Spring 5-20-2019

The Homeostatic Factor Del-1 Promotes Periodontal Bone Regeneration During Resolution of Periodontitis

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UNIVERTITY OF PENNSYLVANIA
SCHOOL OF DENTAL MEDICINE

THE HOMEOSTATIC FACTOR DEL-1 PROMOTES PERIODONTAL BONE REGENERATION DURING RESOLUTION OF PERIODONTITIS

DA-YO YUH

A DISSERTATION

Presented to the Faculties of the University of Pennsylvania

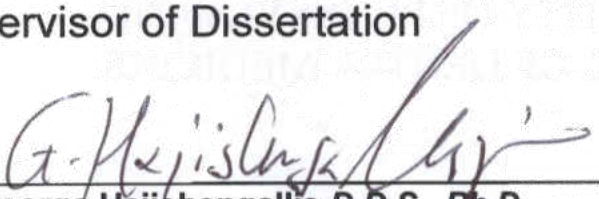
in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Science in Dentistry

2019

Supervisor of Dissertation



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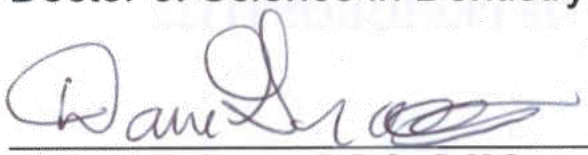

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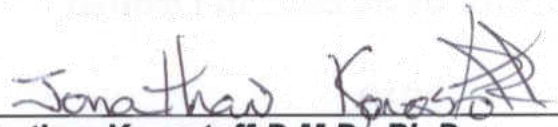

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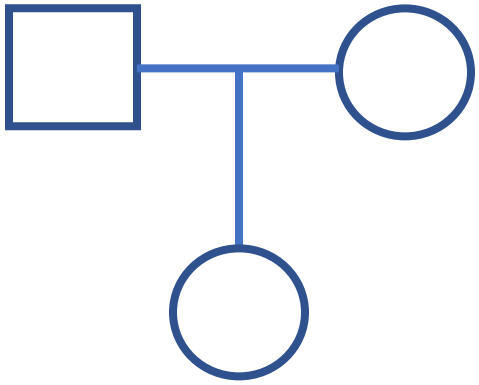
DEDICATION

To my wife Tinghan Chang and our daughter



Agnes

ACKNOWLEDGMENT

I wish to acknowledge my mentor, Dr. George Hajishengallis, for accepting me as his first Doctor of Science in Dentistry (DScD) student, and for his constant guidance in my research. My purpose to pursue further education in basic sciences is to bridge the gap of research between basic science and clinical science in my medical center. Dr. Hajishengallis's broad knowledge and contribution in the research frontier of periodontology made it possible for me to explore the unknown field of the disease. I wish to thank Dr. Jonathan Korostoff for recommending Dr. Hajishengallis's laboratory to me and for his warm discussions. I wish to thank Dr. Henry Daniell with whom I completed my first laboratory rotation and developed lettuce-derived DEL-1. Dr. Daniell's kindly instruction helped me to overcome the embarrassing stage of switching from a clinical periodontist to the first step of being a scientist as well as the transition from animal biologist to plant biologist and genomic scientist. I wish to thank Dr. Dana T. Graves for establishing the DScD program at Penn which made possible for me and my wife to pursue dental research. Dr. Graves's broad knowledge on periodontology and osteoimmunology foster the growth of this thesis. I also wish to thank Dr. Tetsuhiro Kajikawa for his openminded discussion in science with me when the result is not as expected, and provided several technical advices and helped me to carry out the *in vitro* osteoblast mineralization assay, Dr. Jieun Shin for teaching me perform the primary culture of osteoblastic progenitors from mice, Dr. Hui Wang, Dr. Xiaofei Li, Dr. Baomei Wang for teaching me the immunology concept, Dr. Kavita Hosur for

providing DEL-1-Fc and its mutants for my experiments, Ms. Betsy Chang for teaching me the skill to keep laboratory notes as well as to extract knowledge from the previous member's laboratory note, Ms. Chang's specific knowledge in chloroplast genome editing and plant-tissue culturing provides great help in the screening for DEL-1 plasmid and lettuce, Dr. Jieun Park for teaching me overlapping PCR technique, Dr. Chung-Tsai Lee for providing knowledge and help with molecular cloning, Dr. Ziqing Li for providing me with the knowledge of bone multicellular unit and the orthopedics' point of view in bone regeneration, Dr. Qunzhou Zhang for his warm encouragement and time to go through the presentation slides with me and clear my though for better presenting the story, the gingival derived mesenchymal stem cell provided by Dr. Zhang helped me develop the assay to test the receptor-binding function on the prokaryote-derived DEL-1-Fc. I also wish to thank Dr. Anh D. Le for her warm encouragement for me and my wife to pursue dental research at Penn during our first online interview.

I sincerely thank all my friends and my family members in Taiwan and in US for supporting our research, especially Dr. Yeong-seng Yuh, my father, who inspire me to pursue science, and Dr. Langchee Chang, my father in law, who help us take care of Agnes when we were not available. Finally, I would like to thank my wife Dr. Tinghan Chang whose accompany, and support help me struggle through every difficulty I encountered.

ABSTRACT

Background: Periodontitis is the most common osteolytic disease that is characterized by inflammation and bone loss. Although periodontitis can be initiated by microbial dysbiosis, the net bone loss is due to the uncoupling of bone formation from resorption, in turn attributed to unresolved inflammatory host response. Aberrant resolution of inflammation may contribute to many osteolytic inflammatory diseases, thus there is an urgent need for effective regimens to promote resolution and restore positive osteogenic coupling. Recently, an endogenous molecule, DEL-1, has been identified as a homeostatic factor that restrains osteoclastogenesis, regulates integrin-dependent leukocyte recruitment during the initiation of inflammation, and promotes the resolution of inflammation. This study aims to explore the osteogenic role of DEL-1 in bone regeneration during resolution of periodontitis.

Methods: DEL-1-deficient (*Edil3*$^{-/-}$) and -sufficient (*Edil3*$^{+/+}$) mice (*n*=5-6/group) were subjected to 10-day ligature-induced periodontitis followed by ligature removal for 5 days to facilitate resolution. Bone gain was quantified morphometrically and newly regenerated bone was confirmed by modified Masson's-trichrome staining. Primary cultures of *Edil3*$^{-/-}$ and *Edil3*$^{+/+}$osteoblastic progenitors were treated with osteogenic medium and their calcification potential was determined by Alizarin-Red-S staining (*n*=4cultures/group). DEL-1 and mutant versions thereof were tested *in vivo* and *in vitro* to identify critical component(s) of DEL-1 involved in bone regeneration and osteogenic differentiation. The receptor

involved was determined by pull-down assay followed by receptor-knockdown experiments in osteoblastic progenitors.

Results: After ligature removal, periodontal inflammation was resolved and bone was regenerated in *Edil3*$^{+/+}$ mice but not in *Edil3*$^{-/-}$ mice (unpaired *t*-test, $P<0.01$). Consistently, *Edil3*$^{-/-}$osteoblastic progenitors were defective in calcified nodule formation. Local gingival injection of DEL-1 in *Edil3*$^{-/-}$ mice restored bone regeneration (unpaired *t*-test,$P<0.01$). Mechanistic analysis using DEL-1 and mutants thereof showed that bone regeneration and osteogenic differentiation were dependent on interactions between the RGD motif on the *N*-terminal one-third of DEL-1 and the β3-integrin on osteoblastic progenitors. Whereas earlier studies showed that intact DEL-1 is required for efferocytosis and the emergence of pro-resolving macrophages, its RGD-containing *N*-terminal component was sufficient to promote osteogenic differentiation and bone regeneration.

Conclusions: DEL-1 is shown for the first time to promote bone regeneration during resolution of periodontitis, a function that might be exploited therapeutically to treat human periodontitis. This study further provides an effective strategy to also restore bone that has been destroyed due to other chronic osteolytic diseases.

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LIST OF ABBREVIATIONS

ABC	Alveolar bone crest
AgP	Aggressive Periodontitis
α-MEM	Minimum essential medium eagle-alpha modification
Bglap	Bone gamma-carboxyglutamate protein
BMP2	Bone morphogenesis protein 2
CEJ	Cementum-enamel junction
CP	Chronic Periodontitis
DEL-1	Developmental endothelium locus-1 protein
DNA	Deoxyribonucleic acid
Edil3	EGF like repeats and discoidin I like domain 3
EGF	Epidermal growth factor
FBS	Fetal bovine serum
Fc	Fragment crystallizable region of antibody
Fig	Figure
GCF	Gingival crevicular fluid
G-CSF	Granulocyte-colony stimulating factor
GMSC	Gingival-derived mesenchymal stem-like cell
HSC	Hematopoietic stem cell
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-17	Interleukin 17

IL-23	Interleukin 23
ITGB1	Integrin β1
ITGB3	Integrin β3
kDa	Kilodalton (1,000 daltons)
LIP	Ligature-induced experimental periodontitis
LXR	Liver X receptor
M	Molar
MFG-E8	Milk fat globule-EGF factor 8
MSC	Mesenchymal stromal and stem-like cell
MSU	Monosodium urate
PBS	Phosphate-buffered saline
PDL	Periodontal ligament
PDGF	Platelet-derived growth factor
PPAR γ	Peroxisome proliferator-activated receptor γ
qPCR	Real-time and quantitative real-time polymerase chain reaction
RANKL	Receptor activator NFκB ligand
RNA	Ribonucleic acid
RUNX2	Runt-related transcription factor 2
SDS	Sodium dodecyl sulfate
SPMs	Specialized pro-resolving mediators
Tregs	Regulatory T cells
TNF	Tumor necrosis factor

CHAPTER 1. INTRODUCTION

"There may be many ways of treating a disease, but there can be but one correct diagnosis"

Morton Amsterdam, 1974

1.1 Periodontitis as a dysregulated chronic inflammatory disease

Periodontitis affects 47% of United States adults and is characterized by inflammation and bone loss (Eke et al., 2015; Graves et al., 2011; Hajishengallis, 2014a; Maekawa et al., 2014a; Shin et al., 2015). Although it was once considered to be an infectious disease (Mombelli, 2003), recent evidence indicates that "periodontal pathogens" may actually derive from the commensal microbiota in which they are normally present albeit in low numbers. It is now believed that dysregulated periodontal inflammation leads to what has been termed "dysbiosis" of the subgingival microbiota resulting in outgrowth of the periodontal pathogens (Darveau et al., 2012; Dutzan et al., 2018; Lamont and Hajishengallis, 2015; Lamont et al., 2018; Xiao et al., 2017). Dysbiosis represents a state of imbalance in the relative abundance or influence of species within a microbial community associated with inflammatory disease (Lamont and Hajishengallis, 2015). Accumulating evidence indicates a strong association between dysbiosis and the host microenvironment (Hajishengallis, 2014b; Nassar et al., 2017; Xiao et al., 2017; Yost et al., 2017). Thus, periodontitis should no longer be considered as an

infectious disease in the classical sense, rather, it represents a dysbiotic disease in which bacteria are required but not sufficient for the disease to progress (Socransky and Haffajee, 1992). Recognizing the dysregulated inflammatory nature of periodontitis opens up new opportunities for the diagnosis, treatment and long-term management of periodontal disease.

While the evidence in germ-free animal studies reveals that bacteria are required for the initiation of periodontitis (L. et al., 1980; Rovin et al., 1966), the progression of periodontal disease and bone loss depends on the host inflammatory response (Assuma et al., 1998; Hajishengallis, 2014a; Hajishengallis et al., 2014; Pacios et al., 2015). Even though the host inflammatory response facilitates the elimination of certain bacteria, the ability to use inflammatory "byproducts" for anaerobic respiration enhances the growth of others (Nassar et al., 2017). Specifically, certain types of "inflammophilic" bacteria, such as the proteolytic and asaccharolytic organisms, can obtain essential amino acids and iron from degraded collagen and heme-containing compounds (hemopexin, haptoglobin and hemoglobin) generated during tissue-breakdown mediated by inflammation (Hajishengallis, 2014b). The recently formulated polymicrobial synergy and dysbiosis hypothesis and the ecological plaque hypothesis further suggest that host inflammation response can serve as a selective environmental factor which enriches specific pathogenic bacteria (Lamont et al., 2018). Based on this hypothesis, the unresolved chronic inflammatory periodontal environment may further provide nutrition that supports the periodontitis-associated biofilm

(Hajishengallis, 2014b; Xiao et al., 2017) which in turns facilitates the microbial dysbiosis and progression of periodontitis.

Over the years, the clinical observation that the rate of disease progression varies between patients has played a role in the evolution of the terminology used to classify periodontitis. The nomenclature proposed in the 1999 Classification of Periodontal Diseases and Conditions renamed what had previously been referred to as juvenile/early-onset periodontitis as aggressive (AgP), and the more common adult form of periodontitis as chronic periodontitis (CP) (Armitage, 1999). Although the distinction between the two forms of periodontitis was thought to be due to their association with unique bacterial pathogens, more recent evidence demonstrating their common microbiological, immunologic, and histopathologic features (Armitage, 2010; Ford et al., 2010; Smith et al., 2010) resulted in a clinical diagnostic dilemma (Armitage and Cullinan, 2010). Since the declaration of Human Genome Project in 2003, multiple studies have been conducted to further evaluate the diseases. Advances in machine-learning algorithms and human transcriptome analysis, the contribution of host inflammatory response to periodontitis has been further emphasized in a recent clinical study. Through cluster analysis of the gingival tissue transcriptome and clinical parameters from 120 patients, limited dissimilarities were observed between established AgP and CP lesions when patients were classified according the to 1999 criteria (Kebschull et al., 2013). However, after the authors re-categorized the periodontitis patient into groups based on gene expression signatures, the authors found a distinct phenotypic separation between two clusters in whole-mouth periodontal

destruction and the subgingival microbial burden (Kebschull et al., 2014). This finding suggests that there may in fact be distinct host responses that manifest as phenotypically unique forms of disease and provides clues to identify potential biomarkers for precision diagnosis in periodontitis.

1.1.1 The role of the host immune response in periodontal homeostasis and disease

A tooth is a unique organ in the body that penetrates cortical bone and oral epithelium to erupt into the oral cavity. The non-shedding surface of the teeth creates a distinct microenvironment for the colonization of diverse microorganisms which triggered our body to coevolve a unique immune response to interact with the constant challenge from oral microbiota. In response to the microbiota challenge, a large population of leukocytes is recruited to the junctional epithelium area around the tooth, with neutrophils being the most abundant leukocyte (50-70% of total) in gingivitis and periodontal disease. Neutrophils can produce a plethora of antimicrobial peptides and trigger several pro-inflammatory mechanisms to form the first line of defense against pathogenic insults (Hajishengallis and Hajishengallis, 2014). However, excessive activation of neutrophils also has a detrimental effect on periodontium by overproduction of reactive oxygen species (ROS) which not only kills their target microbes but also creates damage on the surrounding extracellular tissue (Matthews et al., 2007; Robinson, 2008). Intriguingly, both insufficient and excessive neutrophils contribute to the pathogenesis of periodontitis (Darveau, 2010; Deas et al., 2003;

Eskan et al., 2012; Hajishengallis and Hajishengallis, 2014; Moutsopoulos et al., 2014; Nussbaum and Shapira, 2011) suggesting that regulating the neutrophil homeostasis may be essential to periodontal health for a reason beyond its antimicrobial functions.

