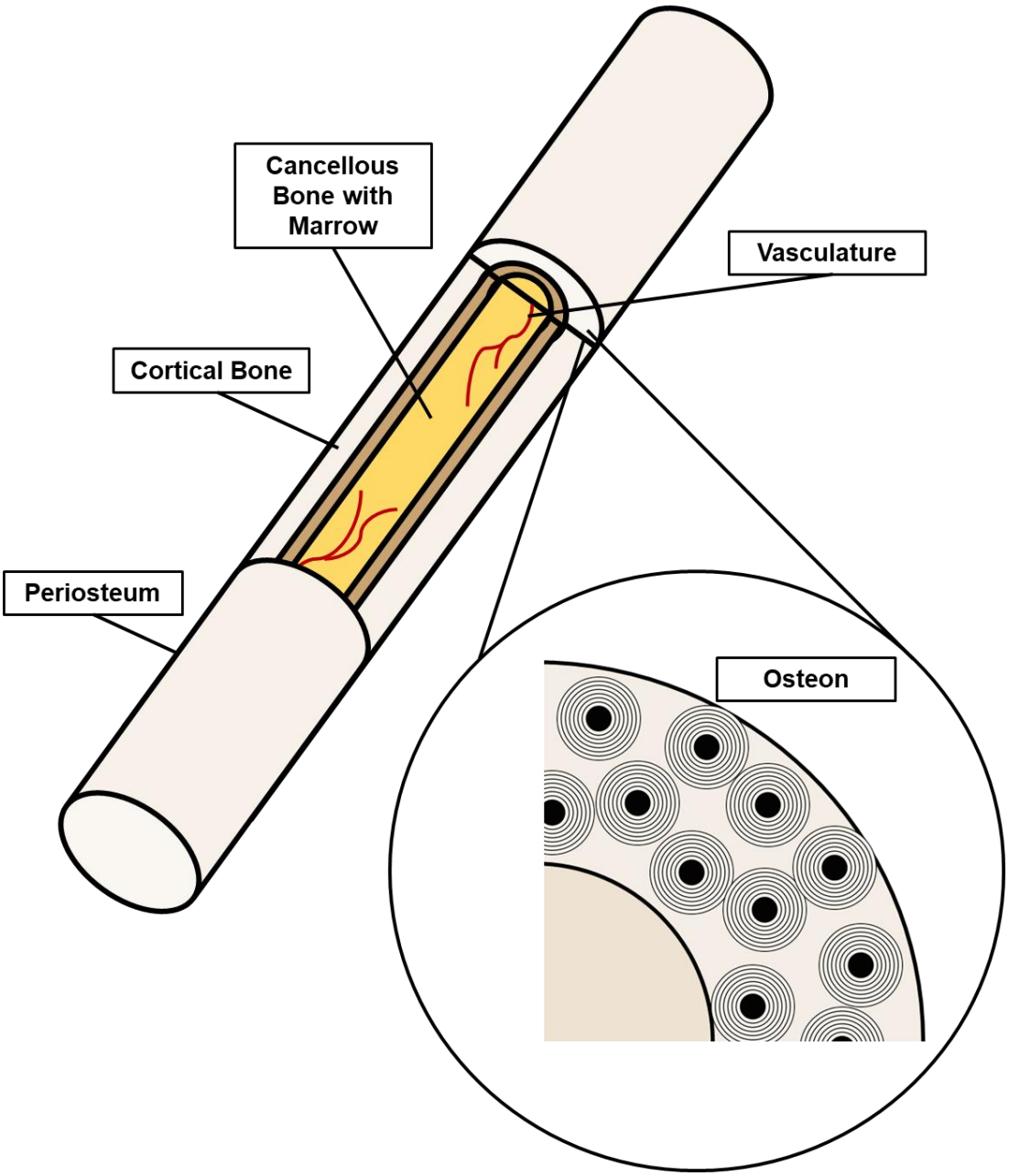

FIGURE 1. Structure of bone. Bone contains three distinct layers: periosteum, cortical bone, and cancellous bone. Periosteum serves as a housing for MSCs. Cortical bone provides mechanical strength through its rigid osteon structure. Cancellous bone is porous and contains bone marrow, various cell type, and vasculature.

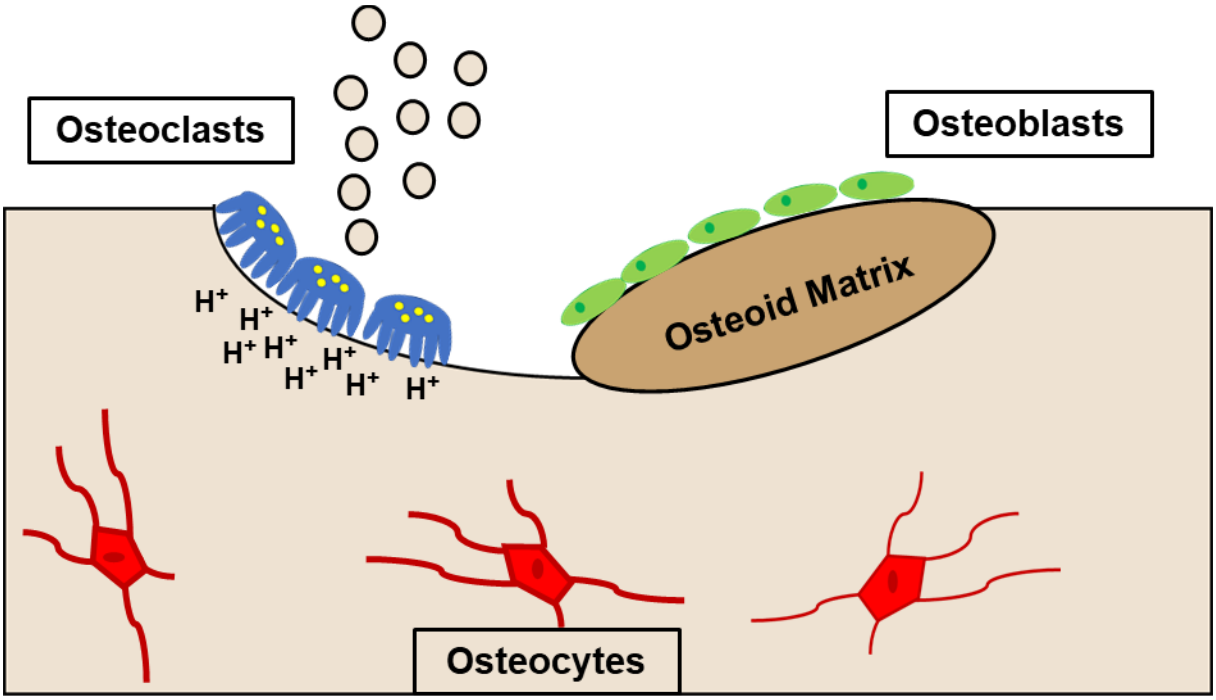

FIGURE 2. Overview of cells involved with bone remodeling. Bone remodeling is controlled by several cell types including osteoblasts, osteoclasts, and osteocytes. Osteoblasts are responsible for secreting the osteoid matrix that is mineralized to form new bone matrix. Osteoclasts degrade the bone matrix that is in need of remodeling. Osteocytes serve as regulators of bone homeostasis.

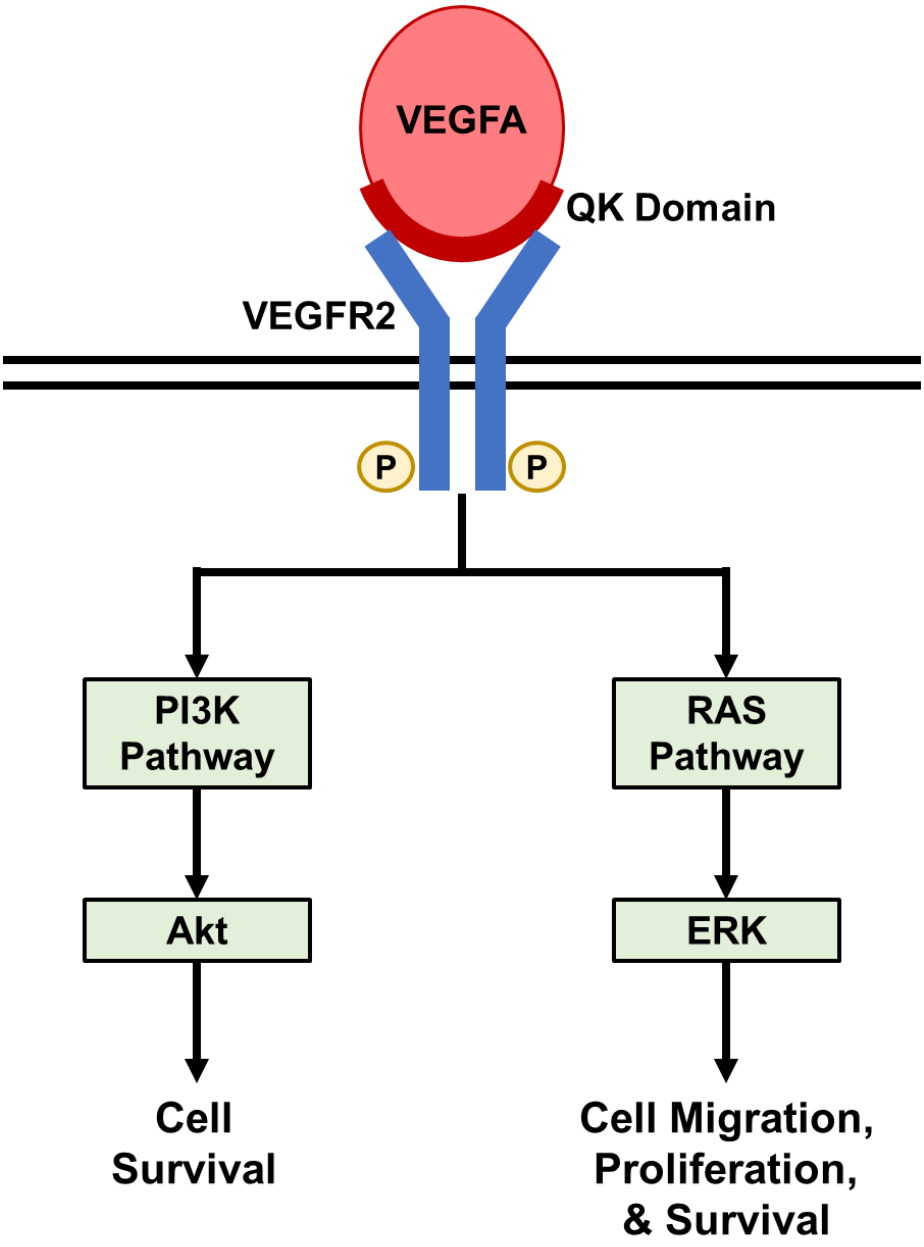

FIGURE 3. The binding of QK/VEGF to VEGFR-2 receptor causes angiogenic signaling cascades in endothelial cells. Activation of the receptor leads to activation of PI3K and RAS pathways which promote cell survival, proliferation, and migration.

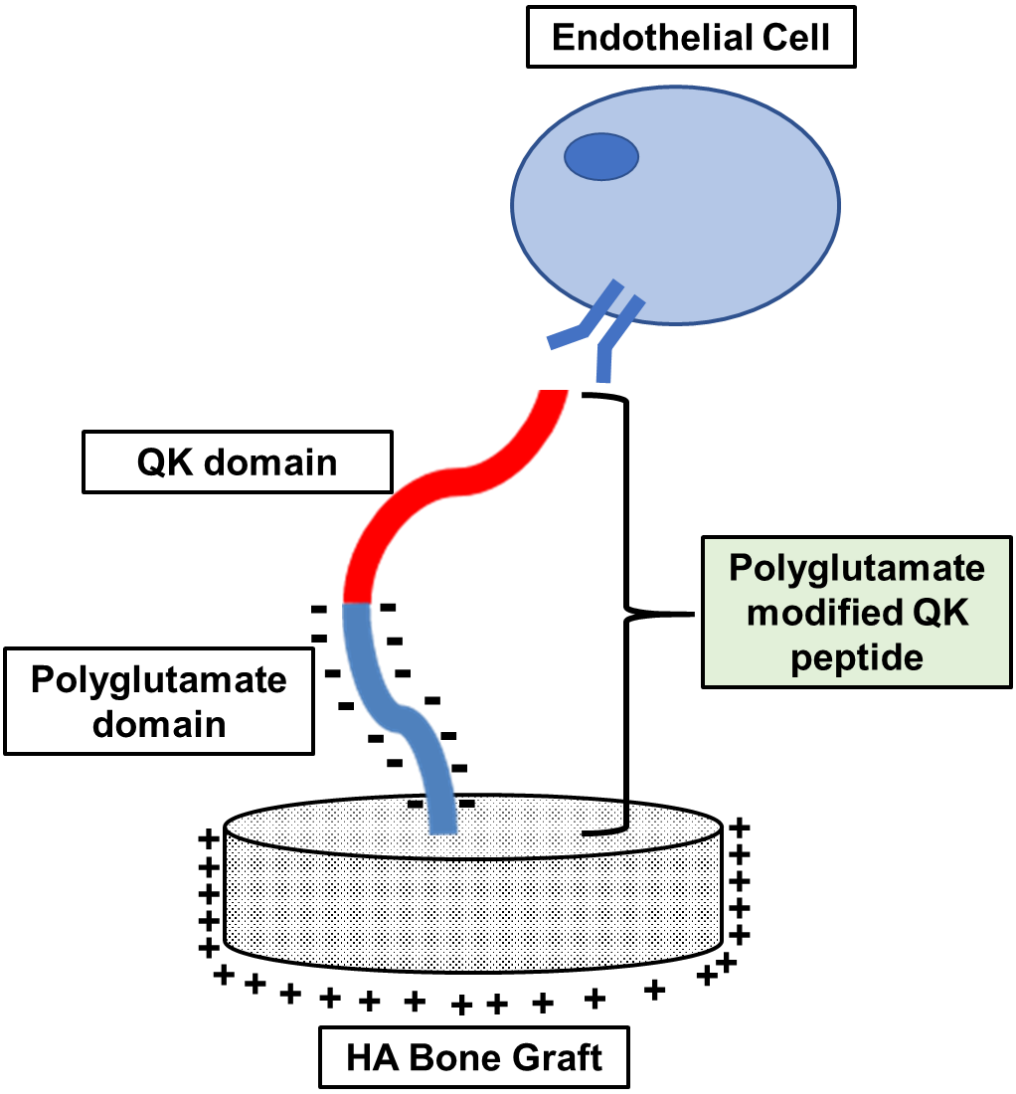

FIGURE 4. Polyglutamate-modified QK peptides anchoring to bone graft. The polyglutamate domain ionically interacts with the mineral HA surface. The bioactive QK peptide is then tightly anchored to the HA due to the polyglutamate domain and can interact with endothelial cells once implanted.

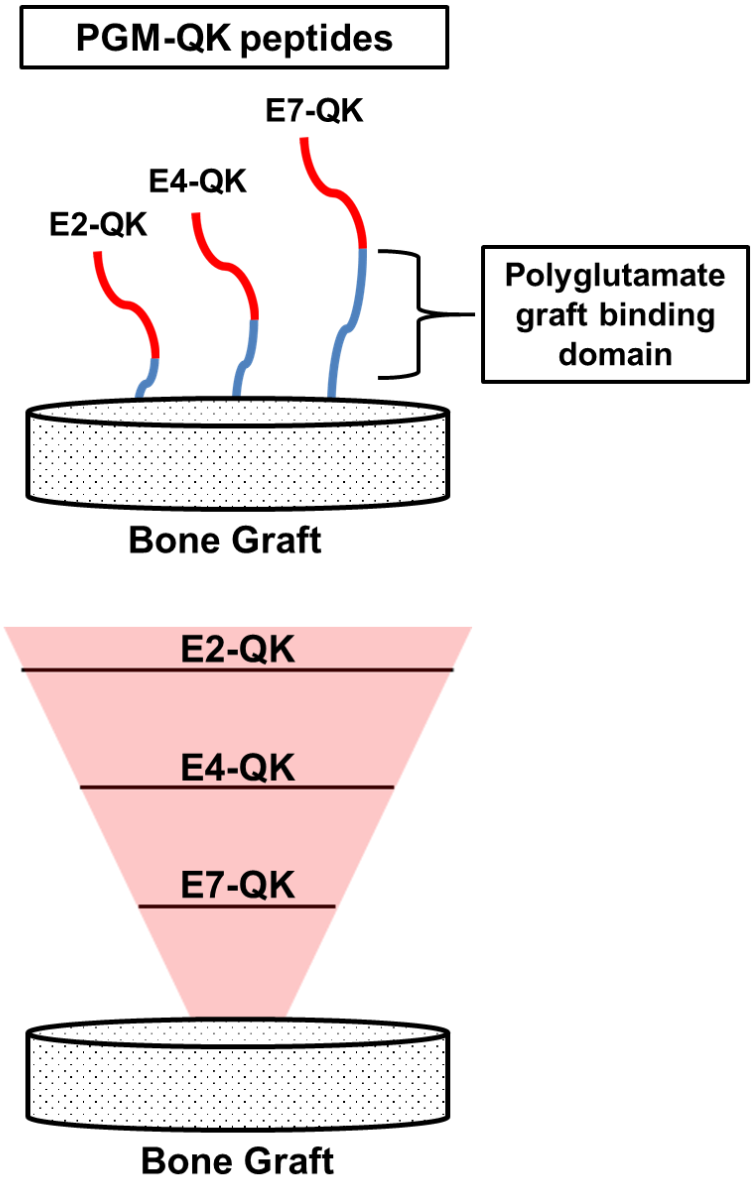

FIGURE 5. Strategy to deliver a gradient of PGM-QK from bone graft. PGM-QK is comprised of E2-QK, E4-QK, and E7-QK peptides. Once coated onto a graft, PGM-QK peptides will release sequentially, thereby maintaining a proangiogenic stimulus.

THE ADDITION OF A POLYGLUTAMATE DOMAIN TO THE ANGIOGENIC QK PEPTIDE IMPROVES PEPTIDE COUPLING TO BONE GRAFT MATERIALS LEADING TO ENHANCED ENDOTHELIAL CELL ACTIVATION

by

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ABSTRACT

Vascularization of bone grafts is vital for graft integration and bone repair, however non-autologous graft sources have limited potential to induce angiogenesis. Accordingly, intensive research has focused on functionalizing non-autologous materials with angiogenic factors. In the current study we evaluated a method for coupling an angiogenic peptide to the surface of two clinically-relevant graft materials, anorganic bovine bone (ABB) and synthetic hydroxyapatite (HA). Specifically, the VEGF-derived "QK" peptide was synthesized with a heptaglutamate (E7) domain, a motif that has strong affinity for calcium phosphate graft materials. Compared with unmodified QK, a 4-6 fold enrichment was observed in the binding of E7-modified QK (E7-QK) to ABB and HA. The E7-QK peptide was then assessed for its capacity to stimulate angiogenic cell behaviors. Human umbilical vein endothelial cells (HUVECs) were treated with solutions of either QK or E7-QK, and it was found that QK and E7-QK elicited equivalent levels of cell migration, tubule formation and activation of the Akt and ERK signaling pathways. These data confirmed that the inherent bioactivity of the QK sequence was not diminished by the addition of the E7 domain. We further verified that the activity of E7-QK was retained following peptide binding to the graft surface. HA disks were coated with QK or E7-QK, and then HUVECs were seeded onto the disks. Consistent with the increased amount of E7-QK bound to HA, relative to QK, markedly greater activation of Akt and ERK 1/2 was observed in cells exposed to the E7-QK-coated disks. Taken together, these results suggest that the E7 domain can be leveraged to

concentrate angiogenic peptides on graft materials, facilitating delivery of higher peptide concentrations within the graft site. The ability to endow diverse graft materials with angiogenic potential holds promise for augmenting the regenerative capacity of non-autologous bone grafts.

INTRODUCTION

More than 2 million bone grafting procedures are performed each year world-wide [1]. Autologous bone is the ideal graft material for these procedures as it retains the osteoinductive growth factors and cells important for effective graft incorporation. However, autologous bone grafts have a number of disadvantages including the risk of secondary surgery site morbidity, as well as the finite amount of donor bone available [2, 3]. To address these issues, non-autogenous graft materials including allograft, xenograft, and synthetic substrates are commonly used as alternatives [4]. These materials are abundant, however, they often lack the critical osteoinductive factors necessary for stimulating graft integration into the surrounding tissue [5]. Without these factors, the potential for complete bone repair is diminished.

Multiple strategies have been pursued to improve the osteoregenerative potential of non-autogenous grafts. One approach is to passively coat the grafts with growth factors that enhance new bone formation such as BMP2, VEGF, PDGF, and FGF [6-12]. However, passively adsorbed growth factors are typically weakly bound to the graft surface, and are therefore rapidly released following graft implantation. This poses several problems. First, inadequate

growth factor binding to the graft precludes sustained delivery of growth factors within the graft site, and secondly, supraphysiologic doses of growth factors are usually required to compensate for the rapid bolus release [7, 13, 14]. Furthermore, the dissemination of high concentrations of growth factors outside of the graft site can cause deleterious side effects. For example, systemic release of recombinant BMP2 (rBMP2) induces inflammation and ectopic calcification [13, 15], whereas high dose rVEGF dissemination can cause increased vascular permeability [16]. For these reasons, improved methods are needed for coupling osteoregenerative factors to graft materials, enabling more controlled and localized delivery.

One promising method for functionalizing graft materials with bioactive factors involves the use of polyglutamate or polyaspartate sequences as binding domains for hydroxyapatite (HA), a calcium phosphate crystal that comprises the principal constituent of bone mineral. These negatively-charged domains, consisting of either repeating glutamate or aspartate residues, bind through ionic interactions with the Ca^{2+} present in HA [17, 18]. Polyglutamate and polyaspartate motifs are found within endogenous bone-resident proteins such as bone sialoprotein and osteocalcin, and their natural function is to localize these proteins to bone matrix [17-20]. To mimic this process, polyglutamate sequences have been incorporated into synthetic bioactive peptides to improve peptide binding to a variety of graft materials including allograft, anorganic bovine bone (ABB), and synthetic HA [21-27]. As an example, our group determined that adding a heptaglutamate (E7) domain to an osteoinductive BMP2-derived

peptide (BMP2pep) significantly increased the amount of peptide that could be loaded onto the graft, as well as retention of the peptide on the graft following implantation [21]. In addition, grafts coated with E7-modified BMP2pep elicited significantly more new bone formation than grafts passively adsorbed with unmodified BMP2pep in a rat mandibular defect model [21]. These results confirmed that better coupling of osteoinductive factors to the graft surface was effective in enhancing the bone regenerative response.

Polyglutamate domains have been primarily used to couple osteoinductive and cell adhesive peptides to graft materials [21, 22, 25, 26], however angiogenic peptides hold considerable potential for augmenting osteogenesis. Angiogenesis plays a crucial role in bone healing [28, 29], and the lack of rapid vascularization into a graft site is one of the major barriers hindering bone regeneration [30]. One of most potent inducers of angiogenesis is VEGFA. VEGFA stimulates the migration and proliferation of endothelial cells through its activation of surface receptors such as VEGFR2 (KDR) [31]. A wealth of studies has established that VEGFA promotes neovascularization within injured tissues [32], and also enhances graft integration and viability [7, 33, 34].

Given the importance of neovascularization in osteoregeneration, the current investigation aimed to functionalize graft materials with an angiogenic peptide derived from VEGFA, referred to as the “QK” peptide [35]. The QK peptide encompasses amino acids 17-25 of the VEGFA protein, a sequence that constitutes the principal domain within VEGFA that binds VEGFR2 [36]. As with VEGFA, the binding of QK to endothelial cell receptors stimulates signaling

events, such as ERK and Akt activation, that promote angiogenic behaviors including cell migration and *in vitro* tubule formation [35, 37]. Moreover, *in vivo* studies have confirmed QK's capacity to induce angiogenesis in a number of animal models [37-39]. In view of these findings, we investigated whether synthesizing QK with an E7 domain would increase peptide association with calcium phosphate graft materials, thereby facilitating more efficient peptide delivery within graft sites. Here we report that E7-modified QK peptides (E7-QK) exhibited significantly better binding than unmodified QK to two types of graft materials, ABB and synthetic HA. Importantly, the increased concentration of E7-QK vs. QK on the graft surface elicited more robust activation of endothelial cells seeded onto the grafts. Collectively these studies highlight the use of E7-QK peptides as a promising therapeutic modality for improving vessel in-growth into bone graft sites.

MATERIALS AND METHODS

VEGF mimetic peptides

All peptides utilized in this study were custom synthesized by Bachem. The QK peptide (KLTWQELQLKYKGI) was synthesized with or without an E7 domain, along with a three-glycine linker sequence to separate the QK domain from the E7 moiety. More specifically, the E7-QK peptide sequence is KLTWQELQLKYKGIGGGEEEEEEE, and the QK sequence is KLTWQELQLKYKGIGGG. For some experiments, E7-QK and QK peptides were also modified with a fluorescein isothiocyanate (FITC) group to facilitate studies

of peptide binding to graft. The FITC tag was chemically conjugated to the N-termini of the peptides. Peptides used for cell signaling studies did not have the FITC tag. Lyophilized peptides were reconstituted in deionized water at a concentration of 1 mg/mL, aliquoted, and stored in -20°C until use. rVEGF (R&D Systems, 293-VE-010) was reconstituted to a 5 µg/mL stock solution, and stored at -20°C.

Graft materials

0.4 g of HA powder (MP Biomedicals, 02150162) were pressed into disks using a 15.875 mm die under 3000 psi as in our prior publications [40]. The HA disks were then sintered at 1000°C in a Thermolyne 48000 series furnace for 4 hrs and allowed to return gradually back to room temperature. Anorganic Bovine Bone (ABB, BioOss) was purchased from Geistlich. ABB graft and HA disks were stored under sterile dry conditions and autoclaved before use.

Endothelial cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from ATCC (HUV-EC-C CRL-1730) and cultured in F-12K media (ATCC 30-2004) with 10% Fetal Bovine Serum (FBS), 0.1 mg/mL heparin (Sigma H3393), 1% antibiotic/antimycotic supplement (Invitrogen), and endothelial cell growth supplement (ECGS, Sigma E0760). Prior to experiments, cells were incubated for 12 hrs in serum-free F-12K media. Cell passages 3-9 were used for all experiments.

Binding of FITC-labeled peptides to bone graft materials

FITC-labeled QK and E7-QK peptides were used to monitor peptide binding to graft materials. Stock solutions of QK or E7-QK were diluted to a final concentration of 1 µM in Tris-buffered saline (TBS). These solutions were used to coat HA disks or 25 mg of ABB for time points ranging from 30 min to 6 hrs. After coating, samples were briefly washed with TBS to remove any unbound peptide and then imaged using a Leica MZ16F fluorescent dissecting microscope. Grafts coated with either QK or E7-QK, along with uncoated controls, were imaged in the same field to enable a direct comparison. Images were captured using a Hamatsu camera system and SimplePCI imaging software. Pixel intensity for the captured images was evaluated using ImageJ software to measure differences in FITC-labeled peptides bound to graft substrate.

Endothelial cell migration

A linear scratch defect model was used to monitor the migration of endothelial cells. HUVECs were seeded at a density of $1x10^5$ cells/well in a 48 well plate, and allowed to grow to confluency. A linear scratch approximately 600 µm wide was introduced into the monolayer, and cells were then incubated at 37°C in serum-free F-12K media containing either 50 ng/mL of rVEGF, or 25 nM of either QK or E7-QK peptide. The scratch wound cultures were incubated in the EVOS FL Auto Cell Imaging System (ThermoFisher Scientific) at 37ºC, and images were taken at 6 and 12 hrs. Relative closure of the scratch wound was quantified using EVOS FL Auto Cell Imaging System Software.