Despite being the most abundant form of circulating leukocytes, neutrophils are relatively short-lived cells with a half-life range from 8 to 12 hours (Mayadas et al., 2014) and can be rapidly replenished by granulopoiesis and egress of neutrophils from bone marrow and peripheral blood vessels, respectively (Edwards, 1994). The ability of human body to maintain a balance between neutrophil production and turnover, while adapting to environmental challenges, implies a homeostatic process for the real-time detection and feedback on the numbers of neutrophils (Mayadas et al., 2014). Ley and colleagues had identified a neutrophil rheostat (neutrostat) that senses neutrophil recruitment and clearance in tissue and regulates neutrophils production in the bone marrow through a negative-feedback loop involving a granulopoietic cytokines cascade, including interleukin-23 (IL-23), interleukin-17 (IL-17) and granulocyte-colony stimulating factor (G-CSF) (Hajishengallis and Korostoff, 2017; Stark et al., 2005). Under steady-state conditions, senescent and apoptotic neutrophils are engulfed by tissue macrophages, which then induce anti-inflammatory signals via expression of peroxisome proliferator-activated receptor γ (PPAR γ) and liver X receptor (LXR). These anti-inflammatory signals, in turn, reduce the steady-state production of IL-23 by macrophages. IL-23 is a well-established inflammatory cytokine that induces IL-17 productions by T cells that in turn further induce the production of G-

CSF by stromal cells, driving granulopoiesis and inflammation (Mayadas et al., 2014). When neutrophils cannot transmigrate to peripheral tissue, such as in leukocyte adhesion deficiency type I (LAD-I), this regulatory circuit breaks down, and may in part explain the unrestrained expression of IL-23 and the downstream cytokines IL-17 and G-CSF in the periodontal tissue (Moutsopoulos et al., 2014). The local overproduction of IL-17 causes inflammation that leads to bone loss and bacterial outgrowth (Hajishengallis and Korostoff, 2017; Moutsopoulos et al., 2014). The concept that LAD-I periodontitis is driven by overactivation of the IL-23/IL-17 axis has led to a successful anti-IL-23 host-modulation therapy for the treatment of periodontitis in an LAD-I patient (Moutsopoulos et al., 2017).

Periodontal health was once considered to represent the complete absence of an inflammatory infiltrate in gingival tissue. The classic work of Page and Schroeder first challenges this concept by providing evidence that the infiltration of neutrophils can be observed in the clinical healthy gingiva in which the initial accumulation of bacteria biofilm was developed within 2-4 days (Page and Schroeder, 1976). More recent studies have extended their findings. Neutrophils were identified in healthy gingiva from germ-free mice (Zenobia et al., 2013). Contemporary histological studies of healthy periodontal tissue from human volunteers also reveal a constant numerical density of neutrophils infiltration from 17.7% to 20.6% was maintained in healthy gingiva (Lang and Bartold, 2018). While it is inevitable to have biofilm accumulation on the non-shedding tooth surface, the observed inflammatory infiltration in healthy periodontal tissue may be explained as homeostatic immunity that maintains a balance between the host and the

microbiota. The homeostatic periodontal inflammatory infiltrates may be controlled by multiple negative regulatory mechanisms such as regulatory T cells (Tregs), and pro-resolving macrophages under conditions of health. Thus, the homeostatic interaction between a regulated host immune response and the microbiota may stabilize the periodontium in a state of health while perturbation of the interactions may result in periodontitis (Douglas, 2018).

Several studies have been recently carried out to dissect causal mechanisms of periodontitis. Complement is one of the major mechanisms that causes periodontitis in which the destructive role of complement has been shown by intervention studies in which pre-existing naturally occurring periodontitis can be blocked by local C3 inhibition (using the drug compstatin Cp40) in cynomolgus monkeys (Maekawa et al., 2016). Although complement can play a protective immune surveillance role in different microbe-driven diseases including periodontitis, complement can also become an effector of tissue destruction if overactivated or dysregulated (Ricklin et al., 2016). Moreover, complement may also be exploited by important periodontitis-associated bacteria (such as *Porphyromonas gingivalis*, *Treponema denticola*, *Tannerella forsythia*, and *Prevotella intermedia*) in ways that promote the dysbiotic transformation of the microbial community (Lamont et al., 2018; Okroj and Potempa, 2018). Furthermore, complement signaling pathways synergize with toll-like receptors in eliciting an inflammatory response that leads to alveolar bone destruction (Abe et al., 2012; Hajishengallis and Lambris, 2011; Hajishengallis et al., 2011; Maekawa et al., 2014a; Maekawa et al., 2014b). Several studies further identified roles of

complement beyond the linear cascade of complement activation, specifically involving cross-talk with other immune and physiological systems, which collectively coordinate host responses to infection or tissue injury. For instance, complement can form a barrier against the spread of invading bacteria by augmenting local clotting (Markiewski et al., 2007) and regulate antigen-presenting cells and the activation and differentiation of T-cell subsets (Dunkelberger and Song, 2010; Hashimoto et al., 2010). However, whether these newly identified mechanisms may also be exploited by periodontitis-associated “inflammophilic” bacteria to induce periodontal inflammation remain to be identified.

On the other hand, through the use of broad-spectrum antibiotics, Dutzan et al have shown that counteracting periodontal dysbiosis reduces the ligature-induced periodontal bone loss and diminishes the expansion of resident memory T helper 17 (Th17) in mice (Dutzan et al., 2018). While the expression of the cytokine interleukin-17 (IL-17) and expansion of Th17 cells has been previously observed in the lesion of human periodontitis and ligature-induced experimental periodontitis (LIP) in mice, Dutzan et al demonstrated that dysregulated IL-17 expression from Th17 cells triggers periodontal bone loss and that genetic or pharmacological inhibition of the expansion of Th17 cells ameliorates the LIP-induced periodontal tissue destruction. Although IL-17 and Th17 cells play an important role in the host defense by inducing anti-bacterial peptides and recruiting neutrophils to promote local inflammation (Ishigame et al., 2009; Iwakura et al., 2011), they also contribute to the pathogenesis of various autoimmune disease as well as periodontitis by causing prolonged inflammation and tissue damage (Maekawa et al., 2015;

Okamoto et al., 2017; Takayanagi, 2012; Zenobia and Hajishengallis, 2015). Interestingly, unlike other epithelial barriers such as the skin and gut, the gingiva-resident Th17 cells can expand, under steady-state conditions, via commensal colonization-independent mechanisms, such as occlusal loading and tissue damage during mastication (Dutzan et al., 2017). However, in periodontitis, a pronounced accumulation of Th17 can be triggered by the dysbiotic oral microbiota, which results in periodontal tissue destruction in mice and humans (Dutzan et al., 2018). In periodontitis, the upregulated IL-17 also induces RANKL expression in osteoblastic cells and periodontal ligament cells which in turn stimulate osteoclastogenesis leading to enhanced periodontal bone loss (Tsukasaki et al., 2018).

1.2 Bone as a homeostatic tissue

Bone is a masterfully programmed tissue that undergoes frequent renewal and has a large capacity for regeneration (He et al., 2004; Xiao et al., 2016a). During adult life, the skeletal mass is sequentially regulated by two key activities occurring on the same surface: bone resorption by hematopoietic lineage osteoclasts and bone matrix formation by mesenchymal lineage osteoblasts -- a process termed remodeling. The group of cells responsible for remodeling is termed the basic multicellular unit (BMU) (Parfitt, 1994). The BMU is a particular structure around the bone remodeling sites which is surrounded by a canopy of bone lining cells/osteoblasts and nearby capillaries. The BMU concept originally included only osteoclasts and osteoblasts, but over recent years, as more cellular contributors to remodeling have been identified (such as T-cells, macrophages, osteocytes, and precursor populations of osteoblasts and osteoclasts), the number of cells in BMU has expanded and more molecules that regulate the balance within BMU have been identified (Sims and Martin, 2015).

Under healthy physiological conditions, an episode of bone resorbed by osteoclasts is precisely restored by the recruited osteoblastic lineage cells which differentiate and deposit just enough new bone at the same place, a homeostatic process referred to as coupling (Parfitt, 1982; Xiao et al., 2016a). While the lifespan of osteoclasts lasts only 2 weeks, the lifespan of active osteoblasts lasts around 3 months (Manolagas, 2000). It has long been of interest to understand how these two distinct cell types, on the same bone surface but at different times, could be linked so that their activities are balanced (Sims and Martin, 2015). Early

studies using timed fluorochrome labeling histological sections identified that bone resorption in iliac crest trabecular BMUs of adult human bone takes approximately 3 weeks (Eriksen et al., 1984b), the formation response 3-4 months (Eriksen et al., 1984a), and between the two activities there is a poorly understood “reversal phase” (Van Tran et al., 1982) of approximately 5 weeks (Eriksen et al., 1984b). In rodents, although the duration of this sequence is compressed, a time delay between resorption and formation still exists: in rat alveolar bone the reversal phase lasts approximately 3.5 days (Vignery and Baron, 1980). These time windows also vary with site, skeletal health, and treatment (Jensen et al., 2014). The increased duration or arrest of reversal phase is also observed in postmenopausal osteoporosis which associates with decreased trabecular bone volume and osteoid formation (Andersen et al., 2013). Exploring the mechanisms by which coupling signals overcome the time delay between bone resorption and bone formation may provide clues in facilitating bone regeneration during resolution of periodontitis.

Under inflammatory conditions, cytokines, such as TNF, RANKL, IL-1, IL-6, and IL-17, promote osteoclastogenesis or activate osteoclasts to resorb bone (Boyle et al., 2003; Graves, 2008; Okamoto et al., 2017) but an equivalent amount of bone formation does not occur, so that resorption and formation are uncoupled (Graves et al., 2011; Parfitt, 1982). The inflammation may affect bone formation through several mechanisms. First, it may stimulate the death of bone lining cells and reduce the number of available progenitor cells that can form new bone (Al-Mashat et al., 2006; Liu et al., 2006). Furthermore, cytokines associated with

inflammation may inhibit the differentiation of osteoblastic progenitors and thus limit bone formation (Behl et al., 2008; Diarra et al., 2007). The third mechanism is reduced bone matrix production due to the impact of inflammatory mediators (Graves et al., 2011; Liu et al., 2006; Tarapore et al., 2016). On the contrary, bone formation may be facilitated through mechanisms that inhibit osteoclast activation (Boyle et al., 2003), sustains the number of available osteogenic progenitors (Stein and Lian, 1993), promotes their differentiation that recapitulates the steps of bone formation during development (Ferguson et al., 1999), and facilitates their deposition of osteoid matrix proteins (Owen et al., 1990) during resolution of inflammation (Claudino et al., 2010; Dresner-Pollak et al., 2004; Stein and Lian, 1993; Xu et al., 1995)

1.3 Resolution of inflammation: a new therapeutic frontier

The correlation between the resolution of inflammation and periodontal bone regeneration has been clinically observed more than 20 years ago, where patients with compromised resolution (higher bleeding on probing index) following periodontal phase I therapy show poorer bone regenerative outcome (Tonetti 1993). Resolution is an active process which starts during inflammation. During the progression of inflammatory events, platelet-leukocyte interactions elicit the formation of lipoxins A4 and B4, which serve as “stop signals” by blocking further neutrophil recruitment from the post-capillary venules. This stop in further neutrophil recruitment to tissues is also temporally associated with a switch of the families of lipid mediators generated from eicosanoids to resolvins of the E and D series as well as protectins (Serhan and Savill, 2005). The resolvins D1, D2, and E1 and lipoxins A4 and B4 further promote the ability of tissue macrophages to uptake apoptotic neutrophils in a non-phlogistic process termed efferocytosis. Efferocytosis in turn causes a switch in macrophage phenotype from inflammatory to pro-resolving and pro-regenerative, i.e., capable to further promote resolution of inflammation and tissue repair (Back et al., 2014; Gyurko and Van Dyke, 2014; Kourtzelis et al., 2017; Recchiuti, 2013). Inability to adequately resolve acute inflammatory condition leads to the development of maladaptive immunity and result in chronic inflammatory diseases (Fullerton & Gilroy, 2016). While current drug development mainly focusses on anti-inflammatory approaches, the pro-resolution strategies intrinsically afford greater scope in treating chronic inflammatory diseases in which these strategies shorten the time required to

restore tissue integrity and function. However, evidence to date suggests that resolution could be tissue- and stimulus-specific, thus, developing a locally deliverable drug may provide an optimal therapeutic effect for the regionally dysregulated chronic inflammatory disease.

1.3.1 Resolution and Integrins

Timely resolution of an inflammatory response is critical to prevent unnecessary tissue damage and promote reconstitution of tissue integrity after acute inflammatory insult (Kourtzelis et al., 2019). Resolution is a tightly regulated process which restricts further leukocytes influx, promotes their clearance from the tissue and restores functional tissue homeostasis (Fullerton and Gilroy, 2016). While dysregulated resolution may contribute to many chronic inflammatory diseases, current regimens to promote resolution are limited.

Recently, a new perspective to regulate resolution has been proposed through mechanisms associated with integrins. Integrins are cell surface heterodimeric receptors that regulate cell-to-cell and cell-to-extracellular matrix interactions. The integrin family comprises 24 members in mammals, formed by the combination of 18 α and 8 β subunits (Kourtzelis et al., 2017) **(Fig. 1)**. These integrins play a critical role in a wide range of important immune

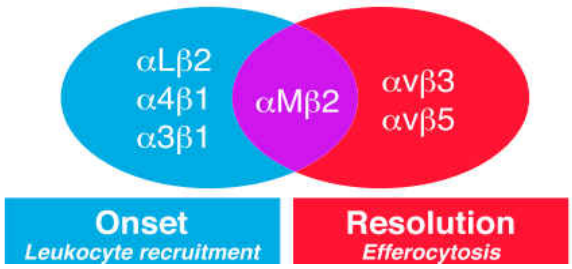

Figure 1. The contribution of integrins to the onset and resolution of inflammation (Kourtzelis et al., 2017).

processes, from the initial recruitment of leukocyte into the sites of inflammation to the clearance of apoptotic cell material in the context of the resolution of inflammation. Recently, an endogenous molecule, DEL-1, has been identified as a homeostatic factor that restrains osteoclastogenesis and β2 integrin-dependent leukocyte recruitment during the initiation of periodontal inflammation (Eskan et al., 2012; Lee et al., 2017; Maekawa et al., 2017) and promote macrophage efferocytosis to facilitate the resolution of inflammation (Kourtzelis et al., 2019). Unlike other endogenous regulators of the leukocyte adhesion cascade, such as pentraxin-3 which is produced by the inflammatory cell itself (Maina et al., 2009), DEL-1 is specifically secreted by cells in the tissue (such as endothelial cells) which indicates its ability to regulate local chronic inflammatory responses and restore functional homeostasis in tissues that express it (Eskan et al., 2012). Here, we present evidence for a novel integrin-dependent mechanism of DEL-1 by which promotes formation of new bone during resolution of periodontitis.

1.3.2 The role of DEL-1 during resolution

DEL-1 is an extracellular matrix protein encoding three Notch-like EGF repeats, an RGD motif, and two discoidin I-like domains (Hidai et al., 1998; Oshima et al., 2014) **(Fig. 2)**. It was first discovered by its unique genetic locus and identified in an enhancer trap event during embryonic vascular development (Hidai et al., 1998; Penta et al., 1999).

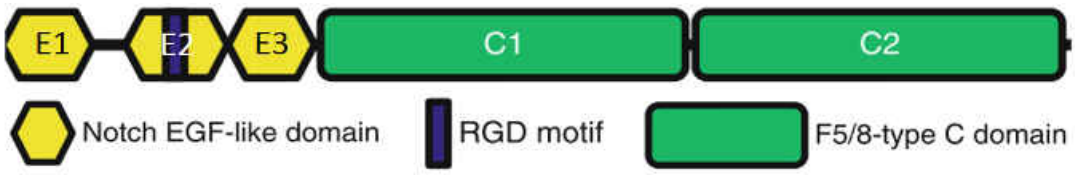

Figure 2. The domain structure of endogenous DEL-1 (Oshima et al., 2014).

The expression of DEL-1 in gingiva is known to be physiologically downregulated during acute periodontal inflammation which allows leukocyte extravasation. (Eskan et al., 2012). Moreover, DEL-1 is downregulated with aging in mice and humans (Eskan et al., 2012; Folwaczny et al., 2017).To study the function of DEL-1 in tissue, a DEL-1-LacZ knock-in transgenic mice model was developed (Yang et al., 2014). In this transgenic, the LacZ gene is inserted into the normal position of DEL-1 and is controlled by the DEL-1 promoter. When bred to homozygosity, the animal becomes functionally deficient in DEL-1. These DEL-1-deficient mice are fertile and live under normal lifespan without apparent vascular or skeletal abnormality. However, these mice do exhibit an accelerated periodontal aging phenotype in which the spontaneous periodontitis starts to develop at a young age in comparison with their wild-type littermates (Eskan et al., 2012).