Endothelial tubule formation

150 µL/well of Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (ThermoFisher Scientific, A1413202) substrate was placed inside a 24 well plate and incubated for 30 min at 37°C to solidify the matrix. Prior to tubule assays, HUVECs were stained with CellTracker Green CMFDA (Life Technologies, C7025) accordingly to the vendor protocol. The labeled HUVECs were then seeded onto Geltrex™ matrices ($1x10^5$ cells/well) in serum-free F-12K media containing 50 ng/mL of rVEGF, or 25 nM of either QK or E7-QK. After a 6 hr incubation, tubule formation was captured from at least 3 random fields/well at 10x magnification using the EVOS FL Auto Cell Imaging System. Network branches and nodes were counted from the collected images to quantify angiogenic network formation.

Activation of signaling cascades in endothelial cells exposed to peptides presented in solution

HUVECs were incubated in serum-free F-12K media containing 25 nM of QK or E7-QK peptide for 10 min. The cells were then lysed in RIPA buffer (ThermoFisher Scientific, 89901) supplemented with 1% protease and phosphatase inhibitors (Sigma). Protein concentration was quantified through BCA analysis (ThermoFisher Scientific, 23209). Protein samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) overnight at 4°C. Membranes were placed in a blocking solution of 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TBST) for 1 hr at 37°C. Blots were probed with primary antibodies specific for either p-Akt

(S473, Cell Signaling, 4060), total Akt (Cell Signaling, 4691S), p-ERK 1/2 (T202/Y204, Cell Signaling 4370L), or total ERK 1/2 (Cell Signaling, 9102S) followed by incubation with HRP-linked secondary antibodies (Cell Signaling, 7074S). Blots were also probed with anti-β-tubulin (Abcam, ab21058) to ensure even loading of protein lysates. Proteins were detected by enhanced chemiluminescence using Clarity Western ECL substrate (BioRad, 170-5060). Densitometric analyses of immunoblots were performed using ImageJ, and the Densitometric Units (DU) measured for the phosphorylated signaling molecule were normalized to the DUs obtained for the respective total amount of protein.

Activation of signaling cascades in cells seeded onto peptide-coated HA disks

HA disks were placed within individual wells of a 24 well plate and then coated for 2 hrs with 0.5 mL of TBS containing 25 nM of either QK or E7-QK peptide. As a negative control, disks were incubated for 2 hr with TBS ("uncoated"). After this interval, the disks were washed briefly with TBS to remove unbound peptide. $5.0x10^5$ HUVECs were seeded onto the HA disks and allowed to attach for 30 min at 37ºC. The disks with adherent HUVECs were then submerged in RIPA buffer containing 1% protease and phosphatase inhibitors for 20 min at 4°C to lyse the attached cells. Cell lysates were concentrated using Amicon Ultra-0.5 Centrifugal Filter Devices (Millipore, UFC500396) and protein concentration was quantified by BCA analysis. Protein samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes overnight at 4°C. Membranes were probed for p-Akt, total Akt, p-ERK 1/2 , total ERK 1/2 and β-tubulin as described previously.

Statistical analysis

Peptide/graft binding experiments were performed three independent times, with each experiment performed in duplicate. Migration assays and endothelial tubule formation assays were conducted in three independent experiments, each experiment executed with triplicate wells. A student's t-test was used to measure the differences between experimental groups. Values were considered significant with a P value of <0.05. Immunoblots shown are representative of at least three independent experiments. Densitometric analysis comparing the relative phosphorylated protein levels versus total levels were averaged between the independent experiments. Relative densitometric values were considered significant with a P value of <0.05.

Results

E7-QK exhibits better binding to bone graft materials than QK.

FITC-labeled QK or E7-QK peptides were coated onto HA disks or ABB particles for time intervals ranging from 30 min to 6 hrs. Fluorescent images of the QK and E7-QK coated materials (as well as uncoated control materials, Unc) were captured at various timepoints to monitor the changes in peptide binding over time. As shown in Fig 1A, a greater amount of E7-QK was apparent on both HA and ABB substrates at all time points when compared to grafts coated with QK peptide or uncoated grafts. To quantify peptide binding to the substrates, images were examined for pixel intensity using Image J (Fig 1B & C). The pixel intensities confirmed that there was a substantial increase in the amount of

bound E7-QK relative to QK. Furthermore, the amount of E7-QK that bound to HA and ABB increased over the 6 hr interval, whereas maximal binding of the QK peptide was observed within ~30 min. These data suggest that the E7 domain is effective in improving the coupling of QK to two distinct graft materials.

E7-QK elicits a proangiogenic phenotype in endothelial cells.

Having verified that the E7 domain improved peptide binding to graft, the effect of E7-QK on endothelial cell behavior was next evaluated. While prior studies have confirmed the angiogenic properties of the QK peptide [35, 37, 38], it was important to insure that the addition of the E7 domain did not negatively impact QK's activity. To assess endothelial cell migration in response to E7-QK, a scratch wound assay was performed. Linear scratch defects were created in confluent HUVEC monolayers, and then cells were incubated with serum-free media ("untreated") or serum-free media containing either rVEGF (positive control), QK, or E7-QK. Cell migration into the scratch wound was monitored in real-time using the EVOS imaging system, and changes in scratch wound width were quantified at 6 and 12 hrs (Fig 2A and B, respectively). At both time points, HUVECs treated with rVEGF, QK, or E7-QK exhibited greater migration as compared with HUVECs in control media (representative images in Fig 2C).

To further evaluate E7-QK activity, an endothelial tubule formation assay was performed. HUVECs were seeded onto GELTREX matrices, and then cells were incubated for 6 hrs with serum-free media (untreated), or serum-free media containing rVEGF, QK, or E7-QK. Images taken at the end of this interval showed a high volume of interconnectivity between HUVECs grown in E7-QK,

rVEGF, or QK solutions (Fig 3A). However, in the absence of any angiogenic stimulus, the cells were unable to form tubules. A quantitative analysis of tubule nodes (Fig 3B) and branches (Fig 3C) revealed no differences in the capacity of E7-QK, rVEGF and QK to stimulate tubule formation, although all three stimuli induced significantly more tubule formation than the untreated control. These studies, combined with the cell migration assays, confirmed that the addition of the E7 domain did not diminish the potency of the QK peptide in stimulating angiogenic endothelial cell behaviors.

E7-QK activates angiogenesis-associated signaling cascades.

The angiogenic activity of E7-QK was also evaluated by monitoring intracellular signaling cascades downstream of VEGFR2 activation, specifically, the phosphorylation of ERK and Akt kinases [41, 42]. HUVECs were treated for 10 min with either serum-free media or serum-free media containing QK or E7-QK. Cells were then lysed and immunoblotted for phosphorylated and total levels of ERK 1/2 and Akt. Cells incubated in QK and E7-QK solutions displayed higher levels of p-ERK 1/2 and p-Akt as compared with untreated HUVECs (Fig 4A). Importantly, QK and E7-QK stimulated equivalent activation of ERK 1/2 (Fig 4B) and Akt (Fig 4C), suggesting that the E7 domain did not impair the ability of the QK sequence to bind and activate VEGF receptors.

Activation of ERK and Akt is increased in cells exposed to HA disks coated with E7-QK versus QK peptides.

We next tested whether E7-QK retained its bioactivity when bound to graft materials. To this end, HA disks were coated for 2 hrs with solutions containing

either QK or E7-QK, or incubated in saline (uncoated) as a control. The disks were subsequently washed to remove unbound peptides. HUVECs were seeded onto the treated disks for 30 min to allow cell attachment and interaction with the peptide-coated surfaces. After this interval, cells were lysed and immunoblotted for activation of ERK 1/2 and Akt (Fig 4D). Densitometric analysis of phosphorylated protein levels normalized to total levels revealed strikingly higher levels of p-ERK 1/2 (Fig 4E) and p-Akt (Fig 4F) in cells attached to QK-coated, or uncoated, disks. Taken together, these data suggest that the E7 domain can be used to concentrate active QK peptides onto bone graft materials.

DISCUSSION

The functionalization of non-autologous bone graft materials with bioactive factors constitutes a highly active area of research. Both osteoinductive and angiogenic factors have been investigated for their potential to improve graft performance, however better methods are needed for coupling these factors to the graft surface [43-46]. In the current study we evaluated a method for increasing the binding of an angiogenic peptide, QK, to the surface of calcium phosphate materials. By adding an E7 domain to the QK peptide, we achieved a 4-6-fold enrichment in the amount of peptide loaded onto two graft materials used in the clinic, ABB and synthetic HA. Similar results were reported by Lee et al., who showed that QK binding to HA biomaterials could be enhanced by adding an HA-binding sequence derived from osteocalcin [43]. In tandem with HA binding domains, the QK peptide has been engineered with sequences that

have affinity for other bone matrix molecules such as collagen I [47]. As an alternative to peptides with matrix binding domains, soluble QK peptides have been encapsulated within hydrogels [39, 48, 49]. Upon implantation, QK either diffuses from the hydrogel, or is released as the hydrogel degrades. While peptide-containing hydrogels have many worthwhile features, bone grafting procedures often require the use of mineralized materials, which have greater mechanical strength, and offer architectural and biochemical properties reflective of native bone [50, 51].

The use of E7-QK to augment the osteoregenerative capacity of graft materials offers several advantages. First, E7-QK peptides can, in theory, be applied to any type of calcium phosphate, providing versatility in clinical applications. While the current investigation focused on ABB and HA, other studies have demonstrated that the E7 domain binds with high affinity to all calcium phosphate materials tested to date including several types of human allograft as well as β-tricalcium phosphate [22, 23]. The E7-QK peptide can be stored as a lyophilized powder and reconstituted in saline for immediate use, suggesting that this coating technique may be readily implemented in the clinic. As another benefit, short, synthetic peptides are simpler, and more cost-effective, to produce than the full-length proteins from they were derived [52]. Recombinant proteins are typically generated via host cell systems, which can introduce contaminants such as cellular by-products or pathogens [53]. In contrast, large amounts of highly pure synthetic peptides can be produced by a commercial peptide synthesizer.

The capacity of the QK peptide to substitute for rVEGF in stimulating neovascularization is well-established. QK stimulates the same endothelial cell behaviors as rVEGF [37], and has comparable angiogenic potency in multiple animal models [37-39]. Consistent with this literature, we find that QK induces endothelial cell migration, tubule formation and activation of key signaling molecules such as ERK and Akt. Importantly, these functions of QK are not diminished by the addition of the E7 domain. E7-QK retains full activity when presented to cells either in solution, or following immobilization onto HA disks. In fact, because significantly more E7-QK than QK binds to HA, endothelial cells seeded onto E7-QK-coated HA disks are strongly activated, as evidenced by ERK 1/2 and Akt phosphorylation. Contrarily, cells exposed to QK-coated HA disks display limited activation of ERK 1/2 and Akt, consistent with the poor binding of QK to HA. The fundamental concept that concentrating QK onto a material surface can enhance endothelial cell activation is supported by other studies. For example, Yang et al. covalently linked QK to electrospun scaffolds, and found that endothelial cells adherent to the QK-conjugated scaffolds had greater viability than cells attached to scaffolds with passively adsorbed QK [54].

In summary, our collective results suggest that the E7 domain serves as an effective tool for concentrating angiogenic peptides on the surface of diverse calcium phosphate graft materials. This, in turn, should enable higher doses of the peptide to be delivered within the graft site, providing a more robust angiogenic stimulus. Given that inadequate, or delayed, vascularization is a major impediment to bone regeneration, the current study offers a promising new

therapeutic modality for enhancing the performance of non-autologous graft materials commonly used in craniofacial and orthopedic procedures.

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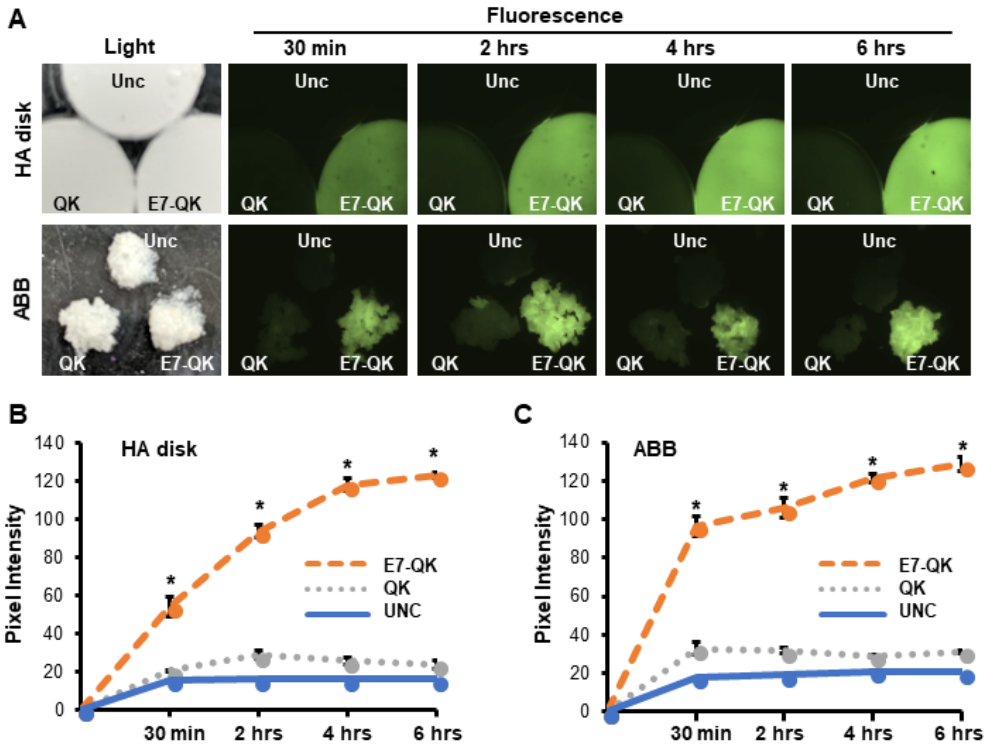

Fig 1. E7 domain directs greater loading of E7-QK onto HA disks and ABB particles. (A) HA disks or ABB particles were incubated with 1 μM of FITC-tagged QK or E7-QK peptides for time points ranging from 30 min to 6 hrs. As a control, samples were incubated in TBS (uncoated, "Unc"). Following these incubations, samples were washed in TBS and imaged by fluorescent microscopy, which revealed greater binding of E7-QK. Unc, QK and E7-QK coated samples were imaged within the same field (as depicted in the light microscopy image) to enable a direct comparison. (B &C) Images from HA disks (B) or ABB (C) were analyzed by Image J to quantify fluorescence intensity. Values represent means and S.E.s from three independent experiments. * denotes $p<0.05$ (relative to Unc samples).

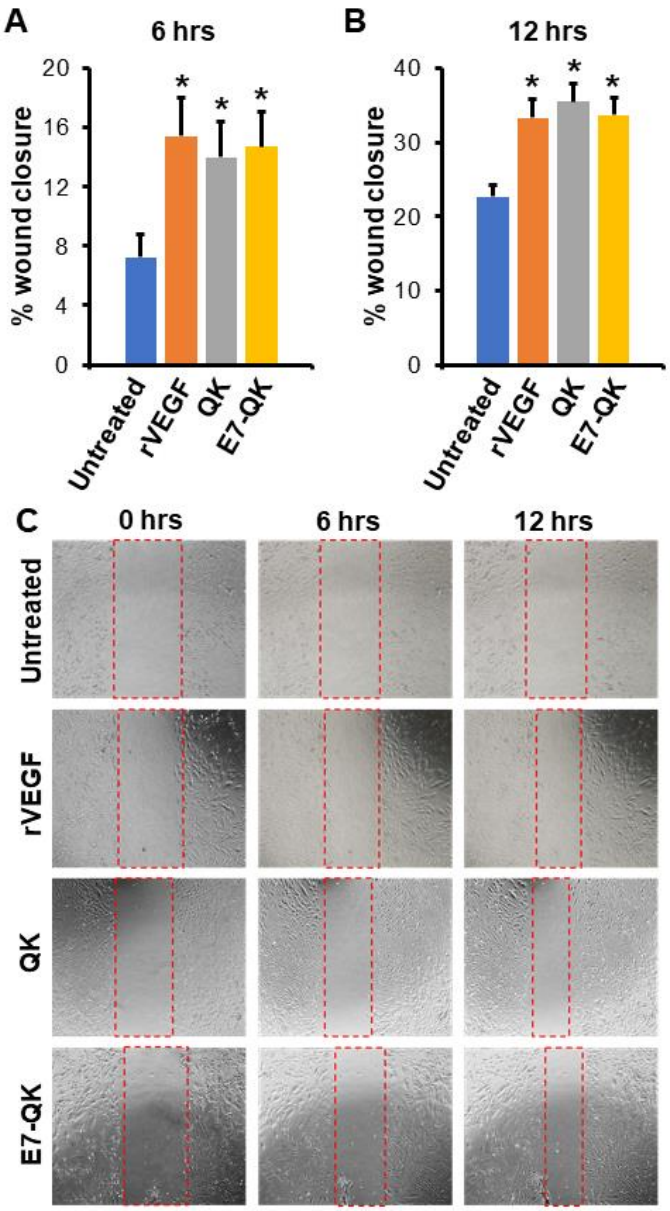

Fig 2. E7-QK stimulates endothelial cell migration. Scratch wounds were introduced into HUVEC monolayers, and then cells were incubated with serum-free media (untreated) or serum-free media containing 50 ng/ml rVEGF, or 25 nM of either QK or E7-QK. (A&B) Analyses of cell migration at 6 (A) and 12 (B) hrs indicated that all of the treatments elicited more robust migration as compared with controls. (C) Representative images with defect area indicated by red dashed lines. Values represent means and S.E.s from 3 independent experiments. * denotes $p<0.05$.

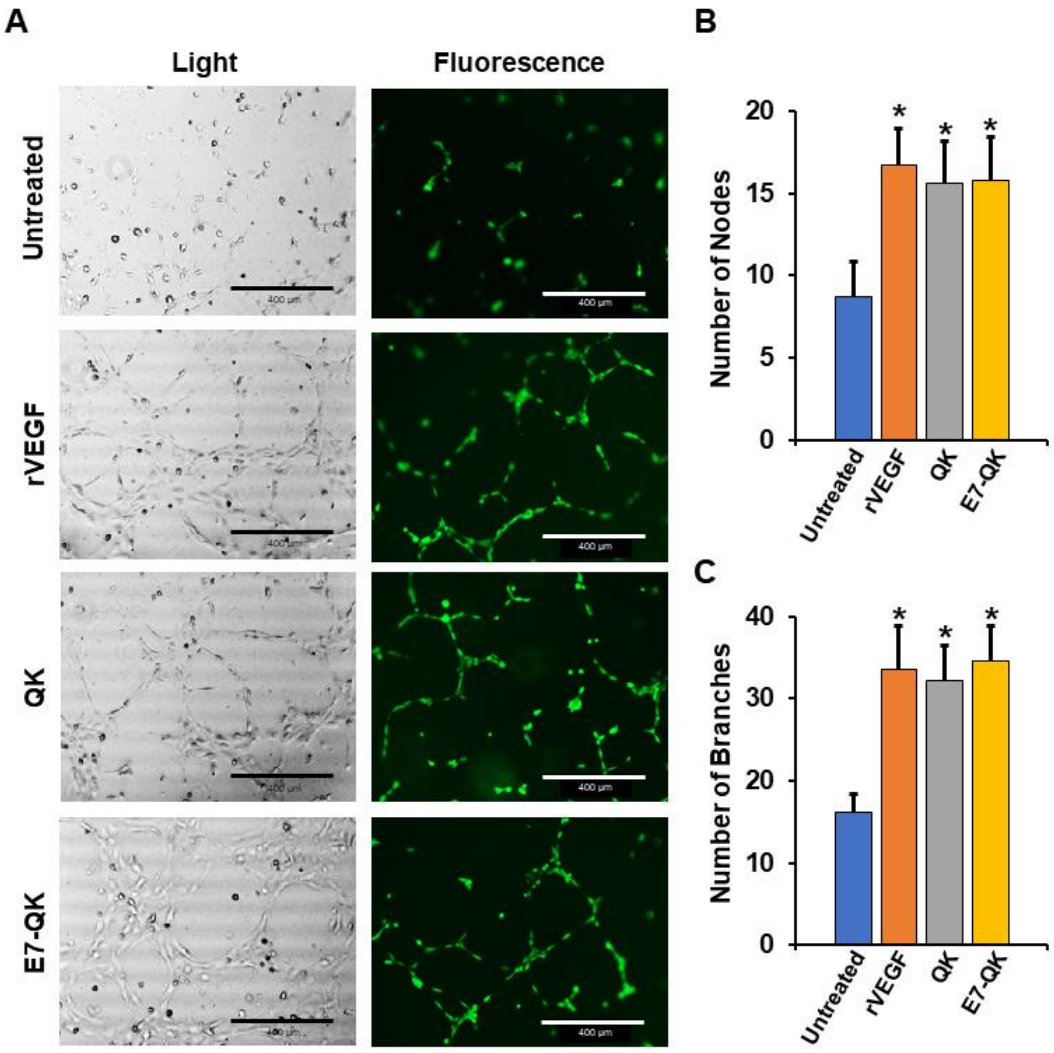

Fig 3. E7-QK induces endothelial tubule formation. HUVECs were pre-labelled with Cell Tracker Green dye, and then seeded onto GELTREX matrices in either serum-free media (untreated) or serum-free media containing 50 ng/mL rVEGF, or 25 nM of either QK or E7-QK peptides. (A) Tubule formation was monitored at 6 hr after cell seeding (phase contrast images in left panels; fluorescent images in right panels). Greater nodal formation (B) and branching (C) were observed in all experimental groups relative to controls. Images are representative of 3 random fields/experimental well. Values represent means and S.E.s from 3 independent experiments, with each experiment performed in triplicate. Scale bar = 400µm.

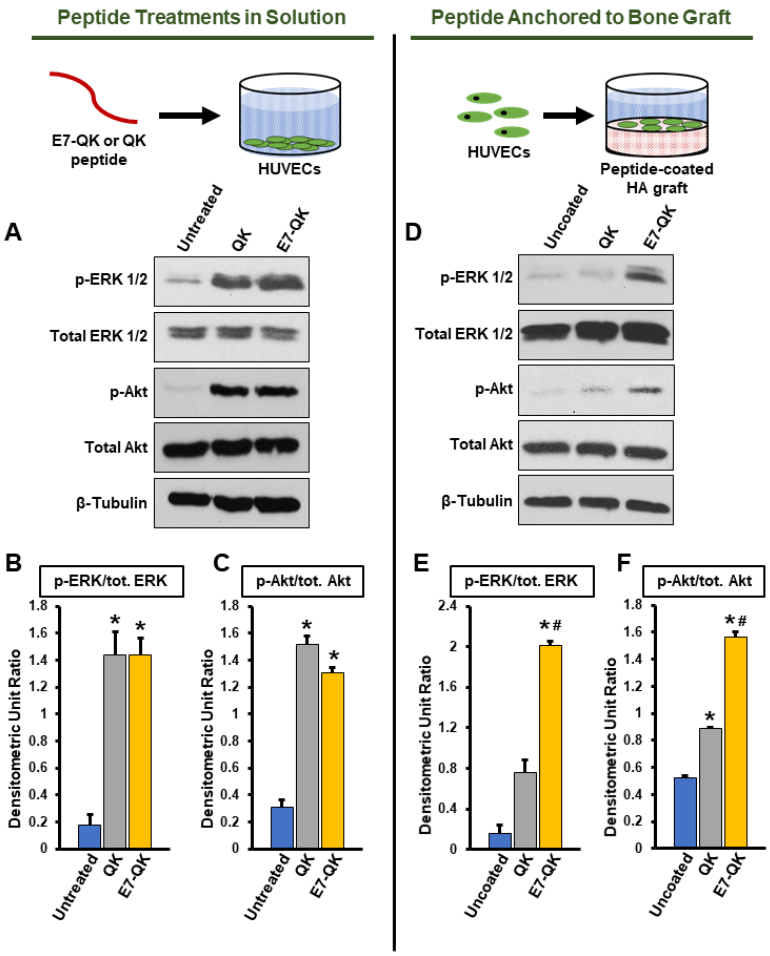

Fig 4. Cell signaling activation in cells exposed to E7-QK in solution, or E7-QK immobilized on HA disks. (A) HUVECs were incubated for 10 min with either serum-free media (untreated) or serum-free media containing 25 nM of QK or E7-QK peptide. Cells were then lysed and immunoblotted for p-ERK1/2, total ERK 1/2, p-Akt, total Akt, or β-tubulin. (B&C) Densitometric analyses of blots were conducted using Image J, and values for phosphorylated ERK 1/2 (B) and Akt (C) were compared to densitometric values for total ERK 1/2 and Akt (Densitometric Unit Ratio). Graphs depict means and S.E.s from three independent experiments. * denotes $p<0.05$ (relative to Untreated samples). (D) HA disks were coated for 2 hrs with either TBS (uncoated) or TBS containing 25 nM of QK or E7-QK peptides. Disks were then washed to remove unbound peptides. HUVECs were seeded onto the treated disks and allowed to adhere for 30 min. The cells were lysed, and after a concentration step, the lysates were immunoblotted for p-ERK 1/2, total ERK 1/2, p-Akt, total Akt and β-tubulin. (E&F) Densitometric analyses of blots were conducted using Image J, and values for phosphorylated ERK 1/2 (E) and Akt (F) were normalized to total protein levels. Graphs depict means and S.E.s from three independent experiments. * denotes $p<0.05$ (relative to Uncoated samples) and # denotes $p<0.05$ (relative to QK peptide coated disks).

SUSTAINED DELIVERY OF THE ANGIOGENIC QK PEPTIDE THROUGH THE USE OF POLYGLUTAMATE DOMAINS TO CONTROL PEPTIDE RELEASE FROM BONE GRAFT MATERIALS

by

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ABSTRACT

Angiogenesis plays a pivotal role in tissue regeneration following bone grafting procedures, however nonautogenous graft materials typically lack critical angiogenic growth factors. While much research has focused on modifying grafts with angiogenic factors, controlled delivery of these molecules remains a challenge. The current study describes a method for sustained delivery of an angiogenic peptide from hydroxyapatite (HA), a common alloplast material. Specifically, VEGF-derived "QK" peptides were synthesized with polyglutamate domains containing varying numbers of glutamates. The rate of peptide release from HA inversely correlated with glutamate number, with diglutamate-QK (E2-QK) released first, followed by tetraglutamate-QK (E4-QK), and finally, heptaglutamate-QK (E7-QK). By coating HA with a mixture of these peptides, termed, PGM-QK (polyglutamate-modified mixture), sequential peptide release was achieved, enabling gradient QK delivery. To evaluate bioactivity, HA disks were coated with PGM-QK and then placed in fresh media for 6 days. Media containing the released peptides was collected at varying time intervals and placed on Human Umbilical Vein Endothelial Cells (HUVECs). Cells were evaluated for activation of angiogenic signaling pathways (ERK and Akt) and cell migration. Results showed that QK peptides were continuously released over the 6-day interval, and maintained their capacity to activate HUVECs. These findings point to a new approach for gradient delivery of an angiogenic stimulus.