The genetic deficiency of DEL-1 not only leads to excessive leukocyte infiltration in mouse gingiva but also causes IL-17-dependent periodontal tissue damage (Eskan et al., 2012; Khader, 2012). IL-17 has been shown to downregulate DEL-1 expression in endothelial cells, but this inhibitory action can be counteracted by resolvins D1 and D2, specialized pro-resolving mediators (SPMs) that promote resolution (Maekawa et al., 2015). Recently, Kourtzelis et al further identified a non-redundant role of DEL-1 to promote resolution of acute inflammation through facilitating macrophage efferocytosis of the apoptotic neutrophils through the binding of its RGD motif to the β3 integrin on the surface of macrophages and the binding of its discoidin I-like domains to the “eat-me” signal phosphatidylserine on

the surface of apoptotic cells (Kourtzelis et al., 2019). Using two different mouse inflammation models (monosodium urate (MSU) crystal-induced peritonitis and ligature-induced periodontitis), Kourtzelis et al moreover showed that while endothelial cell-produced DEL-1 was associated with the inhibition of leukocyte recruitment, macrophage-produced DEL-1 was associated with efferocytosis and inflammation resolution. In this regard, DEL-1-facilitated efferocytosis triggered liver X receptor (LXR) signaling pathway in macrophages and transformed its phenotype to a pro-resolving macrophage which promotes the resolution of inflammation. The findings regarding the compartmentalized functions of DEL-1 have revealed a novel location-dependent homeostatic principle ('location principle') in the spatial regulation of the immune response by DEL-1 (Kourtzelis et al., 2019).

1.4 Current gaps in periodontal therapy

Although the classifications of periodontitis have been constantly changed every one or two decades, the way we treat periodontitis nowadays is still no different from what it was 50 years ago. The discrepancy between diagnosis and treatment lies in the lack of understanding on its pathophysiology and how the functional tissue homeostasis is restored during resolution so that a precise evaluation on the prognosis and a corresponding target therapy cannot be achieved. Current approaches for treating periodontitis focus on removing biofilm from the tooth surface; however, host responses to the treatment vary. While some patients show rapid resolution and reparative bone formation after therapy, others resolve slowly or fail to do so (Jin et al., 2000; Lindhe et al., 1985; Rakic et al., 2018; Van der Weijden and Timmerman, 2002). *Therefore, understanding homeostatic mechanisms that facilitate effective bone regeneration following the treatment of periodontitis is a critical and unanswered issue.*

1.5 Hypothesis

We established an *in vivo* model to investigate quantifiable bone formation during resolution of ligature-induced periodontitis in mice. In this model, we found that DEL-1 expression is upregulated in resolving gingiva as compared to its baseline expression in healthy gingiva. The upregulation of DEL-1 in resolving gingiva suggests a significant physiological role of DEL-1 during the resolution, which we investigated DEL-1 by using DEL-1-deficient (*Edil3*$^{-/-}$) mice. Interestingly, whereas wild-type littermate controls regenerated bone during resolution, DEL-1-deficient (*Edil3*$^{-/-}$) mice failed to regenerate bone during the resolution phase. This deficiency in forming new bone was restored by local injection of recombinant DEL-1 during the resolution phase in *Edil3*$^{-/-}$ mice. *Based on these findings, we hypothesized that DEL-1 may act to promote osteogenesis during resolution of periodontitis.*

While the role of DEL-1 in promoting osteogenesis *in vivo* has not been previously addressed, the unique RGD motif on DEL-1 (Schurpf et al., 2012) provides clues for its potential osteogenic effect. Recent studies have shown that osteoblastic progenitors can be directed to differentiate into osteoblasts and promote mineralization by the ligation of its cell-surface integrins by extracellular matrix proteins with RGD motif (Sens et al., 2017). Whereas intact DEL-1 may be required for efferocytosis and the emergence of pro-resolving macrophages (Kourtzelis et al., 2019), if the hypothesized osteogenic effect of DEL-1 depends on its RGD motif, then the RGD-containing *N*-terminal segment of DEL-1 may be sufficient to promote osteogenic differentiation and bone regeneration. *This study*

proposes to characterize a unique function of DEL-1 in the context of the resolution of periodontal inflammation. Specifically, we hypothesize that DEL-1 promotes osteogenic differentiation to restore functional tissue homeostasis during resolution of periodontitis.

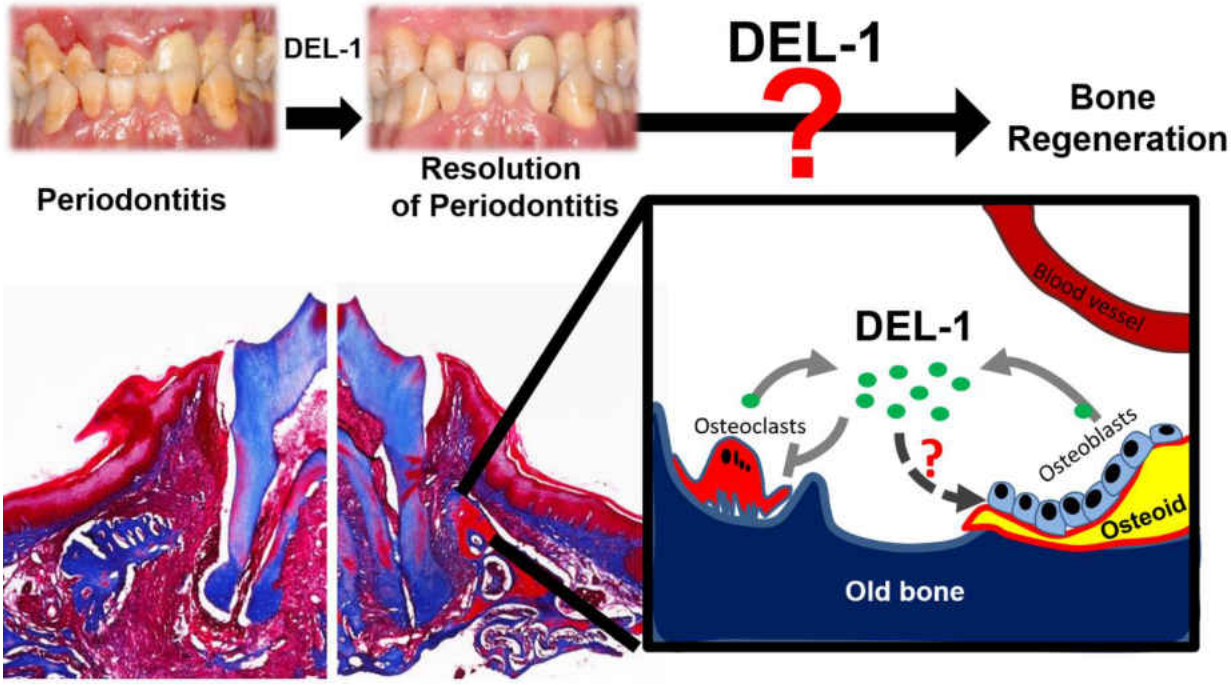

Figure 3. Schematic representation of the hypothesis. This study focuses on interrogating the osteogenic role of DEL-1 during resolution of periodontitis. While it has been shown that DEL-1 can inhibit bone resorption on osteoclasts (Shin et al., 2015), we hypothesized that DEL-1 can directly promote osteogenic differentiation on osteoblasts, and this function promotes formation of new bone during resolution.

Chapter 2. Material and Methods

2.1 Study design

The objective of this study was to understand the mechanisms by which DEL-1 regulates osteogenesis and test its capacity to promote bone regeneration during the resolution phase of ligature-induced periodontitis in mice. All animal procedures were performed according to protocols reviewed and approved by the Institutional Animal Care and Use Committees (IACUCs) of the University of Pennsylvania. *In vivo* experiments in mice involved at least five mice per group including both sexes (determined by GraphPad StateMate power analysis for P=0.05 and a power of 0.80). No animals were withdrawn from the studies according to predetermined criteria in the IACUC protocols or were excluded from any of the analyses. All the *in vitro* and *in vivo* experiments were performed two or more times for verification.

2.2 Animals

Mice were maintained in individually ventilated cages and provided sterile food and water *ad libitum* under specific pathogen-free conditions. They were used for experiments when they were 8 to 10 weeks old. The generation of C57BL/6 DEL-1-deficient (*Edil3* $^{-/-}$) mice was previously described (Choi et al., 2008). Wild-type C57BL/6 mice were purchased from Jackson Laboratory and were bred with DEL-1-deficient mice to establish *Edil3*$^{+/+}$ and *Edil3*$^{-/-}$ littermate controls.

2.2.1 Resolution of ligature-induced periodontitis in mice

Groups of 8-week-old *Edil3*$^{+/+}$ and *Edil3*$^{-/-}$ littermate mice were subjected to experimental periodontitis. A 5-0 silk ligature was tied around the maxillary left second molar for 10 days to induce periodontitis in mice. The contralateral tooth was kept unligated as baseline control. In the resolution group, the ligatures were removed at day 10 to allow 5 days of resolution of inflammation, and the mice were sacrificed at day 15. Periodontal bone loss was assessed morphometrically in defleshed maxillae using a dissecting microscope (40x) fitted with a video image measurement system (Nikon Instruments). Specifically, the CEJ-ABC distance was measured on six predetermined points on the ligated second molar and the affected adjacent regions (Abe and Hajishengallis, 2013). Bone loss was calculated by substracting the six-site total CEJ-ABC distance of the ligated side of each mouse from the six-site total CEJ-ABC distance on the contralateral unligated side. The data was further transformed to indicate bone regeneration (or loss; negative value) by substracting the value obtained from the bone loss generated at 10 days of ligature.

2.2.2 DEL-1 and mutants thereof

To enhance the half-life of recombinant DEL-1 protein, our laboratory has synthesized the chimeric human DEL-1 with the Fc component of human IgG (DEL-1-Fc) (Czajkowsky et al., 2012; Shin et al., 2015). Mutants of DEL-1-Fc including a single amino acid substitution from Glu [E] to Asp [D] in the RGD motif (DEL-1[RGE]-Fc), an N-terminal segment containing only the EGF repeats (DEL-

1-[E1-3]-Fc) were also generated to assist further mechanistic investigation *in vivo* and *in vitro*. It should be noted that DEL-1 performs similar functions (e.g., inhibition of neutrophil transmigration and osteoclastogenesis) across species, probably because human DEL-1 has 96% and 99% a.a. sequence identity with its mouse and cynomolgus counterparts, respectively (Choi et al., 2008; Choi et al., 2015; Eskan et al., 2012; Maekawa et al., 2015; Shin et al., 2015).

2.2.3 Intervention experiments in mice

Groups of 8-week-old *Edil3*$^{-/-}$ mice of both sexes were subjected to experimental periodontitis for 10 days. The ligatures were removed at day 10 to allow resolution of periodontal inflammation. These mice were subsequently daily microinjected (at days 10 to 14) with Fc control (0.31 µg; 12.3 pmol, AG714; Millipore) and various DEL-1-Fc mutants at molar equivalents: Intact DEL-1-Fc (1 µg), DEL-1[RGE]-Fc (1 µg), and DEL-1[E1-3]-Fc (0.54 µg). Microinjections were performed into the palatal gingiva between first and second maxillary molars using a 33-gauge stainless steel needle attached to a micro syringe (Hamilton).

2.2.4 Histological analysis

Coronal sections of the ligated molars were prepared and stained with modified Masson's trichrome staining (# ab150686; Abcam), which stains mature (old) bone and connective tissue in blue; immature new bone (osteoid) and collagen in red (Asonova and Migalkin, 1996).

2.3 In vitro osteogenesis

Osteoblastic progenitors. Primary cultures of osteoblastic progenitor cells were derived from 3-day-old *Edil3*$^{+/+}$ and *Edil3*$^{-/-}$ mice by digesting calvarias in phosphate-buffered saline (PBS) containing 0.1% collagenase (Worthington Biochemical) and 0.2% dispase (Roche Chemical Science) for 20 min at 37^{0}C. The digestion was sequentially performed three times and cells isolated from last two digestions were cultured in α-MEM supplemented with 10% FBS as primary osteoblastic progenitors (Takedachi et al., 2012). The murine osteoblastic progenitor cell line (MC3T3-E1 Subclone 4) was purchased from ATCC (#CRL-2593, Rockville, MD) for receptor knockdown experiments. All cells were maintained in α-MEM supplemented with 10% FBS, penicillin (50 U/ml) and streptomycin (50 mg/ml) prior to further experiments.

Osteogenic differentiation assay. The osteoblastic progenitors were also cultured in osteogenic differentiation medium (contains 50 mg/ml ascorbic acid and 10 mM β-glycerophosphate in α-MEM supplemented with 10% FBS) for 12 to 18 days. The medium was changed every three days. Mineralized bone nodules were detected with Alizarin Red S staining on day 12 or day 18 of osteoblastic differentiation. The cultures were washed twice with PBS, fixed with 4% PFA-PBS for 15min and washed again with PBS and sterile water. Alizarin red staining was performed by covering the cells with 2% Alizarin red S solution (pH 4.1-4.3) for 45 min and then rinsing extensively with water. The plates were scanned, and the calcified nodules were quantified using the Image J software (https:// imagej.nih.gov/ij).

2.3.1 Real-time and quantitative real-time polymerase chain reaction (qPCR) analysis

Following *in vivo* or *in vitro* treatments, total RNA was extracted from gingiva tissue and *in vitro* cultured cells using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Reverse-transcription was carried out using 500ng of total RNA and High-Capacity RNA-to cDNA kit (#4387406, Thermo Fisher Scientific). The level of gene expressed was examined using Taqman Fast Advanced Master Mix (#4444557, Thermo Fisher Scientific) according to the manufacturer's protocol.

The primers used for mouse gene detection include: *Itgb3*(#Mm00443980_m1), *Edil3*(#Mm01291247_m1), *Runx2*(#Mm00501584_m1), *Bglap*(#Mm03413826_mH), *Gapdh*(#Mm99999915_g1)

2.3.2 Western blot analysis

Following treatment, cells were lysed with RIPA buffer supplemented with protease and phosphatase inhibitor, then subjected to SDS-PAGE. Total protein content was quantified and transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore,MA), blocked with 5% non-fat milk in Tris-buffered saline containing 0.05% Tween 20 (TBST), probed with primary antibody (4°C overnight), and incubated with corresponding secondary antibodies (room temperature, 1 hour). After enhanced chemiluminescence (ECL) using Luminata Forte Western HRP substrate (Millipore), the protein bands were imaged using the FlourChem M

imaging system (ProteinSimple) and the band intensities were quantified with the Image J software.

2.3.3 Pull-down assay

Pull-down experiments were performed using Pull-down polyHis protein:protein Interaction kit (#21277; Pierce,IL) as per the manufacturer's instruction. Briefly, MC3T3-E1 cells were cultured in a T75 flask and incubated at 37°C for 48 h. After reaching confluence, the cells (~1 $x10^7$) were released by trypsin digestion, neutralized with medium containing 10% FBS, washed with phosphate buffered saline (PBS) and collected by centrifuging at 500 x g for 5 min. The cells were then lysed, and the cell membrane proteins were isolated using cell fractionation kit (#9038; Cell Signaling Technology). The pull-down washing solution was prepared by mixing 1:1 proportion of TBS: Pierce Lysis Buffer and imidazole stock solution (4M) to adjust to a final concentration of 10 mM imidazole. Cobalt chelate resin (50 µL) was added to the spin column and equilibrated with washing solution. Subsequently, the resin was incubated with 300 µL of histidine-tagged DEL-1-Fc (100 µg) at 4°C with gentle rocking. After incubation for 1 h, the DEL-1-Fc-bonded resins were collected in the spin column by centrifugation at 1250 x g for 1 min, washed 5 times with washing solution, and incubated with the cell membrane lysate of MC3T3-E1 cells for 4 h at 4°C. After washing for additional 5 times, the bound proteins in the resin were eluted with elution buffer (imidazole, 290 mM). The eluted sample was resolved using SDS-PAGE and the captured molecules were identified by immunoblotting with appropriate antibodies, including rabbit anti-

mouse β3 integrin (#13166, CST), β1 integrin (#34971, CST), DEL-1 (#12580-1-AP, Proteintech), and HRP conjugated murine anti-6x-His tag antibody (#R931-25, Invitrogen).

2.3.4 Receptor knockdown by specific shRNA

To knock down integrin β3 in MC3T3-E1 cells, we used the shRNA pLKO.1 construct specific for integrin β3 (TRCN0000009620; Sigma) and, as a control, empty pLKO.1 vector (SHC001; Sigma). Knockdown efficiency was confirmed by qPCR analysis. For transfection of shRNA, cells were plated in 6-well plates at a density of $1.5x10^5$ cells/well, cultured until 70%-80% confluence and transfected with vectors using FuGENE HD® (Roche) transfection reagent following a ratio of 3:1 (volume of FuGENE: µg of DNA). Cells were maintained in nonselective medium for 48 hours post-transfection, and then changed to selection medium containing Puromycin (2 µg/ml). The use of selection medium was continued for 3 weeks with frequent changes of medium to eliminate dead cells and debris until distinct colonies could be visualized. The adherent cells were further released by trypsin digestion and plated in T75 flasks for further propagation. The transfected cells were maintained under selection medium (contained Puromycin 2 µg/ml) for the duration of the experiments.