INTRODUCTION

Tissue revascularization is critical for the healthy repair of bone following bone grafting procedures, as the vasculature provides the necessary growth factors and cells to facilitate osteoregeneration. Delayed or insufficient vessel development impedes repair during the early stages of bone callus formation and recovery [1-3]. One limitation associated with commercial bone graft materials, which are derived from nonautogenous sources, is that they often lack the requisite growth factors to elicit effective neovascularization. Allografts and xenografts require treatment to remove immunogenic or pathogenic material, however growth factors are typically damaged in this process.[3-5]. On the other hand, fully synthetic graft materials, such as hydroxyapatite (HA) or β-tricalcium phosphate (β-TCP), have no organic components [3, 5]. Thus, considerable research efforts have been devoted to reconstituting these various graft materials with pro-angiogenic molecules [6-8].

Several growth factors, such as PDGF, VEGF, and FGF, have been investigated as potential therapeutic agents for stimulating revascularization at graft sites [6, 9-13]. VEGF is one of the most widely studied due to its potent angiogenic activity [6, 9, 14-16]. An extensive literature has shown that delivery of VEGF within bone defects enhances tissue regeneration [15, 16]. However, one challenge related to the clinical use of VEGF is that this growth factor's function is heavily reliant upon its gradient release from a graft carrier [17-19]. In native tissues, a chemotactic VEGF gradient is important for promoting endothelial chemotaxis, and correspondingly, vessel in-growth, into the injured

site [19, 20]. Many investigators have achieved gradient release through the use of hydrogels and other material carriers for VEGF, however, at present, there are few effective approaches for establishing gradient release from mineralized graft materials [21-24]. For many graft applications, mineralized substrates are needed due to their favorable mechanical and architecture features, as well as their high degree of osteoconductivity [25, 26].

One strategy for reconstituting mineralized grafts with growth factors is to engineer recombinant proteins or biomimetic peptides with graft-specific binding domains [7, 27-34]. One such domain is polyglutamate, a stretch of contiguous glutamate residues that bind to HA, the main mineral constituent within bone. The negatively-charged glutamates interact ionically with the Ca^{2+} within the HA crystal [35-37]. In prior studies we demonstrated that a polyglutamate domain comprised of seven glutamates (heptaglutamate, E7) was very effective in anchoring a variety of mimetic peptides to graft materials; these include a BMP2-derived peptide, a VEGF-derived peptide, and the integrin-binding peptides, DGEA and RGD [7, 31-34, 38]. Notably, grafts functionalized with an E7-BMP2 mimetic peptide elicited more new bone formation in a rat mandibular implant model than grafts passively-adsorbed with the unmodified BMP2 peptide [31]. These data support the concept that better coupling of peptides to the graft surface leads to improved osteoregeneration. Furthermore, in a study of the DGEA peptide, we showed that modulating the number of glutamate residues within the polyglutamate domain could be leveraged to control peptide retention

on the graft [39]. More specifically, peptide retention increased in accordance with glutamate number [39].

In the current manuscript we used polyglutamate domains with varying numbers of glutamates to achieve prolonged delivery of the VEGF mimetic peptide, QK. QK has emerged as a very promising reagent for stimulating tissue vascularization. Several *in vivo* studies have suggested that the angiogenic potential of the QK peptide is comparable to that of the full-length recombinant VEGF (rVEGF) protein [40-42]. QK binds to the VEGFR2 receptor on endothelial cells, leading to the activation of signaling cascades such as Akt and ERK1/2 [41]. These VEGFR2-driven pathways subsequently direct angiogenic endothelial cell behaviors including cell migration and tubule formation. To implement sustained delivery of the QK peptide from a synthetic HA scaffold, QK was synthesized with diglutamate (E2-QK), tetraglutamate (E4-QK), or heptaglutamate (E7-QK). These peptides were shown to release from HA sequentially, depending upon the number of glutamates within the polyglutamate domain. In addition, we created a 1:1:1 mixture of the peptides, termed, PGM-QK (PolyGlutamate-modified Mixture), in order to develop a gradient delivery system on HA. Our collective results suggest that the PGM-QK mixture enables the sustained delivery of active QK peptide, which in turn promotes the prolonged activation of endothelial cells.

MATERIALS AND METHODS

Synthesis of HA disks

0.4 g of HA powder (MP Biomedicals, 02150162) were pressed into disks using a 15.875 mm die under 3000 psi as in our prior publications [39]. The HA disks were then sintered at 1000°C in a Thermolyne 48000 series furnace for 4 hrs. and allowed to return gradually back to room temperature. HA disks were stored under sterile dry conditions and autoclaved before use.

Endothelial cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from ATCC (HUV-EC-C CRL-1730) and cultured in F-12K media (ATCC 30-2004) with 10% Fetal Bovine Serum (FBS), 0.1 mg/mL heparin (Sigma H3393), 1% antibiotic/antimycotic supplement (Invitrogen), and endothelial cell growth supplement (ECGS, Sigma E0760). Prior to experiments, cells were incubated for 12 hrs in serum-free F-12K media. Cell passages 3-9 were used for all experiments.

Treatment of HUVECs with polyglutamate-modified peptides in solution

HUVECs were incubated in serum-free F-12K media containing the individual peptides or the PGM-QK mixture. For the single peptide treatments, solutions were comprised of 250 nM QK (the native peptide lacking a polyglutamate domain), E2-QK, E4-QK, or E7-QK. For the PGM-QK treatments, a 1:1:1 peptide ratio was used with a final peptide concentration of 250 nM (i.e., 83.3nM of E2-QK, E4-QK and E7-QK, each). Endothelial cells were treated for 10 min with the peptide solutions, and then cells were lysed in RIPA buffer (ThermoFisher

Scientific, 89901) supplemented with 1% protease and phosphatase inhibitors (Sigma). Protein concentration was quantified through BCA analysis (ThermoFisher Scientific, 23209). Protein samples were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) overnight at 4°C. Membranes were placed in a blocking solution of 5% non-fat dry milk in TBS containing 0.1% Tween 20 (TBST) for 1 hr at 37°C. Blots were probed with primary antibodies specific for p-Akt (S473, Cell Signaling, 4060), total Akt (Cell Signaling, 4691S), p-ERK 1/2 (T202/Y204, Cell Signaling 4370L), or total ERK 1/2 (Cell Signaling, 9102S) followed by incubation with HRP-linked secondary antibodies (Cell Signaling, 7074S). Blots were also probed with anti-β-tubulin (Abcam, ab21058) to ensure even loading of protein lysates. Proteins were detected by enhanced chemiluminescence using Clarity Western ECL substrate (BioRad, 170-5060). Densitometric analyses of immunoblots were performed using ImageJ, and the Densitometric Units (DU) measured for the phosphorylated signaling molecule were normalized to the DUs obtained for the respective total amount of protein. All blots were conducted 3 times, using 3 independently generated cell lysates.

Migration of HUVECs

A linear scratch defect model was used to monitor the migration of endothelial cells. HUVECs were stained using a CellTracker™ Green CMFDA dye (ThermoFisher Scientific). Labeled HUVECs were seeded at a density of $1x10^5$ cells/well in a 48 well plate and allowed to grow to confluency. A linear scratch approximately 600 µm wide was introduced into the monolayer, and cells

were then incubated at 37°C in serum-free F-12K media with or without added peptides. In addition, we evaluated the PGM-QK mixture (a 1:1:1 ratio of E2-QK, E4-QK and E7-QK at a final concentration of 250 nM), as well as peptide-containing conditioned media collected from HA disks pre-coated with QK or PGM-QK. The scratch wound cultures were incubated in the EVOS FL Auto Cell Imaging System (ThermoFisher Scientific) at 37ºC, and images were taken at 12 hrs. Relative closure of the scratch wound was quantified using EVOS FL Auto Cell Imaging System Software.

Binding kinetics of FITC-labeled PGM-QK peptides to bone graft

FITC-labeled QK, E2-QK, E4-QK, and E7-QK peptides were used to monitor the binding properties of each peptide to HA disks. Stock solutions of each peptide were diluted to a 1 µM final concentration in Tris-buffered saline (TBS). HA disks were coated with the peptide solution for 2 or 6 hrs. After incubation, coating solutions were collected. Solution fluorescent intensity was measured for each peptide solution before and after incubation with the HA disk using a BioTek synergy H1 microplate reader. The loss in solution fluorescence was used to quantify the percentage of peptide removed from solution (due to immobilization on the HA disk). Additionally, HA disks were briefly washed after incubation to remove any unbound peptide and then imaged under a Leica MZ16F fluorescent dissecting microscope. Grafts coated with each individual peptide were imaged side-by-side to allow a direct comparison using a Hamatsu camera system and SimplePCI imaging software.

Release kinetics of PGM-QK peptides

FITC-labeled QK, E2-QK, E4-QK, and E7-QK peptides were monitored for release from HA disks. Stock solutions of each peptide were diluted to a 10 µM final concentration in TBS. HA disks were coated with the 10 µM solutions for 2 hrs. A 10 µM concentration was used for these studies (rather than 1 µM as above) in order to saturate each disk with the maximal amount of peptide. Binding percentages were calculated as previously described by measuring solution fluorescence. The coated disks were washed to remove any unbound peptide and fresh TBS was placed on the coated HA disks. Coated HA disks were then placed under gentle agitation for up to 6 days at 4°C using a Labnet Shaker 30 tabletop shaker at 200 RPM. TBS solutions containing the released peptide were collected from the samples 1, 3, or 6 days after the initial coating interval. Solution fluorescent intensity was measured for each collection time point using a BioTek Synergy H1 microplate reader. The cumulative percent of peptide released from graft material was calculated using the solution fluorescence intensities at each collection point as compared with the initial amount of peptide bound to the grafting material. Treated grafts were imaged side-by-side at each timepoint to allow a direct comparison of peptide release over time using a Hamatsu camera system and SimplePCI imaging software.

Statistical analysis

Peptide/graft binding and release experiments were performed three independent times, with each experiment performed in duplicate. Migration assays were conducted in three independent experiments, each experiment

executed with triplicate wells. A student's t-test was used to measure the differences between experimental groups. Values were considered significant with a P value of <0.05. Immunoblots shown are representative of at least three independent experiments. Densitometric analysis comparing the relative phosphorylated protein levels versus total levels were averaged between the independent experiments. Relative densitometric values were considered significant with a P value of <0.05.

Results

PGM-QK peptides retain their capacity to induce angiogenic signaling in endothelial cells.

QK peptides modified with polyglutamate domains were compared with the native QK peptide for their capacity to activate ERK and Akt signaling cascades in endothelial cells. HUVECs were treated for 10 min. with solutions containing QK, E2-QK, E4-QK, or E7-QK. Cells were then lysed and immunoblotted for phosphorylated and total levels of ERK1/2 and Akt (representative experiment in Fig 1A). Results from three independent experiments were quantified by densitometry, comparing the levels of phosphorylated to total protein (Fig 1B). Densitometric analyses indicated that all of the peptides induced significant increases in the activation of ERK and Akt relative to untreated control cells. Furthermore, there were no statistical differences between the activity of the native QK peptide and any of the polyglutamate-modified peptides, indicating that these peptides retain their full bioactivity.

We next examined the effect of the PGM-QK mixture (1:1:1 ratio of E2-QK, E4-QK, and E7-QK) on endothelial cell signaling. HUVECs were treated for 10 min. with a 250 nM final concentration of PGM-QK, or with 250 nM of the original QK peptide, and then evaluated for activation of ERK and Akt. As shown in Fig 2A, PGM-QK and QK induced an equivalent degree of ERK and Akt activation. We further investigated the activity of PGM-QK using a scratch wound assay to study cell migration. HUVECs were grown to confluency, and then a scratch wound was created. Cells were incubated for 12 hrs. in media containing 250 nM PGM-QK or 250 nM QK. PGM-QK and QK induced a comparable degree of cell migration (Fig 2B), confirming PGM-QK's pro-angiogenic activity.

Binding and release characteristics of PGM-QK components from HA graft material are dependent on the number of glutamate residues within the polyglutamate domain.

Polyglutamate-modified peptides were next assessed for binding and retention on HA graft materials. HA disks were coated for 2 or 6 hrs. in solutions containing 1 μM FITC-labeled QK, E2-QK, E4-QK, or E7-QK and then disks were imaged. Representative disks from each of the 4 groups were placed side-by-side in the same microscopic field to allow a direct comparison. Images of the disks (Fig 3A) show a clear relationship between the number of glutamates in the polyglutamate domain and the amount of peptide bound to the graft material, i.e. E7-QK bound better than E2-QK (Fig 3A). These images were corroborated by the calculated percentages of bound peptide, as determined by a solution fluorescence depletion assay. In this assay, the amount of fluorescence in the

solution is depleted as the peptide becomes bound to the HA disk. The solution fluorescence after binding is then compared with the fluorescence values for the starting solution. As shown in Fig 3B, increasing the number of glutamate residues resulted in an enrichment in the amount of peptide bound to the disk. These data confirmed that variable-length polyglutamate domains can be leveraged to tune the binding of peptides to HA.

We further hypothesized that polyglutamate domains would modulate the retention of peptides on the disks. To measure peptide release, the disks were first coated with a high concentration of peptide, 10 μM, to saturate the graft, thereby ensuring that peptides with varying loading efficiencies would have comparable levels of peptide initially bound. After coating, grafts were washed briefly with saline and imaged (Fig 4A, “Initial Coat”). The disks were then placed in fresh saline and incubated under gentle agitation over the course of 6 days. Samples of the solution, containing released peptide, were collected at each time point, and in addition, disks were imaged at these same time points. As shown in Fig 4A, the amount of peptide bound to the disks was comparable after the initial coat, however much of the unmodified QK peptide was released from the disk by day 1. In contrast, most of the E7-QK peptide was retained on the disk for the entire 6-day interval. Solution fluorescence measurements were consistent with these findings (Fig 4B). The rate of peptide release correlated with the number of glutamate residues within the polyglutamate domain, evidenced by the rapid release of QK and E2-QK, but sustained retention of E7-QK.