2.4 Statistical analysis

Data were evaluated with one-way ANOVA and the Dunnett's multiple-comparison test with the InStat program (GraphPad Software). Where appropriate (comparison

of two groups only), two-tailed *t* tests were performed. *P* value <0.05 was considered to be statistically significant.

Chapter 3. Results

3.1 DEL-1 is upregulated during resolution of periodontitis

DEL-1 restrains osteoclastogenesis and inhibits inflammatory bone loss in experimental periodontitis in mice and non-human primates (Shin et al., 2015). Here, we focused on a new function of DEL-1 in the context of periodontitis resolution. Periodontal inflammation resolution was initiated by removing ligatures from C57BL/6 wild-type mice which previously received ligature-induced periodontitis for 10 days (**Fig.4A)**. The bone levels (CEJ-ABC distance values) measured after resolution at day 15 were transformed to indicate bone gain relative to a baseline set by the bone levels measured at day 10 after ligature-induced periodontitis (**Fig.4B-D)**. Ligature removal at day 10 resulted in bone gain 5 days later, whereas the continuous presence of ligatures for 15 days caused further bone loss as compared to baseline (**Fig.4D)**.

The expression of endogenous DEL-1 in gingiva is known to be significantly reduced and downregulated during aging and periodontal inflammation (Eskan et al., 2012). Through quantitative real-time PCR of dissected mouse gingiva, we showed significant upregulation of DEL-1 in resolving gingiva in comparison with the uninflamed baseline ($P<0.05$ vs. non-ligature site; **Fig. 4E).** We reasoned that the upregulated expression of DEL-1 in periodontal inflammation resolution may be involved in the observed bone gain.

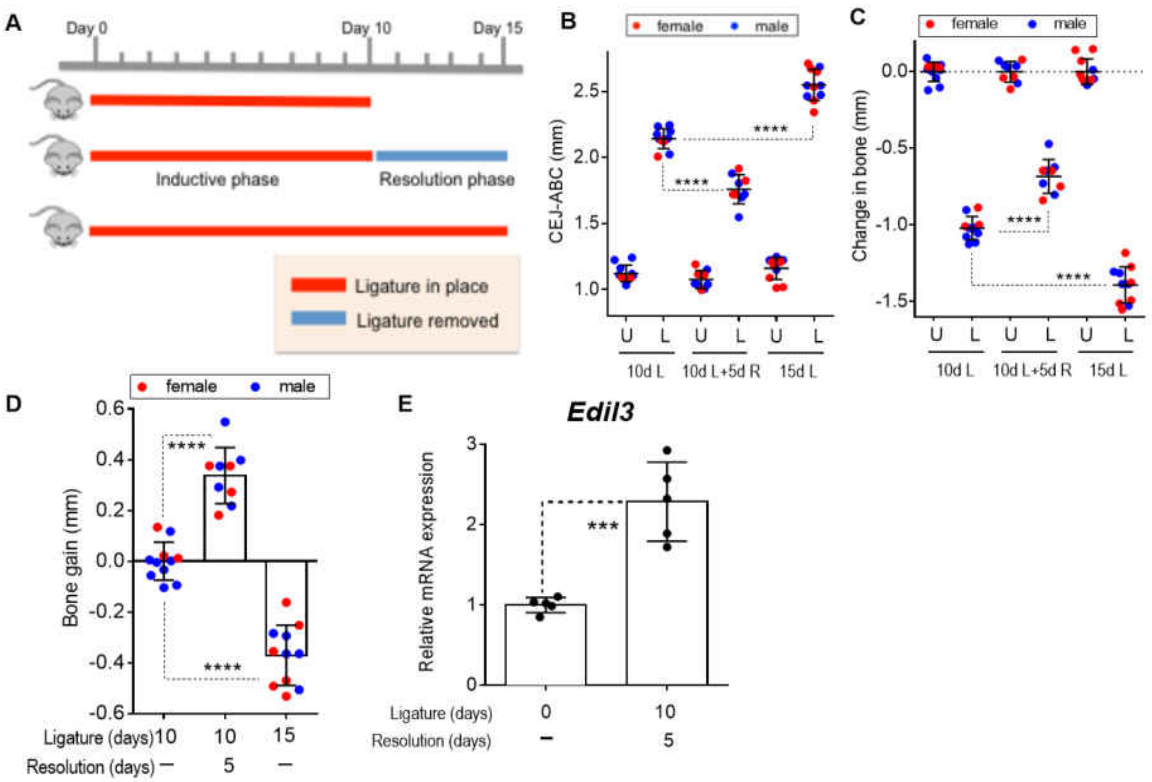

Figure 4. Upregulation of DEL-1 and bone gain during resolution. (A) Model description. (B) Measurement of bone heights (distance from cement-enamel junction to alveolar bone crest; CEJ-ABC) in groups of mice (8- to 10-week old) after 10- or 15-days ligature (10d L or 15d L) or after 10 days ligature followed by 5 days without ligatures to enable resolution (10d L+5d R). (C) Data from B were transformed to indicate bone loss in ligated (L) sites versus un-ligated (U) contralateral sites. (D) Data from C were transformed to indicate bone regeneration (or loss; negative values) relative to the 10d L group which served as the baseline. Data are means ± SD (n=9-11 female or male mice per group). ****P<0.0001 (One-way ANOVA and Dunnett's post-test). (E) Healthy gingiva and gingiva during the resolution phase were dissected from mice and assayed for DEL-1(Edil3) gene expression analysis using qPCR. Data are mean± SD (n= 5 mice/group) ***P < 0.001 (Two-tailed unpaired t-test).

3.2 DEL-1 promotes bone regeneration during resolution of periodontitis

To investigate whether the observed bone gain involved formation of new bone, we performed histological analysis using modified Masson's trichrome staining which stains newly formed osteoid in red and old bone in blue. We then confirmed there was indeed newly regenerated bone on C57BL/6 DEL-1-sufficient (*Edil3*$^{+/+}$) mice during resolution of ligature-induced periodontitis (**Fig.5A**). On the contrary, *Edil3*$^{-/-}$ mice failed to generate new bone although the ligatures were removed (**Fig.5B**). Reduced coronal periodontal attachment but increased gingival swelling was also observed in the resolving phase of *Edil3*$^{-/-}$ mice in comparison with that observed on *Edil3*$^{+/+}$ littermates (**Fig.5B**). The difference in bone regeneration was quantified morphometrically; *Edil3*$^{-/-}$ mice significantly lost ability to regenerate bone during resolution phase ($P<0.05$ vs. *Edil3*$^{+/+}$ littermates; **Fig.6**).

To test whether the deficiency of DEL-1 could be rescued through replenishing the DEL-1 during resolution, we performed daily microinjection of DEL-1-Fc (1μg) versus equal molar amount of Fc control (0.31μg) during resolution phase in *Edil3*$^{-/-}$ mice. As expected, we successfully restored the bone gain in *Edil3*$^{-/-}$ mice by supplying DEL-1-Fc during resolution phase ($P>0.05$; **Fig.6**). To interrogate whether the observed bone gain was contributed by newly regenerated bone, we performed a split mouth experimental design (Antczak-Bouckoms et al., 1990) by performing 10 days LIP and 5 days resolution on both left and right side on the same *Edil3*$^{-/-}$ mice, and locally inject Fc control on the left side and DEL-1-Fc on the right during resolution. The trichrome staining further showed the injection of

DEL-1-Fc not only resolved the gingival swelling but also restored periodontal attachments and new bone formation on *Edil3*$^{-/-}$ mice (**Fig. 5C, D**). In sum, endogenous DEL-1 plays a critical role in formation of new bone during periodontal inflammation resolution which restores functional homeostasis. In contrast, DEL-1 deficiency results in impaired bone regeneration DEL-1 which is restorable through local delivery of DEL-1-Fc during the resolution phase.

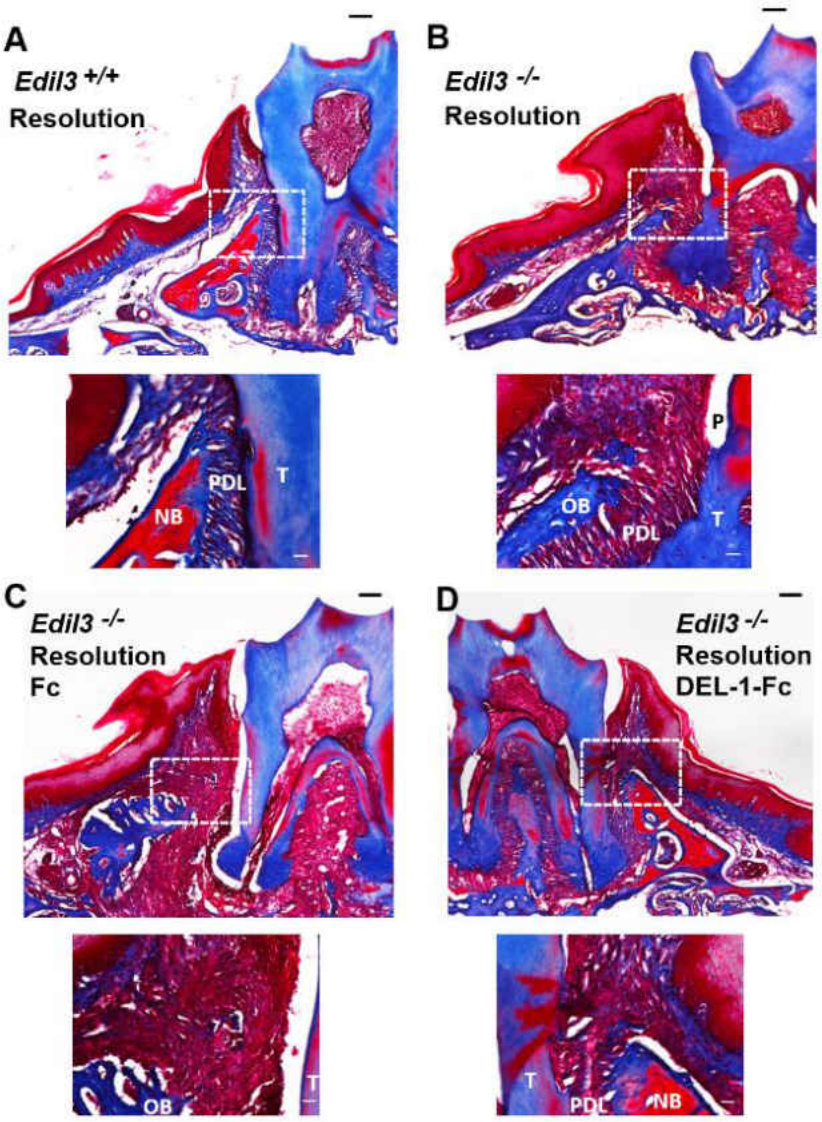

Figure 5. DEL-1-deficient mice fail to regenerate bone during the resolution phase, and the deficiency in bone regeneration can be restored by injecting DEL-1-Fc. Coronal sections of the resolution of ligated molars on **(A)** DEL-1-sufficient (*Edil3*$^{+/+}$) and **(B)** DEL-1-deficient (*Edil3*$^{-/-}$) mice were prepared for histological analysis. Sections from the same *Edil3*$^{-/-}$ mice receiving LIP and resolution on both side but receiving local injection in a split mouth design by injecting Fc control on the left side **(C)** and DEL-1-Fc on the right **(D)**. Sections were stained with modified Masson's trichrome staining, which stains mature (old) bone blue and immature new bone (osteoid) red. Shown are representative images (scale bars, 100 μm) and insets (scale bars, 25 μm) from resolution sites. NB, new bone; OB, old bone; PDL, periodontal ligament; T, tooth; P, Periodontal pocket.

3.3 DEL-1 domains and motifs involved in osteogenesis

We next examined the role of DEL-1 and its structural features in bone regeneration. To this end, we microinjected mutants of DEL-1-Fc into gingiva during resolution, including a single amino acid mutation in the RGD motif (DEL-1[RGE]-Fc), and an N-terminal segment containing only the EGF repeats (DEL-1[E1-3]-Fc). Interestingly, the single amino acid mutation on the RGD motif completely abolished its ability to promote bone regeneration on intact DEL-1-Fc, and the N-terminal segment of DEL-1 (DEL-1[E1-3]-Fc) was sufficient to induce bone regeneration ($P<0.05$ vs. Fc control; **Fig.6**). This finding suggests that DEL-1 promotes bone regeneration through a mechanism dependent on its RGD motif.

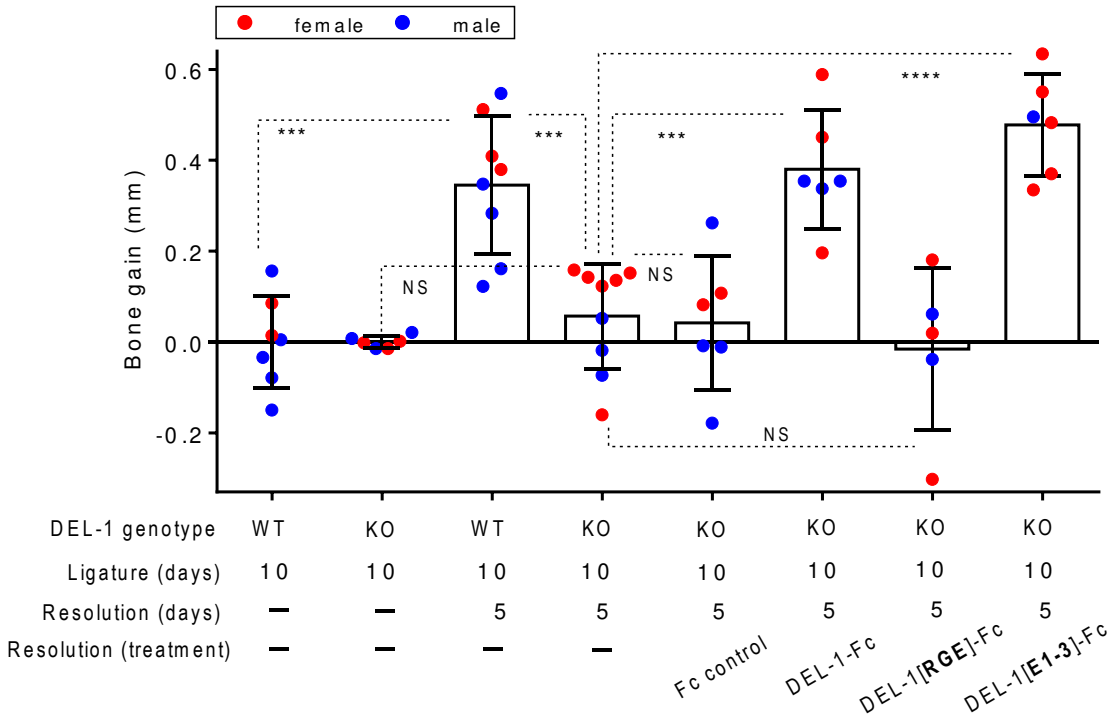

Figure 6. DEL-1 promotes bone regeneration during resolution in an RGD-dependent manner. *Edil3*$^{+/+}$ and *Edil3*$^{-/-}$ littermates (8- to 10-week old) were subjected to ligature-induced periodontitis for 10 days followed (or not) by 5 days resolution, with or without local treatment with DEL-1-Fc (1 µg) or equal molar amounts of Fc control or mutants. Treatments were performed daily (days 10-14) in *Edil3*$^{-/-}$ mice. CEJ-ABC data were transformed to indicate bone regeneration as outlined in Fig.1. Data are means ± SD (*n*=5-9 female or male mice per group). ***P<0.001, ****P<0.0001. WT, *Edil3*$^{+/+}$ mice; KO, *Edil3*$^{-/-}$ mice; NS, not significant (One-way ANOVA and Dunnett's post-test for comparing treatments to untreated KO; two-tailed unpaired *t*-test for the other indicated comparisons, *e.g.*, WT mice with resolution vs. WT mice without resolution).