Prolonged delivery of PGM-QK from graft materials promotes endothelial cell activation.

The PGM-QK mixture, containing an equal ratio of E2-QK, E4-QK, and E7-QK, was coated onto HA disks. Based on data in Fig 4, we hypothesized that nearly all of the E2-QK peptide would be released from the disk between day 0 and day 3, whereas the E7-QK would continue to be released between days 3 and 6. After coating, disks were placed in serum-free media with gentle agitation for 1 day and then the media, with released peptide, was collected (day 0-1). Disks were then placed in fresh media and solutions were collected at 3 days after initial coating (days 1-3). The same disks were again placed in serum-free media, and solutions were collected at 6 days following initial coating (days 3-6, see diagram in Fig 5A). As a control, solutions from disks coated with the umodified QK peptide were collected at the same intervals, days 0-1, 1-3 and 3-6. The peptide-containing solutions were then used to treat HUVECs. Cells were treated with the solutions for 10 min., and then lysed and immunoblotted for activated ERK and Akt. As shown in Fig 5B, ERK and Akt activation were comparable in cells treated with the day 0-1 QK and PGM-QK, consistent with the rapid release of QK and E2-QK from the HA disks. However, for the day 3-6 solutions, PGM-QK elicited greater ERK and Akt activation than QK, in line with results in Fig 4B showing that E7-QK continues to be released during this time interval. Densitometric results in Fig 5C indicated that the degree of QK-induced cell activation decreases from day 1 through days 3 and 6, whereas cell activation by PGM-QK is sustained through all the time points. These data are

further supported by scratch wound assays. HUVECs treated with the PGM-QK solutions collected at the 3 time points exhibited greater migration into the scratch wound compared with cells treated with the QK-containing solutions (Fig 5D). In fact, none of the QK solutions elicited a statistical improvement in migration over the untreated cells. These results suggest that PGM-QK delivered on graft materials holds potential for facilitating sustained activation of endothelial cells.

DISCUSSION

One of the shortcomings associated with nonautogenous bone graft materials is their limited potential to stimulate vessel in-growth into the site. Accordingly, there is much interest in functionalizing these materials with angiogenic factors [6-8]. The VEGF-derived QK peptide is a robust activator of neovascularization, and has therefore been a focus of many tissue regenerative strategies [7, 21, 22, 43, 44]. In multiple preclinical animal models, the QK peptide and rVEGF have been reported to induce a comparable degree of angiogenesis [41, 42]. In seminal work by Finetti et al., angioreactors containing either QK or rVEGF stimulated equivalent vascular infiltration after subcutaneous implantation into mice [41]. Other investigators have tethered the QK peptide to scaffold materials and similarly shown a vast improvement in neovascularization [22]. In one such study, higher capillary density was observed in animals injected intramuscularly with a QK-delivering hydrogel [42]. However, despite these successes, delivery of QK from mineralized scaffolds poses a challenge due to the limited functional

groups available on HA for peptide coupling. There is clear need to control the delivery of angiogenic factors from mineralized grafts. First, VEGF, and derivatives such as QK, should optimally be delivered as a gradient to facilitate endothelial migration and directional vessel in-growth into the graft site [17-19]. Secondly, weak binding of an angiogenic factor to a scaffold typically leads to rapid, bolus release of the factor, which can cause off-target effects. As an example, uncontrolled bolus release of rVEGF results in the formation of insufficient, or leaky, vascular networks (although this has not yet been reported for the QK peptide) [45].

To address the need for controlling the delivery of QK, we modified the peptide with variable-length polyglutamate domains (E2, E4 and E7), and confirmed that the number of glutamates within the domain correlated with the rate of peptide release. We also verified that the bioactivity of the QK peptide was not adversely affected by the addition of glutamate residues. Subsequently, we generated a mixture of the various peptides, PGM-QK. We hypothesized that this mixture would recapitulate a temporal gradient, with E2-QK released first from the graft, followed by E4-QK, and then, E7-QK. The goal was to engineer sustained release of the active QK signaling domain, leading to prolonged activation of endothelial cells. To test this hypothesis, conditioned media was collected at varying time points from HA disks coated with PGM-QK or QK, and then HUVECs were incubated with the media containing the released peptides. Results from these experiments showed that components of the PGM-QK mixture were still actively released from the HA up to 6 days following graft

coating, and importantly, the released peptides activated HUVEC signaling and cell migration. In contrast, solutions collected from QK-coated disks had negligible activity following day 3 after coating, consistent with results suggesting that all of the QK peptide was released from the disk by day 3.

Domains other than polyglutamate have been explored for their potential to enhance coupling of bioactive molecules to mineralized materials [29, 46-49]. In one study, a polyaspartate motif was added to estradiol or levofloxacin, and these modified therapeutics were shown to localize to bone after systemic injection [46]. Additionally, proteins have been engineered with bisphosphonate moieties to promote anchoring to mineral substrates [29, 49]. In the aggregate, these investigations offer novel and effective methods for enhancing the binding of target molecules to bone or bone graft materials. However, the studies do not address the need for controlled release of the therapeutic. Angiogenic molecules such as VEGF are known to be more effective when released as a gradient.

As another advantage of the polyglutamate approach, the delivery of PGM-QK on bone graft materials should be readily amenable to clinical translation. As shown in binding experiments, loading of the PGM-QK components can be achieved in a few hours, facilitating same-day graft preparation and placement. Moreover, synthetic peptides such as QK are less expensive to synthesize than recombinant proteins, which require host-cell systems to produce. Host-cell systems can also introduce contaminants such as immunogenic cellular byproducts or pathogens [50]. PGM-QK peptides are synthesized using a commercial peptide synthesizer, and can be stored long-term as a lyophilized

product. Finally, we and others have shown that polyglutamate domains bind to a wide array of calcium phosphate graft materials including various alloplast (HA and β-TCP), xenograft (BioOss), and allograft (MinerOss, OraGraft) substrates, offering adaptability in the type of graft material used for clinical procedures [7, 31, 32, 34, 38]. Taken together, results in this study describe a relatively simple, and cost-effective, methods for the sustained delivery of the angiogenic QK peptide from HA-containing bone graft materials.

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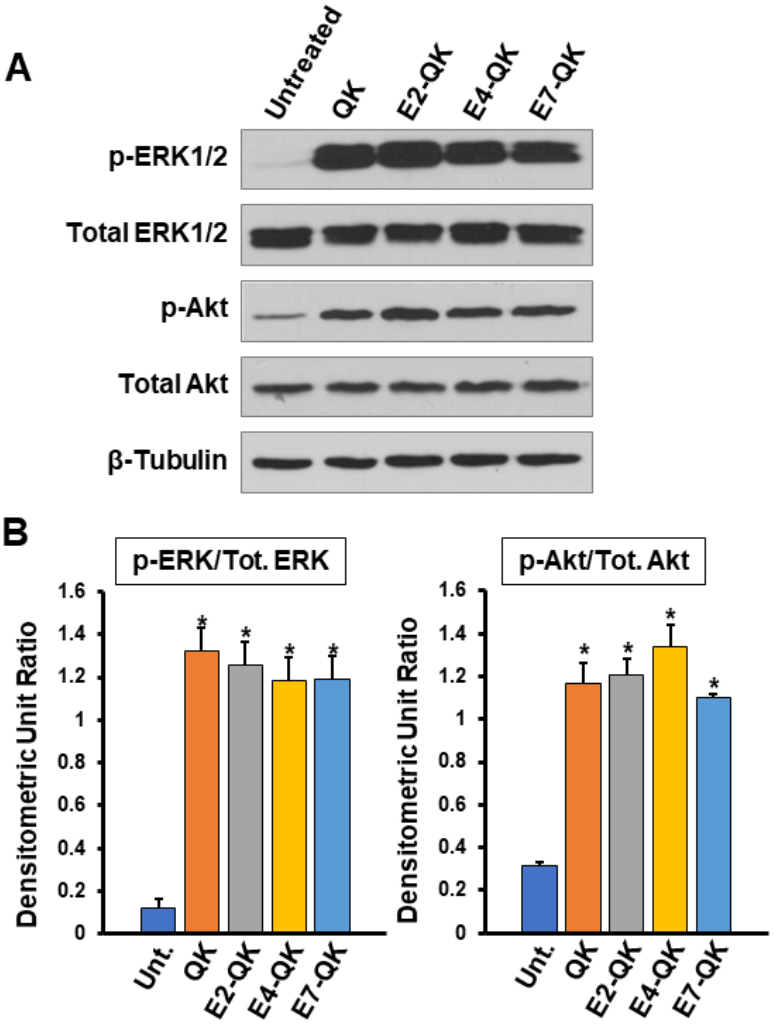

Fig 1. Polyglutamate modified QK peptides retain bioactivity. HUVECs were incubated for 10 min. with either serum-free media (Untreated, Unt.) or serum-free media containing 250 nM of QK, E2-QK, E4-QK, or E7-QK. Cells were lysed and immunoblotted for p-ERK1/2, total ERK1/2, p-Akt, total Akt, or β-tubulin. Densitometric analyses of 3 separate blots, generated from 3 independent cell lysates, were conducted using Image J. Values for phosphorylated ERK 1/2 and Akt were compared to densitometric values for total ERK1/2 and Akt (Densitometric Unit Ratio). All treatment groups showed activation of ERK1/2 and Akt, indicating that each polyglutamate-modified peptide retains bioactive function. Values represent means and S.E.s from 3 independent experiments. * denotes $p<0.05$ as compared with Untreated.

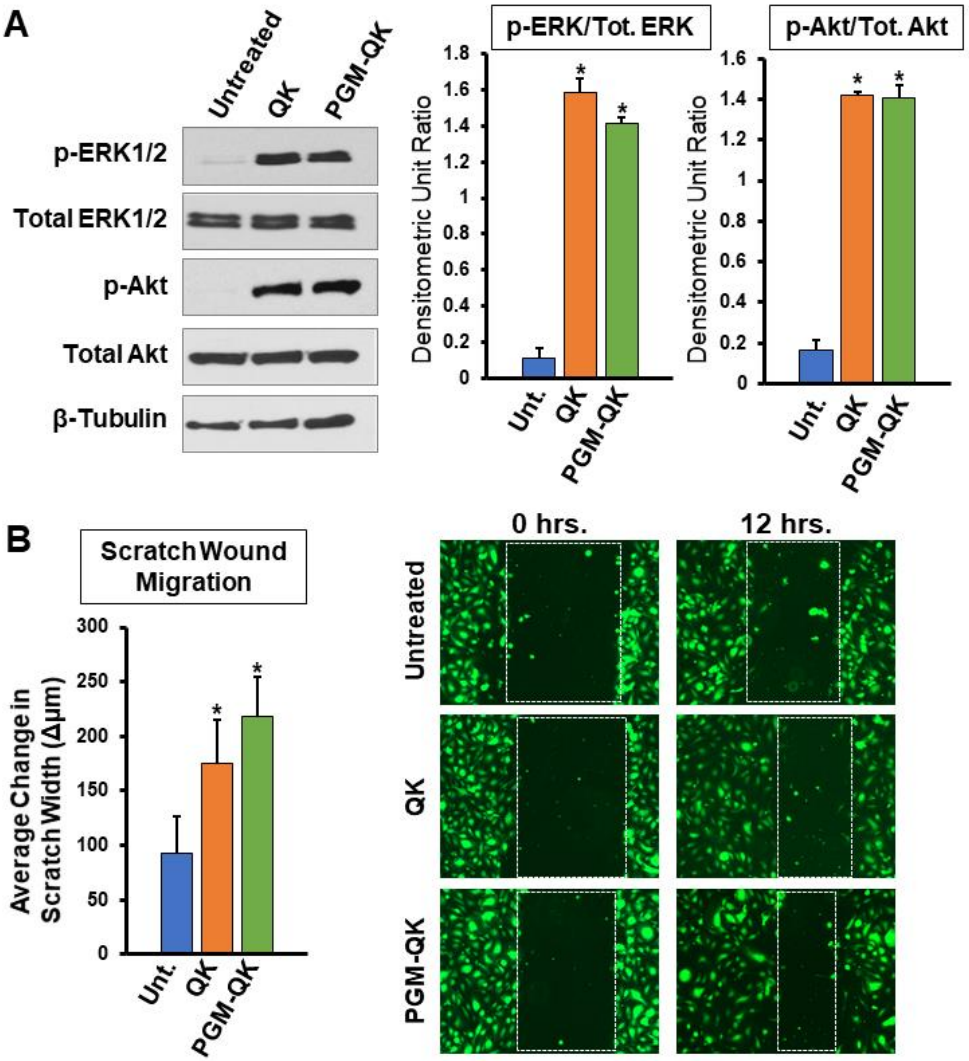

Fig 2. PGM-QK stimulates endothelial cell signaling activation and migration. (A) HUVECs were treated for 10 min. with serum-free media (Unt.) or serum-free media containing 250 nM of QK, or 250 nM PGM-QK (1:1:1 mixture of E2-QK, E4-QK and E7-QK). Cells were lysed and immunoblotted for p-ERK1/2, total ERK1/2, p-Akt, total Akt, or β-tubulin. Densitometric analyses of 3 independent blots were conducted using Image J, and values for phosphorylated ERK1/2 and Akt were compared to values for total ERK1/2 and Akt (Densitometric Unit Ratio). (B) HUVECs prelabeled with CellTracker Green were grown to confluency. Scratch wounds were introduced into the monolayers, and then cells were incubated for 12 hrs in serum-free media (Unt.), or serum-free media containing either 250 nM of QK, or 250 nM of PGM-QK. Analyses of scratch wound closure indicated that cells exposed to QK or PGM-QK exhibited greater migration than untreated cells. Representative images are shown with the defect area indicated by white dashed lines. Values depicted in the graph represent means and S.E.s from 3 independent experiments. * denotes $p<0.05$ vs Unt.

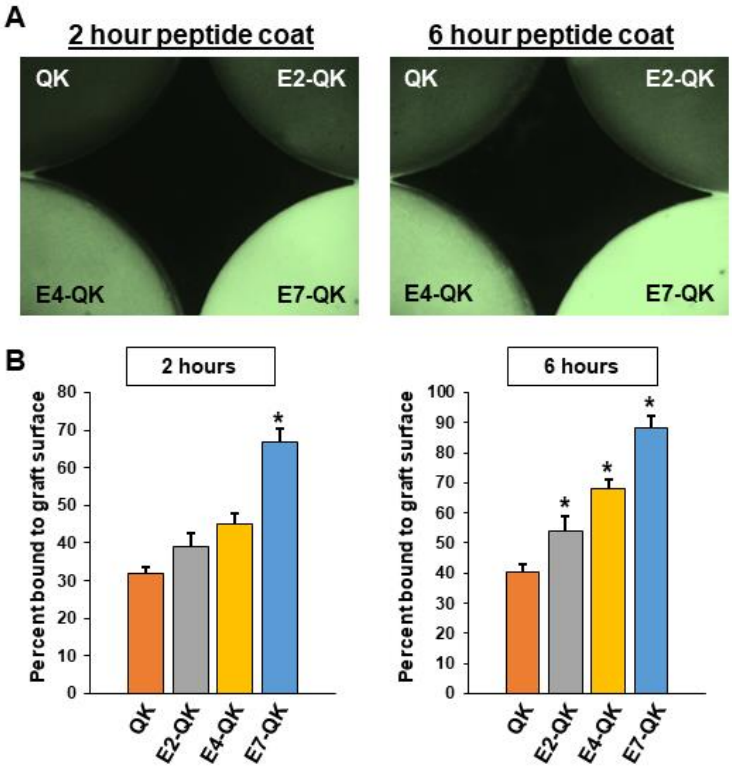

Fig 3. The number of glutamates within the polyglutamate domain regulates the amount of peptide bound to the graft material. HA disks were coated in 1 μM of FITC-tagged QK, E2-QK, E4-QK, or E7-QK for 2 or 6 hrs. After coating, samples were washed briefly in TBS and imaged fluorescently. (A) One disk from each of the 4 peptide groups was placed in the same microscopic field to allow a direct comparison. Images of the coated HA disks show a correlation between the length of polyglutamate domain and the binding efficiency of the peptide to graft material. (B) Solution fluorescence assays were used to quantify the percentage of the peptide within the initial coating solution that adhered to the HA disks. Specifically, the amount of FITC-tagged peptide in the solution, both before and after coating, was measured on a microplate reader. Values represent means and S.E.s from three independent experiments. * denotes $p<0.05$ vs QK.

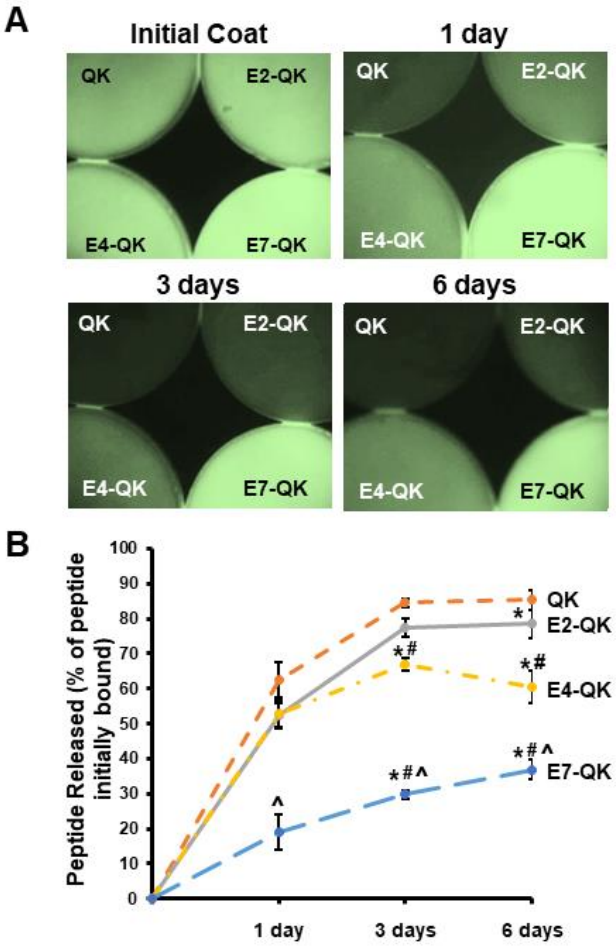

Fig 4. Polyglutamate domains modulate the release of peptides from HA grafts. HA disks were coated for 2 hrs. with a saturating concentration, 10 μM, of FITC-tagged QK, E2-QK, E4-QK, or E7-QK. After coating, disks were placed in fresh TBS under gentle agitation for 6 days to monitor peptide released from graft. (A) Graft were imaged fluorescently immediately after initial coating, as well as at 1, 3 and 6 days following placement in TBS. Images suggest that the peptide release kinetics are determined by the length of the polyglutamate domain. (B) Solutions were collected from the disks at 1, 3, and 6 days, and fluorescence measured to determine the amount of peptide released into solution over time. The cumulative percentage of peptide released was determined by comparing the fluorescence of peptide released at the various time points with the amount of peptide initially bound (determined by solution fluorescence assays). Values represent means and S.E.s from three independent experiments. * denotes $p<0.05$ vs QK; # denotes $p<0.05$ vs E2-QK; ^ denotes $p<0.05$ vs E4-QK.

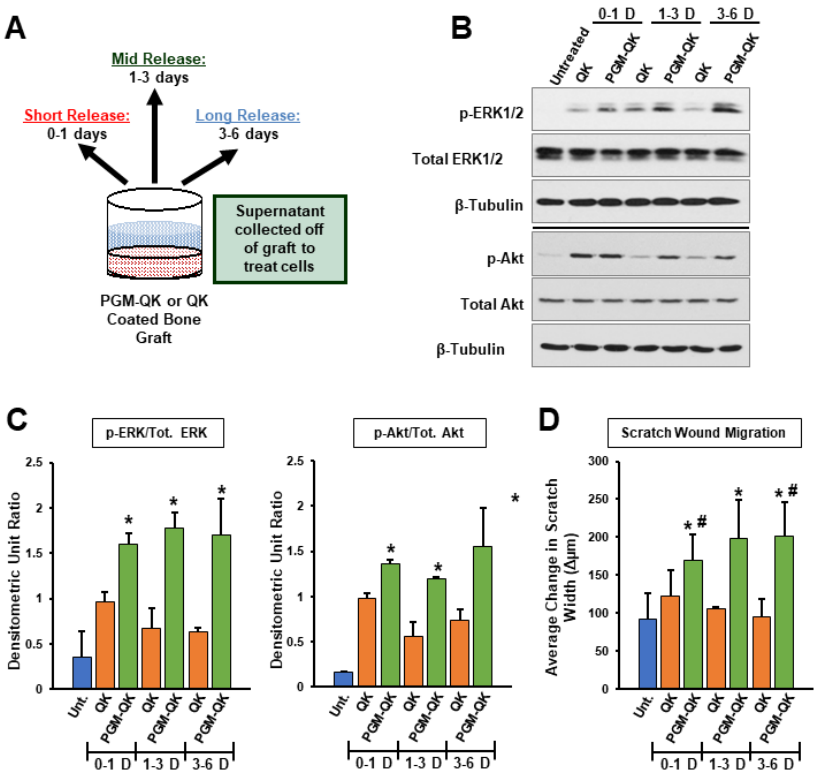

Fig 5. PGM-QK coated grafts facilitate sustained delivery of active QK peptide. (A) HA disks were coated with serum-free media containing 250 nM of QK or PGM-QK for 2 hrs. Coated disks were briefly washed to remove unbound peptide, and placed under gentle agitation in fresh serum-free media for intervals up to 6 days. Media from the disks were collected at 1 day following coating (day 0-1), and then disks were placed in fresh media, and the media collected again at day 3 following coating (days 1-3). After this interval, the same disks were placed in fresh media, and the media collected at day 6 following initial coating (days 3-6). (B) HUVECs were incubated for 10 min. with serum-free media (Untreated, Unt.) or media containing QK or PGM-QK that had been released from the graft at 0-1, 1-3, or 3-6 days. Cells were lysed and immunoblotted for p-ERK1/2, total ERK1/2, p-Akt, total Akt, or β-tubulin. (C) Densitometric analyses of blots were conducted using Image J, and values for phosphorylated ERK1/2 and Akt were compared to densitometric values for total ERK1/2 and Akt (Densitometric Unit Ratio). (D) HUVECs prelabeled with CellTracker Green were grown to confluency. Scratch wounds were introduced into the cell populations, and then cells were incubated for 12 hrs in either serum-free media (Unt.) or media containing QK or PGM-QK released from grafts at 0-1, 1-3, or 3-6 days. Analysis of scratch wound closure indicated that the PGM-QK solutions collected at all time points stimulated significantly greater migration greater untreated cells. Contrarily, none of the QK-containing solutions elicited greater migration than the untreated group. Values for cell signaling and cell migration experiments represent means and S.E.s from three independent experiments. * denotes $p<0.05$ vs control. # denotes $p<0.05$ vs QK.

DISCUSSION

Non-autogenous bone grafting materials have been commercially successful due to their abundant availability and relative effectiveness in bone regeneration. However, these grafting materials still have yet to produce a healing response comparable to autogenous grafts. Autogenous grafting materials remain the “gold standard” biologically as they retain their native cells and growth factors, enhancing bone healing post implantation. Despite these triumphs, clinicians forego their use in favor of more readily available commercial grafting materials due to the limitations in quantity and secondary surgery site complications [28, 29]. Non-autogenous grafts derived from allograft, xenograft and synthetic sources have been widely popular within the clinic and have been shown to enhance bone regeneration [26]. The HA mineral within these grafting materials plays a crucial role in the regenerative capacity of the graft site. HA grafting materials closely mimic the native mineral composition of bone and resorb in sync with the deposition of newly formed bone [125]. Moreover, it has been speculated that the substrate stiffness and microarchitecture of scaffolds derived from allograft and xenograft have important roles in the MSC attachment and osteoblastic differentiation for enhanced bone regeneration [45].

Despite the benefits of using HA grafting materials, there is still a deficit in their bone regenerative capacity in comparison to autogenous bone graft. The most recognized shortfall is the lack of bone healing factors present within the commercial graft [27]. The injury response in bone has been well-characterized and requires coordinated efforts from various signaling factors and cell types to

facilitate a healthy bone regenerative response [21]. Researchers have sought to reincorporate these factors back into grafting materials to improve the regenerative capacity of implanted commercial materials through the delivery of osteogenic, angiogenic, and cell adhesive proteins on grafting materials [114, 126-128]. However, there has been limited success with regard to transferring these therapies to the clinic, leaving a vast need in the field for improvement.

In the clinic, peptides or growth factors such as QK are passively coated onto grafting materials. These peptides/proteins are unable to efficiently localize to the mineral graft substrate using this passive coating technique, however. And without a reliable means for peptides/proteins to interact with the graft material, there have been serious questions raised about the overall efficacy of delivering these molecules on grafting materials. One prime example is the use of rBMP-2 with graft material. Although rBMP-2 has been shown to be a potent inducer of osteogenesis, there have been several reports questioning its overall efficacy within the clinic [129]. It has been suggested that the ineffectiveness of the protein is not due to the lack of cell signaling potential, but rather the inability to be delivered reliably and then subsequently retained within the graft site for its intended purpose [129, 130]. The poor delivery efficiency of protein on graft surfaces is not unique to rBMP-2 however, and impacts the delivery of several other signaling factors and peptides such as rVEGF, PDGF, FGF, RGD, and DGEA on mineralized materials [11, 131]. To address this issue, several of these proteins have been modified with graft binding domains to enhance their delivery within the body. In one study, VEGF was modified with a hydrophobic

HGFI fusion protein, which enabled the protein to localize to electrospun poly(ε-caprolactone (PCL) scaffolds [132]. There has been a great deal of progress functionalizing proteins/peptides to bind to graft carriers in the tissue engineering field as a whole. However, there have been few strategies designed to deliver factors in conjunction with bone grafting materials specifically. Our group has used a polyglutamate domain to enrich the concentration of a number of peptides on HA grafting materials [53, 61-63, 70, 71, 124]. Namely, this dissertation describes a method for achieving a 4-6-fold increase in the amount of QK peptide coated onto HA and anorganic bovine bone (ABB) grafting materials using a E7-QK peptide. Meanwhile, we have also shown in previous studies that the use of these polyglutamate domains retains the peptide on the grafting surface for long periods of time after implantation [61, 62]. In one specific study, we reported that E7-DGEA coated ABB particles retrieved after 1 and 3 months of subcutaneous implantation retained the E7-DGEA within the graft site, whereas unmodified DGEA was undetectable [62]. Similarly, we have shown E7-BMP2pep coated graft materials were retained onto the graft surface after 2 months of subcutaneous implantation, further demonstrating the utility of polyglutamate domains [61]. This enrichment after coating, and controlled delivery of peptides implanted, could potentially mitigate the need for supraphysiological doses required of recombinant protein therapies on grafting materials. Moreover, the polyglutamate modified peptides are biologically active when coated on the graft. E7-QK coated graft materials were able to elicit proangiogenic activation of p-Akt and p-ERK 1/2 of endothelial cells directly seeded onto graft materials. This

finding is important for several other bone healing mimetic peptides such as BMP2 mimetic peptide that would need to be retained onto the surface of the graft for extended periods while still being biologically-active.