3.4 DEL-1 promotes osteoblast differentiation through its RGD motif

The ability of DEL-1 to promote bone regeneration could be explained by direct action on osteoblastic cells or indirectly by DEL-1 enhancement of β3 integrin-mediated efferocytosis which promotes inflammation resolution as demonstrated recently (Kourtzelis et al., 2019) thus enhancing osteoblast function and bone formation. The latter mechanism is consistent with the RGD motif requirement as the efferocytic function of β3 integrin depends on this motif (Hanayama et al., 2004; Kourtzelis et al., 2019). However, efferocytosis also requires the involvement of the *C*-terminal discoidin-like domains of DEL-1, which bind phosphatidylserine, a major 'eat-me' signal on the apoptotic cell surface (Hanayama et al., 2004; Kourtzelis et al., 2019). To interrogate whether DEL-1 can directly interact with osteoblastic progenitors to promote bone gain, we compared the osteogenic capacity of DEL-1 on cultured *Edil3*$^{-/-}$ versus *Edil3*$^{+/+}$ primary osteoblastic progenitors. *Edil3*$^{-/-}$ osteoblastic progenitors exhibited a limited ability in forming calcified nodules (**Fig.7A**). Consistently, this mineralization deficiency was restored by supplying DEL-1-Fc (2μg/ml) every three days in the osteogenic medium in comparison with Fc control (0.62μg/ml). Treatments with DEL-1-Fc mutants used at the same molar concentration DEL-1 showed that the *Edil3*$^{-/-}$ progenitors treated with DEL-1[RGE]-Fc failed to generate calcified nodules, but the progenitors treated with DEL-1[E1-3]-Fc could form calcified nodules as when treated with intact DEL-1-Fc. Further examination on the osteogenic markers showed that the N-terminal part of DEL-1 upregulated osteogenic genes, *Runx2* and *Bglap,* and facilitated osteogenic differentiation in an RGD-dependent manner

(*P*<0.05 vs. Fc control; **Fig.7B**). These *in vitro* experiments further support our findings *in vivo* and indicate that DEL-1 promotes bone regeneration during resolution through directly regulating osteogenic differentiation on osteoblastic progenitors.

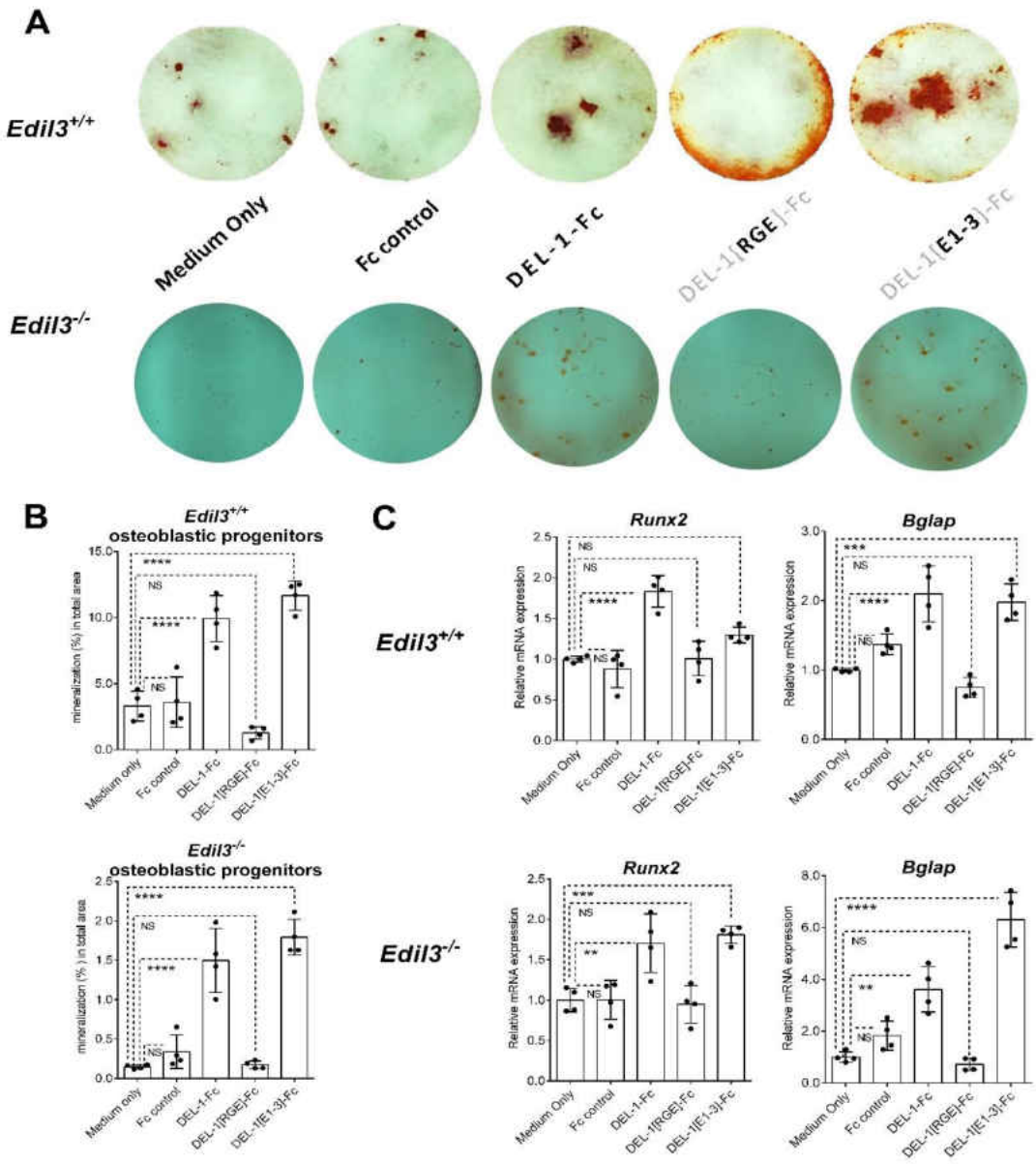

Figure 7. DEL-1 promotes osteoblast differentiation through its RGD motif. Primary osteoblastic progenitor cells were isolated from the calvariae of 3-day-old *Edil3*$^{+/+}$ and *Edil3*$^{-/-}$ mice. The isolated progenitors were cultured in osteogenic differentiation medium in the presence or absence of intact DEL-1-Fc (2 μg/ml) or equal molar amounts of Fc control or mutants. **(A)** Representative mineralized nodules detected with Alizarin Red S staining on day 18 of differentiation. **(B)** The quantified mineralization at day 18, and **(C)** the expression of osteogenic regulated by DEL-1-Fc or mutants were compared at 12

days of differentiation. Upper and lower panel indicated for the osteogenic markers expressed from *Edil3*$^{+/+}$ and *Edil3*$^{-/-}$ progenitors, respectively. Data are means ± SD (n=4 cultures per group). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, NS, not significant (one-way ANOVA and the Dunnett's multiple-comparison test).

3.5 DEL-1 interacts with β3 integrin on osteoblastic progenitors to promote mineralization.

To determine the receptor(s) DEL-1 may use to promote osteogenic differentiation, we performed pull-down assay through binding poly-histidine-tagged DEL-1-Fc onto the polyHis pull-down column and incubated with cell membrane lysate isolated from the murine osteoblastic progenitor cell line (MC3T3-E1 subclone 4). The pull-down results revealed that DEL-1 preferentially interacted with β3 but not β1 integrin on osteoblastic progenitors (**Fig.8A**). To confirm whether DEL-1 promotes osteoblasts mineralization through a β3 integrin-dependent manner, we stably inhibited β3 integrin expression using *Itgb3* shRNA in MC3T3-E1 cells ($P<0.0001$ vs. control shRNA; **Fig.8B**). In MC3T3-E1 cells, DEL-1-Fc induced the expression of the master transcription factor *Runx2*, and *Bglap* (Osteocalcin), typical early, and late osteogenic markers respectively ($P<0.05$ vs. Fc control; **Fig.8C**); however, these upregulatory effects of DEL-1-Fc were abrogated in β3 integrin shRNA-transfected cells ($P>0.05$ vs. Fc control; **Fig.8C**). Using the above-mentioned mineralization assay, we further confirmed that the ability of DEL-1 to promote mineralization was abolished in *Itgb3* knockdown transfectants ($P>0.05$ vs. Fc control; **Fig.8D**).

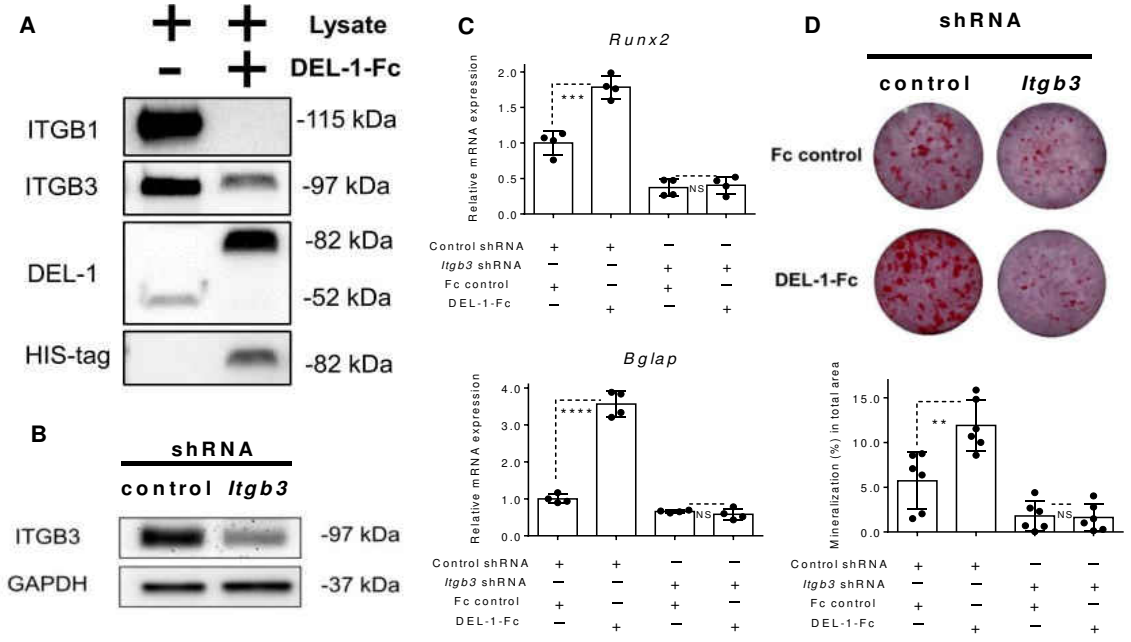

Figure 8. DEL-1 promotes osteoblast mineralization through β3 integrin-dependent mechanism. (A) PolyHis pull-down assay was performed using polyhistidine-tagged DEL-1-Fc (100μg) bound to the cobalt-chelate resin column. Cell membrane protein lysates from MC3T3-E1 cells (1 x10^7 cells) were loaded into the DEL-1-Fc-bound column, and the interacted proteins were eluted with imidazole and resolved using SDS-PAGE followed by immunoblotting with antibodies to the indicated molecules. Lysates represent 10% input. MC3T3-E1 cells were further transfected with control versus β3 knockdown shRNA. **(B)** The knockdown of β3 integrins was confirmed by immunoblotting. **(C)** MC3T3-E1 cells were cultured in osteogenic medium in the presence of DEL-1-Fc (2μg/ml) or Fc control (0.62 μg/ml). The cells were analyzed for the expression of *Runx2* (at day 6), *Bglap* (at day12). **(D)** Representative images of Alizarin Red S staining, and the quantified mineralization results. Data of mRNA expression level were normalized to those of *Gapdh* mRNA and expressed as fold change in transcript level relative to control shRNA-treated cells, which were assigned an average value of 1. Data are means ± SD (*n*= 4-6 cultures per group). $^{**}P < 0.01$, $^{***}P < 0.001$, not significant (two-tailed unpaired *t*-test).

Chapter 4. Discussion and Conclusion

4.1 DEL-1 as a novel osteogenic factor: Implications and future directions

While the murine periodontal resolution model has been previously developed to interrogate the mechanism by which DEL-1 promotes inflammation resolution (Kourtzelis et al., 2019), the current model has been further developed to investigate the ability of DEL-1 to mediate a novel function during inflammation resolution, namely bone regeneration. Our findings show for the first time that DEL-1 promotes formation of new alveolar bone during resolution of experimental periodontitis in mice. Mechanistically, we showed that DEL-1 promotes osteoblastic differentiation by acting via its RGD motif on β3 integrin in osteolineage progenitors.

Since regulating inflammation may also have a profound effect on bone formation, the possibility that DEL-1 may indirectly promote bone formation during resolution through its anti-inflammatory effect cannot be ignored. DEL-1 has been shown to regulate inflammation through at least two distinct mechanisms: inhibiting neutrophil recruitment to infected or inflamed tissues (Choi et al., 2008; Eskan et al., 2012) and promoting efferocytosis (Kourtzelis et al., 2019). The latter mechanism contributes critically to inflammation resolution and is thus likely to secondarily enhance osteoblast function and bone formation (Matzelle et al., 2012). However, we have conclusively shown that DEL-1 can have direct effects on osteogenesis. First, DEL-1(E1-3)-Fc, which lacks the *C*-terminal discoidin-like domains, successfully promoted *in vivo* bone regeneration in our model (**Fig.6**),

whereas it failed to promote efferocytosis (Kourtzelis et al., 2019). Second, DEL-1 *in vitro* was capable of promoting the differentiation of osteoprogenitors into osteoblastic cells with mineralization capacity. Consistently, both the above-mentioned *in vivo* and *in vitro* activities of DEL-1 were absolutely dependent upon its integrin-binding RGD motif and the interactive partner on osteoprogenitors is likely the β3 integrin. An earlier study, which also used MC3T3-E1 osteoprogenitor cells, an established model of osteoblast differentiation and function (Addison et al., 2015; Quarles et al., 1992), implicated α5β1 integrin as the DEL-1 receptor (Oh et al., 2017). However, the authors found that DEL-1-induced matrix mineralization was inhibited equally well by antibodies to either β1 or β3 integrin. A future experiment utilizing osteoblastic lineage-specific β3 integrin knockout mice may conclusively implicate this integrin as the DEL-1 receptor in osteoprogenitors. If injecting DEL-1-Fc fails to promote bone regeneration in these conditional knockout mice, one may conclude that bone regeneration is contributed by the direct effect of DEL-1 on β3 integrin in osteoblastic lineages.

The association between DEL-1 and osteogenesis has been previously inferred by genetic (Layh-Schmitt et al., 2017) and proteomic (Rose-Martel et al., 2015) mineralization screenings. The osteogenic effect of DEL-1 is also consistent with relevant studies on another glycoprotein, the milk-fat globule-epidermal growth factor 8(MFG-E8), which is structurally similar to DEL-1 (MFG-E8 and DEL-1 both contain two discoidin-like domains and RGD motif, although DEL-1 has three EGF domains and MFG-E8 only two). Indeed, deficiency of MFG-E8 in mouse

osteoblastic progenitors resulted in compromised bone formation *in vivo* and *in vitro* (Sinningen et al., 2015). While the RGD motif of DEL-1 and MFG-E8 has been shown to bind to αvβ3 integrin in different cell types, direct evidence for interaction with αvβ3 integrin on osteoblastic progenitors has been shown for DEL-1 only, in our present study. In this regard, our pull-down assay indicated a direct interaction of DEL-1 with β3 on osteogenic progenitor (MC3T3-E1) cells. Another research group has also shown that DEL-1 can induce Runx2/ERK signaling pathway to induce osteogenic differentiation in MC3T3-E1 cells (Oh et al., 2017).

The periodontal ligament (PDL) contains a heterogeneous mesenchymal stromal and stem cell (MSC) population which is involved in periodontal tissue regeneration, including the extracellular matrix and adjacent mineralized tissues such as the alveolar bone. Multipotent stem cells in the PDL can differentiate into distinct lineages including osteoblasts that form alveolar bone (Bassir et al., 2016; Fournier et al., 2013; Ren et al., 2015; Seo et al., 2004; Washio et al., 2010; Yamamoto et al., 2014). Future studies can address whether DEL-1 in the PDL regulates the MSC niche to promote bone regeneration and to identify the underlying mechanisms. In this regard, DEL-1 was shown to regulate the bone marrow stem cell niche. Specifically. DEL-1 was shown to be a crucial regulator of the hematopoietic stem cell niche (HSC) niche, i.e., the supportive micro-anatomical environment in the bone marrow where HSCs reside. In the HSC niche, DEL-1 is expressed by arteriolar endothelial cells, cells of the osteoblastic lineage and a specialized mesenchymal stromal cell (MSC) type, the CXCL12-abundant reticular cells (Mitroulis et al., 2017a). Similar to our studies presented

here, DEL-1 was shown to interact with β3 integrin on HSCs and to promote their proliferation and myeloid differentiation (Mitroulis et al., 2017b).

4.2 Application of current work to the clinical periodontal therapy

The physiological upregulation of DEL-1 and its novel osteogenic property during resolution of periodontitis renders DEL-1 an ideal biomarker to monitor and predict patient's response during reevaluation phase of periodontal therapy. This concept has been successfully demonstrated in our recent work in which the upregulation of DEL-1 can be detected in gingival crevicular fluid (GCF) of patients following periodontal therapy (scaling and root planing) (Kourtzelis et al., 2019). Further application of DEL-1 as a pro-resolution and osteogenic protein drug may be suitable to apply in specific patient populations (such as the elderly), in which poor resolution response to the scaling and root-planing as well as lower level of DEL-1 in GCF is detected, as customized precision medicine to restore functional bone regeneration in these patients.

Most of the current periodontal regenerative strategy requires surgical intervention so that a direct application of osteoinductive protein drug (such as BMP2, PDGF-BB, and Enamel Matrix Derivatives) on the root surface may be feasible. However, such kind of surgical strategy underestimates the regenerative potential in our body in which the functional bone regeneration is achievable under proper homeostatic regulation. The law of bone remodeling, commonly referred to as *Wolff's law,* asserts that the internal trabecular bone adapts to the external loadings, reorienting with the principal stress trajectories to maximize mechanical efficiency creating a naturally optimum structure (Boyle and Kim, 2011), in which "the remodeling of the bone occurs in response to physical stress or lack of stress" (Oppenheimer et al., 2008; Wolff, 1986). As cervical periodontal ligaments and

fibers play critical role in mediating the principal occlusal stress to alveolar bone (Nomoto et al., 2006; Saffar et al., 1997), transplanting tooth with intact cervical periodontal fibers further regenerates crestal bone in periodontal defect while transplanting tooth with defective cervical periodontal fibers result in crestal bone loss in the previous study (Polson and Caton, 1982). The traditional periodontal bone-grafting surgery apply the bone graft to the periodontal bony defect in crestal stress bearing area; however, this procedure is against to *Wolff's law* because any bone graft interrupts the stress trajectories will be prone to resorption (Oppenheimer et al., 2008), besides, the periodontal incisions during bone-grafting surgery unavoidably dissect or remove cervical periodontal fibers which may interrupt the distribution of the occlusal force and contribute to crestal bone loss (Carnevale and Kaldahl, 2000; Nomoto et al., 2006; Pfeifer, 1965; Wood et al., 1972). In comparison with traditional bone-grafting surgery, local injection of DEL-1 provides an osteogenic strategy without interrupting the load-bearing structure of pre-existing cervical periodontal fibers. Through the comparison of local injecting Fc control or DEL-1-Fc in *Edil3*$^{-/-}$ mice, we have demonstrated that DEL-1 injection regenerates the crestal tooth-supporting bone in the context of resolution and restore similar functional tissue structure to its DEL-1-sufficient littermate. This physiological bone regenerating strategy may allow regenerating the functional structure of periodontal bone in the patient with insufficient of DEL-1 and reduce the need for bone-grafting periodontal surgery.

The proposed pro-resolving DEL-1 therapeutic strategy for periodontitis is intended to be applied as a complementary approach to augment the therapeutic

effect following the standard phase I periodontal therapy (i.e. oral hygiene instruction, scaling and root planing. Interestingly, DEL-1 promotes the production of certain resolvins (e.g. RvD1 and RvE1) during inflammation resolution in periodontitis (Kourtzelis et al., 2019). Reciprocally, resolvins can contribute to the rising levels of DEL-1 during the resolution of inflammation, for instance, by counteracting inhibitory mechanisms that would otherwise suppress DEL-1 expression (Maekawa et al., 2015). Moreover, DEL-1 is a non-redundant effector of inflammation resolution acting downstream of resolvins (Kourtzelis et al., 2019). Together, these findings suggest the operation of a positive feedback loop that connects DEL-1 and resolvins and reinforces inflammation resolution. As the traditional plaque control strategy has been well-established for most periodontitis patients, DEL-1 therapy applied on top of plaque control may further improve treatment quality through facilitating resolution of inflammation and functional tissue regeneration.

4.3 Conclusion

Our findings support the notion for a direct effect of DEL-1 in promoting osteoblastogenesis and formation of new bone through a β3 integrin dependent mechanism. Whereas intact DEL-1 is required for efferocytosis and the emerging of pro-resolving macrophages (Kourtzelis et al., 2019), the RGD-containing *N*-terminal part of DEL-1 is sufficient to promote osteogenic differentiation and bone regeneration. In conclusion, DEL-1 is shown for the first time to promote bone regeneration during resolution of periodontitis, a function that may be adjunctively exploited in combination with the current periodontal therapy to facilitate bone regeneration during resolution of human periodontitis.

Chapter 5. Translational Application of the Research

5.1. Introduction

5.1.1 The current hurdle of regenerative periodontal therapy

More than 30 years have passed since the first successful application of regenerative therapy for the treatment of periodontal diseases (Lin et al., 2015). Despite being feasible, a complete restoration of structure and function of diseased periodontium is still an unpredictable task and the relevance of comparing costs and outcomes of periodontal treatment has increasingly and repeatedly been emphasized in the periodontal literature (Braegger, 2005; Cochran et al., 2015; Listl and Birch, 2013; Pennington et al., 2011; Pennington et al., 2009). While the health care resources (such as dentists and dental assistant's time, surgical instruments, diagnostic equipment, and regenerative materials to be applied) are limited, doctors are forced to make the best use of those resources to compose a treatment plan within patient's affordability which limits the availability of optimal treatment outcome. Although the rapid advance in medical technology often leads to a more effective treatment option, such option often involves higher costs than existing therapeutic approaches and piles up the treatment cost. Hence, developing an effective, efficient, yet affordable treatment strategy is a critical and unmet need for translational periodontal research.

Historically, periodontal regeneration has focused on applying barrier membrane and scaffold such as bone substitutes to provide for defect fill and selected cell repopulation of the lesion (Cochran et al., 2015). Over the past two

decades, numerous studies have explored the potential of using biologic proteins and peptides in periodontal regeneration, and several have been approved for clinical use (Lin et al., 2015). While emerging studies continuously discover new protein candidates for their pharmaceutical application in customized periodontal regeneration and precision medicine, the expense of protein synthesis, purification, and preservation had piled up the cost of these therapeutic strategies. However, a precision medicine cannot be precise enough if the patient who need it cannot afford for the treatment. A novel strategy in protein drug synthesis, preservation and delivery remains unavailable from the market. Recent advance in biopharmaceutical research has developed the technique to reduce the cost of protein drug manufacturing through genetic modification on the chloroplasts to synthesize pharmaceutical proteins.

5.1.2 Chloroplast as a bioreactor for affordable protein drug synthesis

Chloroplasts are one of the ultimate symbiotic collaboration between prokaryotic microbe (cyanobacteria) and its eukaryotic host (plant cells). This collaboration eventually sustains life on earth by converting solar energy to carbohydrates through the process of photosynthesis and oxygen release (Daniell et al., 2016). Beyond photosynthesis, chloroplasts also play several critical roles in plant physiology and development, including synthesis of amino acids, nucleotides, phytohormones, fatty acids and production on some multitude of secondary metabolites and the sulfur and nitrogen assimilation (Pfannschmidt and Yang, 2012). Chloroplasts possess their own genomes, ribosomes, tRNAs and protein

synthetic machinery; they also import thousands of nuclear encoded proteins synthesized in the cytoplasm (Daniell et al 2016 A, B). Chloroplast genome contains operons like prokaryotes but also several types of introns like eukaryotes. Thus, chloroplasts possess both prokaryotic and eukaryotic features.

During evolution, hundreds of genes of the endosymbiont have been transferred to the host nuclear genome (Chen et al., 2018); thus most chloroplast proteins are synthesized in the cytosol and imported into the chloroplast for self-assembly or along with subunits synthesized by the chloroplast genome (Daniell et al 2016 A, B). This unique collaboration makes chloroplast a specialized organelle for protein synthesis, folding and several posttranslational modifications (PTMs) in plant cells. Specifically, the endogenous disulfide transferase in chloroplast can facilitate protein folding and stabilize the tertiary structure to maintain the proper functional protein confirmation (Zhang et al., 2017). On the other hand, recent studies also reveal that chloroplast can perform several PTMs such as phosphorylation, acetylation, methylation, and may be able to collaborate with endoplasmic reticulum (ER) and Golgi apparatus to achieve N-glycosylation via vascular Golgi-to-plastid transport pathway (Kitajima et al., 2009; Lehtimaki et al., 2015; Zhang et al., 2017). The independence of chloroplast genome and maternal inheritance characteristic makes chloroplast an ideal bioreactor for a low cost and rapid scale-up biopharmaceutical production, and minimizes the concern of outcross of introduced foreign genes (Daniell, 2007). While biologically active enzymes and vaccines generated by this technique has been shown to be functional despite keeping in room temperature, this innovative protein synthesis

strategy provides an ideal platform in translating active periodontal regenerative protein into affordable regenerative treatment modality.

As DEL-1-Fc has been shown to be an effective protein drug to restrain IL-17-associated bone loss in non-human primates (Shin et al., 2015), the current study further confirms its potential in promoting bone regeneration during inflammation resolution. However, the ultimate goal of our scientific work is not just to provide effective medicines that improve current treatment, but also to make it affordable so that the majority of the patient in need could benefit from it. Thus, at the end of this thesis, we performed a translational study by sub-cloning DEL-1-Fc sequence into chloroplast vectors and aiming to transform the lettuce chloroplast genome to generate genetically modified plants expressing DEL-1-Fc so that periodontitis patients may benefit from this research.

5.2 Materials and Method

5.2.1 Subcloning DEL-1-Fc plasmid targeting lettuce chloroplasts

To target the lettuce chloroplast for DEL-1-Fc protein expression, we developed a plan to insert DEL-1-Fc sequence into the lettuce-chloroplast targeting plasmid (pLs-MF). The pLs-MF backbone vector was previously constructed by Henry Daniell's laboratory (Daniell et al., 2019; Kumari et al., 2019) which contains the lettuce chloroplast genome flanking sequences including partial 16S (16S rRNA), isoleucine tRNA(*trnI*), alanine tRNA(*trnA*) genes, and partial 23S (23S rRNA) to facilitate homologous recombination of the transgene cassette into native lettuce chloroplast genomes (**Fig.9**). The unique expression cassette of the pLs-MF contains a pair of direct repeat *atpB* sequences (649 bp) flanking the *aadA* selection marker gene (encoding aminoglycoside 3'-adenylyltransferase) to confer initial spectinomycin selection and later on marker gene excision (Daniell et al., 2019; Kumari et al., 2019).

The original sequence of DEL-1-Fc was acquired from a pSecTag2-DEL-1-Fc plasmid constructed by George Hajishengallis's laboratory (Choi et al., 2008). This DEL-1 plasmid (pSecTag2-DEL-1-Fc) included nucleotides for 6 histidine sequences at its 3' end so that this His-tagged recombinant protein could be further purified by Ni-NTA column. However, for the translation propose, in order to accommodate the regulation from Food and Drug Administration (FDA), that antigenic epitope should be removed to avoid potential immunogenic reaction. Thus, the His-tag was removed from Del1-Fc using a reverse primer covering the

3’ end of Fc sequence, as well as the stop codon and restriction site suitable for sub-cloning. The DEL-1-Fc insert was amplified by using specific primer sets to create the restriction sites suitable for insertion into pLS-MF (Daniell et al., 2018). In brief, the DEL-1-Fc insert was amplified with forwarding primer (5’- ATT TTA CAT ATG AAG CGC TCG GTA GCC -3’) containing NdeI cutting site and reverse primer (5’- CAA TAA GAC CAA AGT CTC TAG ATC ATT TGC CCG GGC TCA GGC T -3’) containing PshAI cutting site (**Fig.10**). The 3’ end of DEL-1-Fc insert was further extended by overlapping PCR (Kwon et al., 2018) to create NotI cutting site using additional reverse primer (5’- CAG CCA CCG CGG TGG CGG CCG CTT TC -3’) followed by complete digestion on NotI and partial digestion of NdeI restriction enzymes, respectively. The high-fidelity polymerase (#M0491S, NEB) was applied to the abovementioned PCR reactions to avoid mutations when amplifying insert.

For subcloning of DEL-1-Fc into the lettuce marker-free chloroplast vector pLs-MF, it was digested with NdeI (#R0111S, NEB) and NotI (#R0189S, NEB) restriction enzymes, respectively. DEL-1-Fc fragment was further sub-cloned into the linearized vector using NEBuilder® HiFi DNA Assembly (#E5520, NEB). The constructed plasmid was then transformed into TOP10 *E. coli* cell and selected on the Lennox LB broth agar plate with spectinomycin (100 mg/L) overnight (Kwon et al., 2018). Screening for recombinant plasmids was done by colony PCR (Bergkessel and Guthrie, 2013; Verma et al., 2008) using forward (5’- CAA GCC TTT AGT TTT CCA TTT GAA AAT TCG TGC GC -3’) and reverse (5’- TTT CTG AAA TCA AA GA A ATA AAT AAT AAA AAT TTT CAT TTT TAT CT -3’) primers.

The positive clones were further cultured in 5ml of ampicillin-containing (100 mg/L) LB broth (Lennox) medium overnight. The plasmids were extracted by the alkaline lysis method (Green et al., 2012) to confirm the presence of the appropriate insertion by restriction enzyme digestion map using PstI (#R0140S, NEB) and PvuII (#R0151S, NEB) dual cutting enzymes. The DEL-1 protein expression from double positive clones was further confirmed by western blots using monoclonal rabbit anti-human DEL-1 antibody [ab190692, Abcam]. The sequence on both ends of the expression cassette (pLs-MF-DEL-1-Fc) was confirmed by Sanger sequencing using the forward (5'- CAA GCC TTT AGT TTT CCA TTT GAA AAT TCG TGC GC -3') and reverse (5'- TTT CTG AAA TCA AAG AAA TAA ATA ATA AAA ATT TTC ATT TTT ATC T -3') primers, respectively. Finally, the sequence-confirmed plasmids were transformed into TOP10 *E. coli* cell and extracted using PureYield™ Plasmid Miniprep System (Promega, Madison, WI).

5.2.2 Fc-Based Receptor-Binding Functional assay

To confirm protein function before transforming to lettuce, a receptor-binding immunoprecipitation assay was performed. Overnight cultured *E. coli* cells in ampicillin and spectinomycin (100mg/L) -containing Lennox LB broth medium (#BP1427-500, Fisher Scientific) were pelleted, re-suspended in 4ml of extraction buffer (#78248, Thermo Scientific) containing 8µl of lysozyme (L-6876,Sigma) and 8µl of DNase I (#4716728001, Sigma) and 20mg of EDTA-free protease inhibitor (#A32955, Thermo Scientific) per gram of cell pellet and incubate at room temperature for 15 minutes to break down cells. The cell lysate was further

centrifuged at 15,000 x g for 5 minutes to separate soluble proteins from the insoluble proteins and performed western blot to identify which component contains DEL-1-Fc protein. After identifying the DEL-1-Fc in the insoluble portion (inclusion body) (**Fig.13A**), the insoluble proteins were further dissolved by inclusion body solubilization reagent (# 78115, Thermo Scientific), and dialyzed in 1L of 6M urea with 5mM DTT in 4^{o}C for 24 hours and refolded *in vitro* by adding 250ml of 25mM Tris-HCL (pH 7.5) every 12 hours until the final volume become 3L. Finally, the dialysis solution was replaced with 25mM Tris-HCl (pH 7.5) and 150mM NaCl for another 6 hours to elute the protein. After elution, the refolded proteins (50 µg) were first mixed with the protein-G conjugated magnetic beads (10003D, Invitrogen) at 4^{o}C for 2 hours, and then transfer to the magnetic separation rack (7017, CST) to purify the refolded DEL-1-Fc utilizing the affinity of its Fc component with Protein G. The protein G-purified DEL-1-Fc as well as same amount of negative control protein (50 µg, human IgG-Fc, AG714, Millipore) and positive control protein (50 µg, human cell-derived DEL-1-Fc) were further reacted with the 50µg of cell-membrane protein isolated from human gingival-derived mesenchymal stem-like cells (hGMSC) which was isolated by cell fractionation kit (#9038, CST). The immunoprecipitated complex was then washed by RIPA buffer four times and re-suspended with 1x Laemmli buffer. The unreacted total protein was also collected and re-suspended with 1x Laemmli buffer. All buffer-containing samples were heated at 70^{o} C for 15 minutes and loaded in SDS-PAGE for western blot.