In this dissertation I sought to develop a method to deliver an angiogenic factor on bone graft materials. Angiogenesis is one of the most significant healing cascades involved in the regeneration of bone. However, there has yet to be a successful angiogenic therapeutic that can be used in conjunction with commercial bone grafting materials. Therapies using rVEGF have had limited success due to the limited half-life of rVEGF and reliance on gradient rVEGF delivery to develop new vasculature [102, 106]. To address the limited half-life of rVEGF, researchers have begun to experiment with alternative methods to induce angiogenesis aside from rVEGF. One promising inducer of angiogenesis is the peptide mimetic to rVEGF, QK. Identified and characterized by the D'Andrea group, the QK peptide was shown to activate VEGFR-2 receptors on endothelial cells, supporting the notion that QK can induce angiogenesis [117]. Additionally, they also noted that QK peptide's half-life far exceeded rVEGF in a serum-stability analysis, speculating that this improvement could greatly extend the exogenous angiogenic stimulus provided by QK peptide in the body [118]. Several groups have delivered QK on scaffold material to extend its potency and have shown vast improvements in tissue vascularization as a result [118-120]. One example is a study performed by Leslie-Barbick et al. where a PEG-QK modified hydrogel was implanted into a mouse cornea micropocket. After implantation, they observed drastic increases in vessel branch points and overall

vessel number [133]. One benefit of the QK peptide is its simplicity to be modified with binding domains as well. Although possible, the process of altering full-length proteins requires complicated production procedures, which can ultimately disrupt the signaling function of the intended recombinant protein [75-77]. In contrast, peptides can be easily altered with new functional groups using a commercial peptide synthesizer and still retain full bioactivity. In this dissertation, I have shown that the addition of E7 domain to the QK peptide has no effect on the QK peptide's ability to activate endothelial cells when E7-QK is attached to grafting material or in free solution. In fact, we have also used this domain with other peptides and have yet to note a loss in the peptide's signaling function caused by addition of a polyglutamate domain [61-64]. Moreover, the QK peptide has been modified with other binding domains and remained bioactive. In one study performed by the Yu group a collagen mimetic peptide (CMP) was synthesized to QK peptide(QKCMP), which allowed the peptide to integrate within a collagen scaffold [120] . These QKCMP were shown to induce endothelial network formation in HUVECs seeded onto the QKCMP-loaded collagen scaffolds, giving further support that the QK is amenable to modifications [120, 134].

Another unique aspect of angiogenic factors is their reliance on establishing a chemokine gradient. Historically, the gradient release of these factors is achieved through the degradation of their hydrogel carrier [135]. Hydrogels are efficient deliverers of growth factors, however they are more suited for soft tissue applications and do not possess the structural characteristics

required for bone grafting applications [136]. Additionally, the degradation rates of the scaffold for release of growth factor are too rapid for the bone matrix deposition to fill the void adequately [137]. Without the necessary scaffolding filling the defect, a non-union fracture could remain unresolved. Other techniques have evolved broadly from the tissue engineering field for the purpose of gradient delivery scaffolds. One such technique for immobilizing proteins onto scaffolds is through the use of heparin binding domains. The Lee group conjugated these heparin binding domains to a number of proteins, including rVEGF, so that they can localize to the scaffold. In this design the gradient release of rVEGF from the implanted scaffold is established as heparinases cleave the linkage between the protein and implanted scaffold. [138]. Similarly, there have been investigations modifying proteins with matrix metalloproteinase(MMP)-cleavable domains to deliver a gradient on scaffolds. These MMP-cleavable modified proteins are chemically coupled to scaffolds. Once implanted the MMP-cleavable sites are cleaved and a gradient of protein is delivered. [139]. While these methods perform well in establishing a gradient, they have not been translated to work with current bone grafting materials. Our group has developed a method to deliver a gradient specifically from bone grafting materials using polyglutamate domains. The strength of ionic coupling between polyglutamate modified peptides and graft material has been shown to be dependent on polyglutamate domain length. Each component of PGM-QK (E2-QK, E4-QK, and E7-QK) was shown to bind and release from graft respective to the length of the polyglutamate domain. Combined as a PGM-QK mixture, we were able to

achieve activation of endothelial cells from the peptides released from coated graft materials up to 6 days as shown in both the cell signaling and migration assays. Moreover, the composition of PGM-QK can be easily modified and optimized for *in vivo* experimentation. To alter the release gradient, the ratios of PGM-QK could be modified to change the release course of peptide from implanted graft material. Meanwhile, any number of glutamate residues can be synthesized to the QK peptide to create new peptides (i.e. E5-QK) within the mixture to further optimize the gradient delivery *in vivo*.

Overall, this dissertation offers a unique method to deliver a proangiogenic gradient of PGM-QK peptide on commercial graft materials. Using the polyglutamate domain modified QK peptides we were able to enrich the concentration of peptide on graft surface. Additionally, the peptides remain bioactive graft-bound and in solution, stimulating proangiogenic responses in endothelial cells. We have also shown that each individual component of PGM-QK binds and release from graft material based upon the length of polyglutamate domain. The combined mixture, PGM-QK, coated onto grafts was able to deliver a sustained release of angiogenic factor capable of stimulating endothelial cells for an extended time course. These results indicate that PGM-QK is a strong candidate for use within the clinic for coating bone grafts with an angiogenic therapeutic (Figure 6).

Future Directions

In moving forward with PGM-QK delivered on bone graft, there are several milestones yet to be achieved before it would be ready for the clinic. In our studies outlined in this dissertation, it was shown that PGM-QK released from bone graft successfully maintained a proangiogenic stimulus *in vitro*. There are several future *in vivo* studies necessary to determine if this angiogenic gradient delivered by PGM-QK coated graft material will increase vasculature. Moreover, we also need to assess how this increased vascularization will improve overall bone healing as well. To address if PGM-QK delivered on bone graft materials induces angiogenesis, a subcutaneous implant model can be performed. Implantation of the treated bone grafts into subcutaneous pouches minimally damages the tissue surrounding giving a clear analysis of what new vascularization is a direct result of the PGM-QK released into the surrounding tissue. Sections retrieved from the excised tissue can be stained immunohistochemically (IHC) for blood vessels markers such as CD-31 and CD-34. Different concentrations and ratios of individual components of PGM-QK can be experimented with using this model to optimize the angiogenic response *in vivo*. Once a gradient of PGM-QK delivered on graft is shown to successfully induce angiogenesis, a shift in focus can made to assess the resulting bone regeneration in response to implanted PGM-coated bone graft in a critically sized bone defect. PGM-QK coated graft implanted into a critical defect, can be monitored over time for bone density and vessel network formation using microCT and angio-CT, respectively. Excised tissues can be sectioned, stained,

and evaluated by IHC for vascularization (CD-31 & CD-34), bone matrix deposition (Masson's Trichrome), and mineralization (Van Kossa & Alizarin red). Future studies would also require PGM-QK to be evaluated in conjunction with several bone graft types and applications, as well. Studies have shown differences in integration of bone grafting materials due to material source (allograft vs xenograft), composition (cortical vs cancellous), and application (load bearing vs non-load bearing) [26, 40, 41, 125]. Although vascularization of a healing bone site is critical for all bone graft applications, there may be differences in the response to PGM-QK based upon the particular bone graft application. One major consideration would be the comparison between cortical and cancellous bone graft applications. Cortical bone has been shown to reintegrate much slower due to high mineral content and lack of cell invasion. PGM-QK coating of these materials may not be as beneficial due to the lack of cell integration within the graft. Once these considerations have been carefully explored, PGM-QK delivered on bone grafts should be a strong candidate for clinical evaluation.

Outside of the ongoing studies for PGM-QK delivered on bone graft, there are several applications for the polyglutamate domain that have yet to be uncovered. The goal of our lab is to produce a comprehensive peptide therapy that can be delivered on bone grafting material to enhance bone regeneration. In this dissertation, PGM-QK delivered on graft material was shown to maintain a proangiogenic stimulus critical for new vascularization during bone repair. However, there are several other healing cascades that are required in

coordination with angiogenesis for healthy bone regeneration. It has been shown that the co-delivery of factors such as rVEGF and rBMP-2 have synergistic effects on bone regeneration [140, 141]. The polyglutamate-modified peptides could potentially improve this effect by reliably delivering mimetic peptides for both factors. More importantly, since the release of the peptide is dependent on the polyglutamate domain length and not scaffold design, we could selectively deliver a gradient of PGM-QK while also retaining the BMP-2 mimetic peptide on the grafting surface.

The use of the polyglutamate domain to deliver bioactive peptides on grafting materials is a technology easily transferred to the commercial market. The polyglutamate-modified peptides are synthesized by a commercial synthesizer, which produces a purer and cheaper product in comparison to the production of recombinant proteins in host cell systems. The peptides can be lyophilized and stored for long-term use. Once needed, the peptide is then reconstituted in saline for immediate coating of bone graft. Additionally, the coating of bone grafts with polyglutamate peptides does not require any new training for clinicians. Grafts are coated in a solution of reconstituted peptide similar to the passive coating practices of graft materials with recombinant proteins, lending itself to be easily adopted in the clinic. Our group has shown that polyglutamate modified peptides readily bind and localize to several different HA-containing commercial grafting materials, giving clinicians their preference of grafting material, as well.

The polyglutamate domain was shown to localize to HA due to the Ca^{2+} ions present within HA, which serve as the ionic coupler to the glutamic acids [68]. There are several other biomaterials yet to be tested that also contain positive charges that could interact with the polyglutamate domain. One prime example for the potential use of polyglutamate domains outside of bone grafting is to coat resorbable metal stents composed of Fe^{3+} and Mg^{2+} alloys with peptides. The polyglutamate domain could be a method to coat these stent materials with peptides or drug compounds aimed at reducing restenosis. Meanwhile, we have shown that these polyglutamate domains can localize other products to bone graft material. In one study, we incorporated short E2 polyglutamate domains within the shell of a nanocage particle. The nanocages were found to localize to HA-containing material similar the polyglutamate modified peptides [142]. These particles could potentially deliver several therapeutics to bone including hormones, cancer therapies, and microRNA to treat a variety of bone related illnesses. Aside from coating techniques, the polyglutamate domain may have some potential applications for injectable therapies. In previous studies, we have shown that E7-DGEA injected through the tail vein of Sprague-Dawley rats localized to the skeleton after 24 hours of injection [62]. These findings indicate that the polyglutamate domain could offer an alternative method to target the skeletal system similar to bisphosphonate groups for several bone-related therapies [84, 143].

In sum, the full utility of the polyglutamate domain has yet to be uncovered. This dissertation demonstrates the use of the polyglutamate domain

to deliver angiogenic factors on graft material. As an ultimate goal, these PGM-QK peptides will become a part of a comprehensive peptide mixture aimed at improving bone graft regeneration. Moreover, there are several other applications for the polyglutamate domain outside of the bone graft niche, further emphasizing the potential applicability of these domains in biomedical research in the years to come.

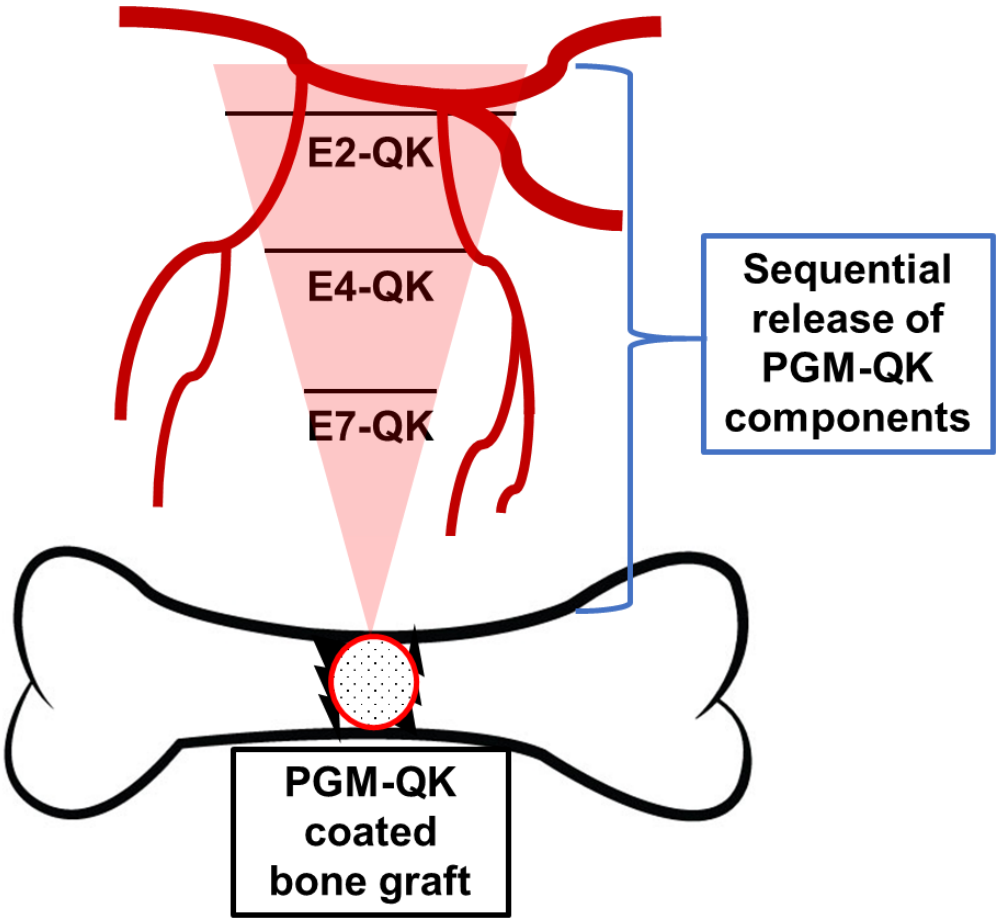

FIGURE 6. Overall goal for implanted PGM-QK coated materials. A mixture of PGM-QK peptides are coated onto the bone graft and implanted within the bone defect. Each component (E2-QK, E4-QK, and E7-QK) will release from the bone graft based upon its respective polyglutamate domain length. Sequential release of each component will deliver a constant angiogenic gradient needed for new blood vessel formation near the bone graft implant site.

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