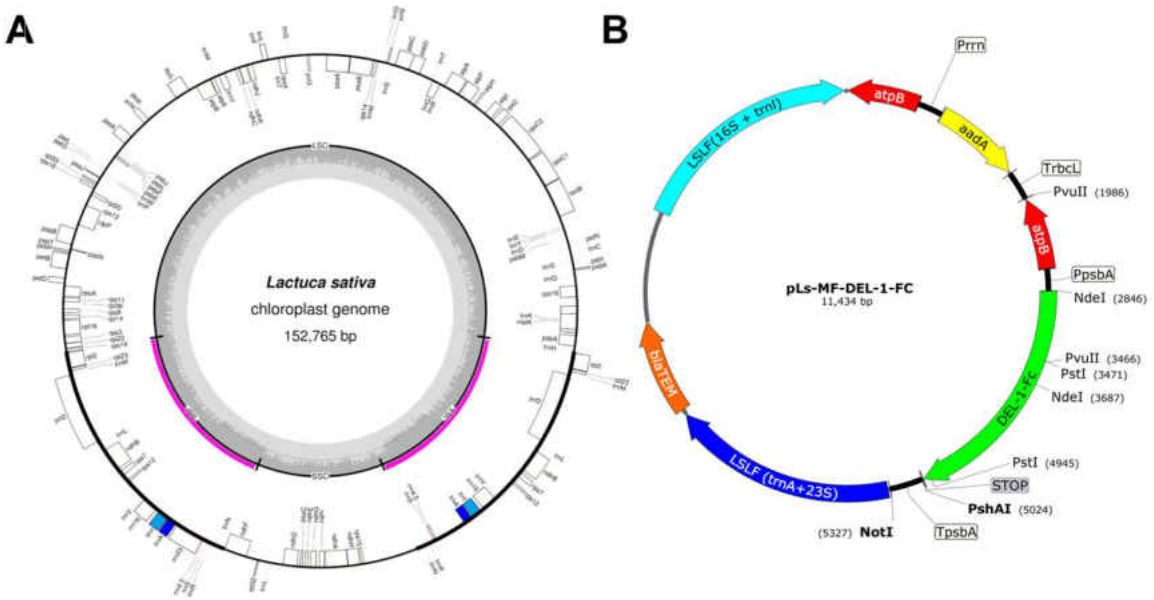

Figure 9. Maps of the Lettuce chloroplast genome and pLs-MF-DEL-1-Fc marker-free chloroplast vector. A. Map of lettuce (*Lactuca sativa*) chloroplast genome (accession number: AP007232). The dark and light blue marked the location of *trnA* and *trnI* genes, respectively. **B.** Marker-free chloroplast vector DEL-1-Fc: All components of the lettuce marker-free chloroplast vector were previously developed in the Daniell laboratory (Daniell et al., 2019; Kumari et al., 2019). The *trnA* and *trnI* genes are respectively shown in dark and light blue; the inverted repeat sequences of the *atpB* gene are shown in red; the *aadA* and *blaTEM* genes are shown in yellow and orange for spectinomycin and ampicillin resistance genes, respectively. The DEL-1-Fc gene is shown in green, and the restriction sites used for sub-cloning shown on the plasmid map. The *Prrn* and *PpsbA* are chloroplast promoters; the *Trbcl* and *TpsbA* at 3' end of the *aadA* and *DEL-1-Fc* genes are 3'UTRs. *STOP* shows the stop codon. The lettuce chloroplasts genome map was originally developed in this thesis using OGDRAW online software (https://chlorobox.mpimp-golm.mpg.de/OGDraw.html) using lettuce chloroplast sequence downloaded from NCBI database. The plasmid map was developed using SnapGene software (https://www.snapgene.com/).

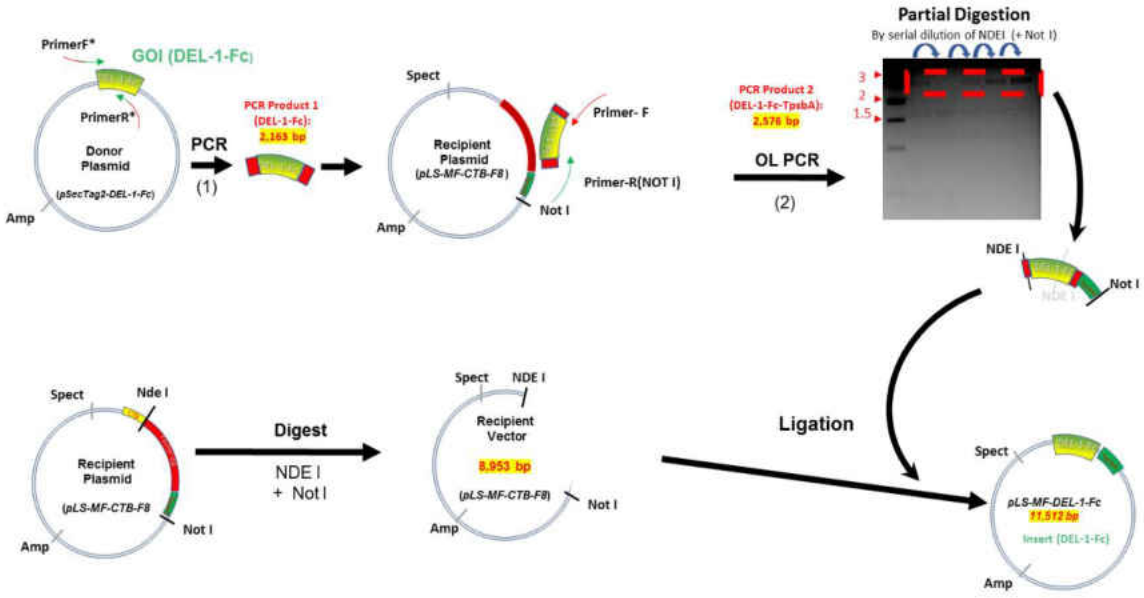

Figure 10. Cloning strategy for constructing DEL-1-Fc plasmid (pLs-MF-DEL-1-Fc) targeting chloroplast in lettuce. The DEL-1-Fc fragment (2,163 bp) was amplified with forward primer containing NdeI cutting site and reverse primer containing PshAI cutting site. The 3' end of DEL-1-Fc insert was further extended by overlapping PCR (OLPCR) to approach NotI cutting site using additional reverse primer, followed by a complete digestion on NotI and a partial digestion of NdeI restriction enzymes, respectively. The pLs-MF vector was digested with NdeI and NotI to yield the insert fragment: PpsbA and TpsbA using NdeI and NotI. DEL-1-Fc insert (2,576 bp).

5.3 Results

5.3.1 Subcloning of pLS-MF-DEL-1-Fc plasmid

The constructed plasmid was evaluated using previously established protocol (Verma et al., 2008) in which putative clones were screened by colony PCR (Bergkessel and Guthrie, 2013; Verma et al., 2008), restriction enzyme digestion maps (Verma et al., 2008), and the expressed protein size using western blot (Kwon et al., 2018) (**Fig.11**). After digesting the vector and insert with the restriction enzyme, a vector fragment without insert was also transformed into competent cells as a negative control to rule out potential carry-over of undigested backbone plasmid (**Fig.11A**). One out of 28 positive clones obtained from spectinomycin (100µg/mL), selection plate shows the predicted band size (2,281bp) under the screen of colony PCR (**Fig.11B**). The positive clone was further amplified in Lennox LB broth medium containing ampicillin (100µg/mL). The plasmid was further extracted and digested with restriction enzymes PstI and PvuII (**Fig.9B**). The PstI enzyme digested both in the middle and the 3'end of DEL-1-Fc sequence, creating the fragments of 1,474 bp and 10,038 bp; on the other hand, the PvuII enzyme digested at the end of *Trbcl* and in the middle of DEL-1-Fc sequence, creating the fragments of 1,480 bp and 10,032 bp (**Fig.11C**). After confirming by enzyme digestion, the recombinant protein in the positive clone was further extracted for western blot analysis. The total cell-lysate from the untransformed competent cells (Top10 *E. coli*) and competent cells transformed with original DEL-1 plasmid (pSecTag2-DEL-1-Fc) were included as the negative and positive

control of DEL-1 expression, respectively (**Fig.11D**). Potential nonspecific binding of the monoclonal rabbit-anti-human DEL-1 antibody was identified between 75 and 100 kDa in the untransformed cell-lysate while possible true-positive signal between 50 and 75 kDa was observed in cells transformed with original DEL-1 plasmid (pSecTag2-DEL-1-Fc). The DEL-1-Fc expressed from the positive clone (pLs-MF-DEL-1-Fc) was strongly detected at the same size as detected in the positive control.

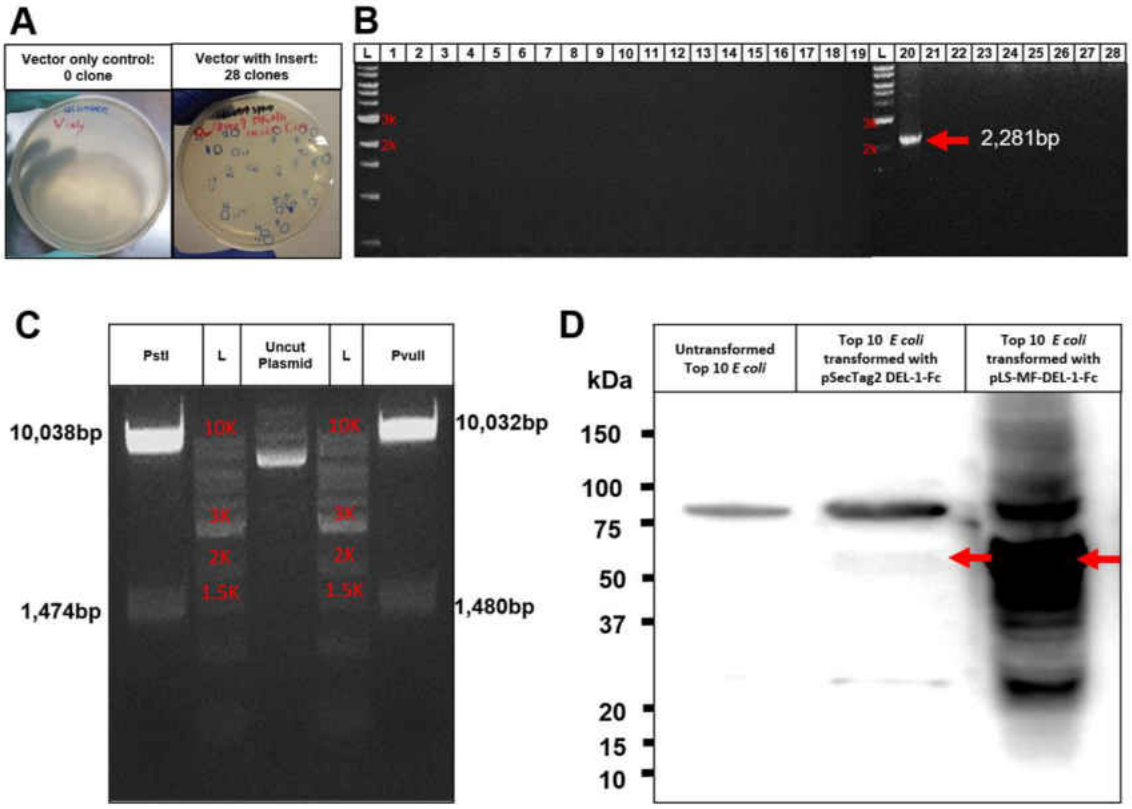

Figure 11. Evaluate the constructed plasmid. A. The competent cells transformed with digested vector without or with insert fragment were applied on the plate containing spectinomycin(100μg/mL). The outgrowth clones were marked and picked up for colony PCR screening. **B.** The colony PCR screening revealed one positive clone with the amplified band close to the predicted size indicated by the red arrow (2,281bp). L: 1kb DNA ladder (#N3232L, NEB). **C.** The undigested plasmid and the plasmid digested by PstI or PvuII were compared on the electrophoresis gel. The predicted bands were identified under PstI (1,474bp; 10,038bp) or PvuII (1,474bp; 10,038bp) enzyme digestion, respectively. **D.** The western blot revealed strong expression of recombinant DEL-1-Fc on the size similar to the positive control (indicated by red arrows).

5.3.2 *Sanger sequencing confirms seamless integration of DEL-1-Fc sequence into pLs-MF plasmid*

The internal fragment between PstI restriction sites on the positive clone was removed and inserted with the corresponding fragment digested from the original DEL-1 plasmid (pSecTag2-DEL-1-Fc) to avoid mutations during PCR amplification (**Fig.12A**). The re-ligated plasmid was further transformed into competent cells and the outgrowth clones were screened and picked-up for Sanger sequencing using forward and reverse primers on the backbone (pLs-MF) vector. The forward sequencing validates the junction between *PpsbA* and *DEL-1* gene, and an intact 5'PstI restriction site (**Fig.12B**). The reversed sequencing validates the junction between *TpsbA* and *IgG Fc* gene as well as intact 3'PstI restriction site (**Fig.12C**).

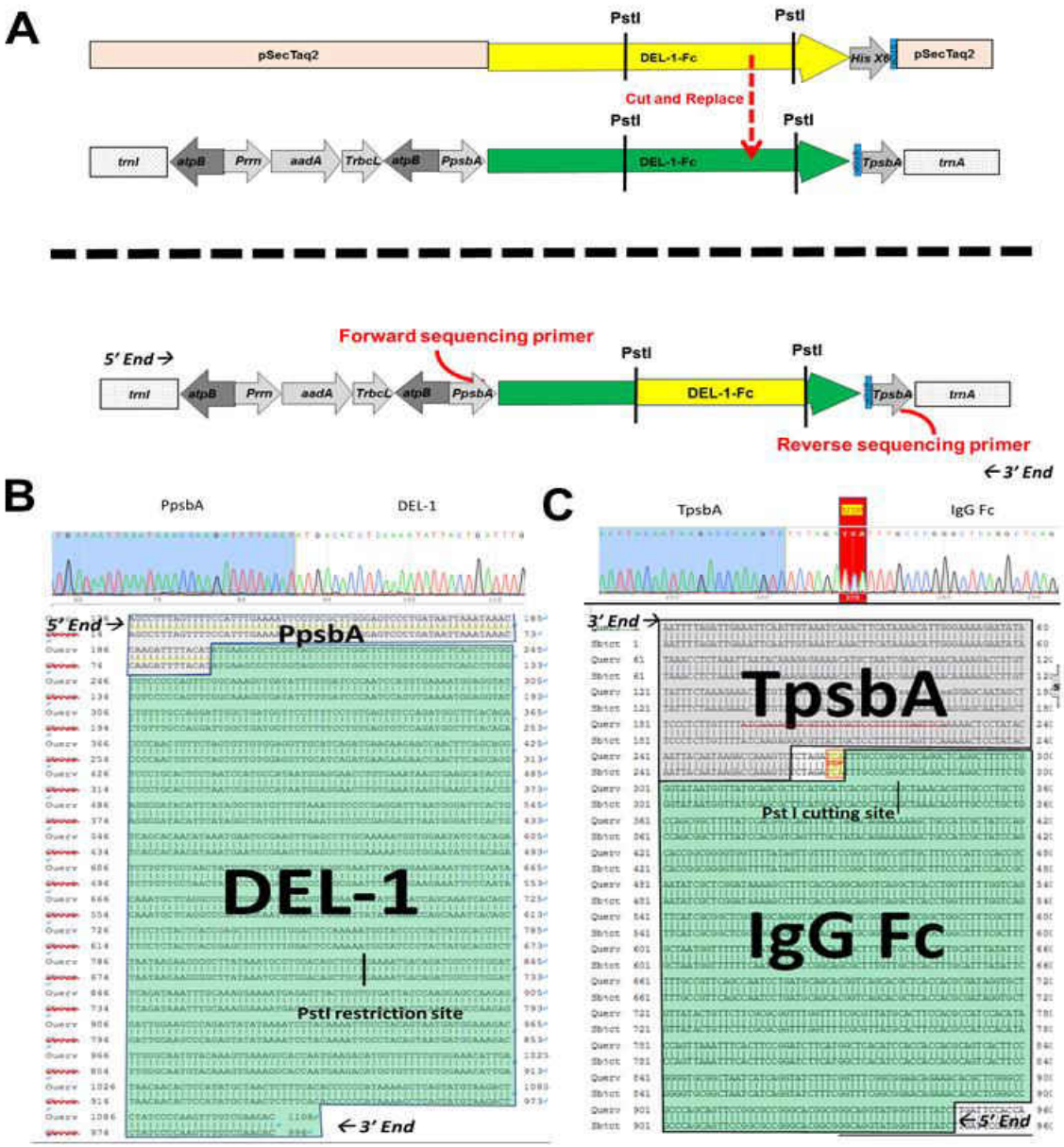

Figure 12. Sequencing result indicated no mutation was introduced. A. The internal fragment between PstI restriction sites on the constructed plasmid (pLs-MF-DEL-1-Fc) was replaced by the corresponding fragment on the original DEL-1 plasmid (pSecTag2-DEL-1-Fc). **B.** The forward sequencing validates the junction between *PpsbA* and *DEL-1* gene, and an intact 5'PstI restriction site. **C.** The reversed sequencing validates the junction between *TpsbA* and *IgG Fc* gene as well as intact 3'PstI restriction site.

5.3.3 Identify and purify E. coli-derived DEL-1-Fc protein from inclusion body

To interrogate whether the constructed protein may have a biological function, the E. coli-derived DEL-1-Fc was further extracted for functional assay. However, after comparing the protein extracted from the whole cell homogenate, supernatant, and insoluble cellular debris in the pellet, we found most of the recombinant DEL-1-Fc protein expressed in E. coli was in the insoluble pellet fraction, indicating the potential aggregation in the inclusion body (**Fig.13A**). After resolubilizing and refolding the protein aggregates from the inclusion body, the refolded DEL-1-Fc was further purified through specific binding affinity of its Fc component to the protein-G beads (**Fig.13B**).

5.3.4 Utilizing immunoprecipitation to interrogate receptor-protein interaction

To interrogate whether the DEL-1-Fc derived from prokaryotic system may serve similar receptor binding function as the human cell-derived DEL-1-Fc, we further developed an *in vitro* receptor-binding assay to interrogate this concept. The Protein G-purified DEL-1-Fc was further mixed with the cell membrane proteins extracted from human gingival-derived mesenchymal stem-like cells (hGMSC) to allow receptor-ligand immunoprecipitation (**Fig.13B**). Utilizing the recombinant human IgG-Fc as negative control and human cell-derived DEL-1-Fc as positive control, we further found that prokaryote-derived DEL-1-Fc (generated by *pLS-MF-DEL-1-Fc* plasmid in *E coli*) may serve similar receptor-binding function as those derived from human cells (**Fig.13C**). Similar to the MC3T3-E1 cells, a basal level

of endogenous DEL-1 (52kDa) was also detected on the hGMSC cell membrane and the preferential interaction of DEL-1-Fc to β3 integrin (but not to β1 integrin) was also observed in both human and E coli-derived DEL-1-Fc. The negative control (IgG Fc) does not precipitate the β3 integrin receptor suggesting that the observed β3 integrin binding function from prokaryote-derived DEL-1-Fc may be specific.

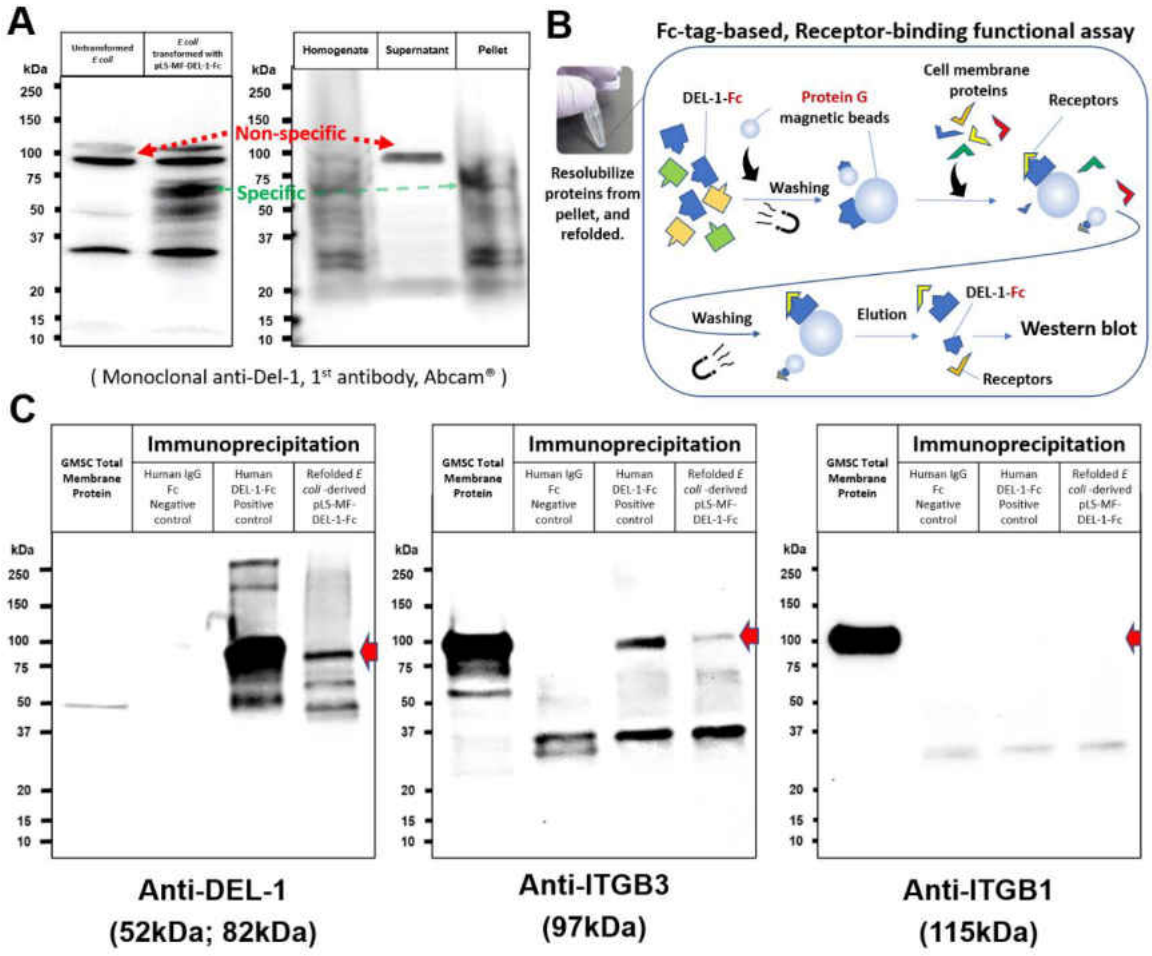

Figure 13. Functional assay for prokaryote-derived DEL-1-Fc protein. A. Specific DEL-1 signal was identified through the comparison between total cell-lysate derived from untransformed and pLs-MF-DEL-1-Fc transformed competent cells. The comparison between total cell-homogenate, soluble supernatant, and insoluble pellet indicated most non-specific *E. coli* proteins that react with monoclonal DEL-1 antibody were kept in the supernatant, while most of the recombinant DEL-1-Fc proteins were retained in the insoluble pellet. **B.** The crude DEL-1-Fc protein recovered from the pellet were purified by the Protein-G magnetic beads and performed immunoprecipitation with the 50μg of cell membrane proteins (receptors) isolated from human gingival-derived mesenchymal stem-like cells (hGMSC). **C.** Comparison of receptor-binding function between 50μg of crude proteins containing prokaryote (*E coli*)-derived DEL-1-Fc, human IgG-Fc (negative control) and human cell-derived DEL-1-Fc (positive control) on the receptor between the recombinant DEL-1-Fc to the β3 integrin and β1 integrin.

5.4 Discussion

Chloroplast transformation technology (CTT) has been developed more than 30 years and has multiple pharmaceutical applications aiming on the treatment of hemophilia (Herzog et al., 2017; Kwon et al., 2018; Su et al., 2015), pulmonary hypertension (Shenoy et al., 2014), diabetes (Boyhan and Daniell, 2011; Kwon et al., 2013; Posgai et al., 2017) and vaccination (Chan et al., 2016; Davoodi-Semiromi et al., 2010). While many traditional biopharmaceutics was produced by tobacco (Liu et al., 2016; Xiao et al., 2016b) and some of them contains poly-histidine tags (Arlen et al., 2007; Koya et al., 2005) for the benefit of large biomass production and for the ease of protein purification, the potentially addictive nature of tobacco (due to its nicotine content) and the potential antigenic effect of the poly-histidine tags have limited its clinical application. The currently constructed pLs-MF-DEL-1-Fc plasmid utilizing the latest strategies in Dr. Henry Daniell's laboratory to conquer the above mentioned concerns: First, the plasmid was specifically designed for transforming the lettuce chloroplast to avoid the potential side effect from tobacco; second, the current plasmid has removed the poly-histidine tag to avoid the antigenic effect, the histidine tag-based purification step can be replaced by the affinity purification utilizing the Fc component of DEL-1-Fc which binds to protein A/G and other materials following the well-established process for the purification of commercially available monoclonal antibodies (Choe et al., 2016). In addition, the currently designed plasmid targets the *trnI*, and *trnA*

genes within both inverted repeat regions on lettuce chloroplast genome (**Fig.9A**) which doubles the expression per chloroplast once the homoplasmy is achieved.

The PsbA promoter (*PpsbA*) engineered on the current pLs-MF-DEL-1-Fc construct has been shown to be a more efficient promoter in hyper-expression of foreign protein in *E.* coli in comparison with traditional T7 promoter (Brixey et al., 1997), which may explained the observed hyperexpression of DEL-1 signals in pLs-MF-DEL-1-Fc plasmid in comparison with the original (pSecTag2-DEL-1-Fc) plasmid (**Fig.11D**). On the other hand, while the DEL-1 is a glycoprotein originally produced in a eukaryotic system, lack of glycosylation in *E. coli* or chloroplast raises a concern on structure or function of Del1. Although the functional comparison between un-glycosylated and glycosylated DEL-1 protein has not yet been fully investigated, one recent study found the pro-resolution activity of MFG-E8, a protein shares 70% structure similarity with DEL-1, proportionally decreased by the increased glycosylation under hyperglycemic condition, suggesting the un-glycosylated DEL-1 may have a better pro-resolution function (Das et al., 2016). Our immunoprecipitation result (**Fig.13C**) further suggested that the prokaryote (*E.* coli)- derived DEL-1-Fc may preferentially bind to the β3 but not β1 integrin receptor like the eukaryotic (human)-cell derived DEL-1-Fc (**Fig.8A**). This receptor-binding immunoprecipitation assay may also be applied in the lettuce-derived DEL-1-Fc to interrogate its function.

While the *E. coli*-derived DEL-1-Fc protein was recovered from the insoluble protein aggregates (inclusion bodies), it raises a concern regarding structural

differences in the refolded DEL-1 protein. As the structure of DEL-1 contains 12 disulfide bonds, 2 O-glycosylation and 1 N-glycosylation (http://www.uniprot.org/uniprot/O43854), the complex structure may heavily rely on the post-translational modification for proper folding. However, as Anfinsen's experiment indicated (Anfinsen, 1973; Fernandez-Reche et al., 2018), the sequence of the peptide dictated the information required for the tertiary protein folding thermodynamically which partially explains our finding that refolded protein obtains a specific binding function to β3 but not β1 integrin. This receptor-binding assay may be further applied as an efficient functional assay which can easily test the chloroplast-derived protein function to screen for the lettuce clones with optimal biological function, at least in part.

The fused immunoglobin Fc domain on DEL-1 (DEL-1-Fc) not only serves the function to increase the plasma half-life of DEL-1 but has also been reported as a carrier to assist oral drug delivery through the transcytosis event in young mice intestine utilizing the neonatal Fc receptor (FcRn) (Pridgen et al., 2013; Shi et al., 2018). The chloroplast-derived protein drug is bioencapsulated by plant cell wall which can protect from degradation by gastric enzymes/acid and can be released by cellulolytic microbe in the gut for drug delivery (Kwon and Daniell, 2016). Utilizing chloroplast as bioreactor for DEL-1-Fc synthesis and delivery may provide an affordable precision treatment option to improve the homeostatic bone regeneration in poorly responsive periodontitis patient due to insufficient DEL-1 expression.

5.4.1 Relationship of current work to periodontal specialty

Aging is an inevitable process which may gradually leads to a scenario of unresolved inflammation (Arnardottir et al., 2014) and impaired bone regeneration (Ambrosi et al., 2017). While the current advance in medical technology has significantly prolonged the lifespan for mankind, an increasing need to improve the quality of these expanded lives has been underestimated and unmet by traditional treatment philosophy. Although the goal of periodontal treatment is to preserve or regenerate the periodontal attachment for the maintenance of periodontal health, it is also inevitable to have periodontal attachment loss and bone loss with age (Billings et al., 2018). Thus, establishing a diagnostic system that identifies endogenous periodontal factors diminished with age and establishing a supplementary age-associated periodontal treatment strategy will ultimately improve the quality of life for periodontitis patient. Endogenous DEL-1 in periodontal tissue has been identified to be diminished with age and contribute to age-associated periodontal bone loss (Eskan et al., 2012). The first part of this thesis further identified the upregulation of DEL-1 in periodontal tissue is necessary for resolving bone regeneration during inflammation resolution (**Fig.4F, Fig.5, Fig.6**). While the level of DEL-1 in gingival crevicular fluid (GCF) has been shown to be associated with periodontal resolution (Kourtzelis et al., 2019), DEL-1 may become a suitable GCF biomarker to determine aging-associated patient response during periodontal reevaluation (Tonetti et al., 2018). This chapter further provides an affordable strategy to replenish DEL-1 which complements the proposed therapeutic strategy from innovative diagnosis to precision medicine. Benefiting

from the chloroplast transformation technology, the DEL-1-Fc produced by transplastomic lettuce will be easy to preserve, affordable to supply and eventually provide a therapeutic option for the aging-associated poorly resolved periodontitis patient.

5.4.2 Conclusion

Based on our previous findings *in vitro*, we successfully established a receptor-binding immunoprecipitation assay to test the function of recombinant DEL-1-Fc *in vitro*. This functional assay may be further applied as quality control for the lettuce-derived DEL-1-Fc to screen for optimal protein quality prior to further application. This chapter introduces a translational application by transforming DEL-1-Fc into the chloroplast genome so that an effective, efficient, and affordable pro-resolution periodontal regenerative treatment strategy may be achieved in the near future.

Research Limitations

While many conclusions can be drawn from the presented studies, a few limitations deserve mention. To investigate the resolution mechanism in clinical periodontitis patients, an ideal intervention studies should be performed on clinical patients or non-human primates. However, given by the limitation of ethics and research budget, we establish and utilize a murine periodontal resolution model to approximate the clinical scenario of periodontal resolution. The fundamental difference of susceptible microbiota between murine and primates may limit our ability to make broader generalization from current results. Secondly, although our *in vitro* findings suggest DEL-1 have a direct osteogenic effect on osteoblastic progenitor cells, whether DEL-1 directly contribute to the observed periodontal resolving bone regeneration through osteogenic progenitors *in vivo* remains to be investigated. While resolution of inflammation may have a profound effect on bone regeneration, whether the RGD motif of DEL-1 contributes to other immune regulatory mechanism(s) that indirectly promote bone regeneration is still unknown in the current study. Future experiments using osteoblast (*Sp7-Cre*)- or osteocyte (*Dmp1-Cre*) -specific β3 integrin (*Itgb3*) conditional knockout mice may allow the lineage-specific interrogation of DEL-1 to investigate whether DEL-1-promoted bone regeneration is contributed by it direct osteogenic effect *in vivo.* Finally, the cost of manufacturing and purifying high-quality DEL-1-Fc and its mutant proteins limit the availability of experimental materials for further investigations and clinical applications. To alleviate this limitation, we provide a novel strategy in Chapter 5

to reduce the cost of DEL-1-Fc manufacturing using the chloroplast transformation technology. Nevertheless, as the *E coli* and chloroplast- derived proteins may contain certain portions of endotoxins (such as lipopolysaccharide and lipid A) which may limit their application to endotoxin-sensitive functional assay, current study using modified receptor-pulldown assay to suggest the potential function of prokaryote-derived DEL-1-Fc. However, future application using endotoxin-removal assay (#88270, Thermo Scientific) to remove the endotoxin in prokaryote-derived DEL-1-Fc may allow for more functional cell- and tissue-specific assay *in vitro* and *in vivo*.

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