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INVITED MEDICAL REVIEW

WILEY ORAL DISEASES

Histatins, wound healing, and cell migration

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Wounds in the oral mucosa heal faster and more efficiently than those in the skin, although the mechanisms underlying these differences are not completely clear. In the last 10 years, a group of salivary peptides, the histatins, has gained attention on behalf of their ability to improve several phases of the wound-healing process. In addition to their roles as anti-microbial agents and in enamel maintenance, histatins elicit other biological effects, namely by promoting the migration of different cell types contained in the oral mucosa and in non-oral tissues. Histatins, and specifically histatin-1, promote cell adhesion and migration in oral keratinocytes, gingival and dermal fibroblasts, non-oral epithelial cells, and endothelial cells. This is particularly relevant, as histatin-1 promotes the re-epithelialization phase and the angiogenic responses by increasing epithelial and endothelial cell migration. Although the molecular mechanisms associated with histatin-dependent cell migration remain poorly understood, recent studies have pointed to the control of signaling endosomes and the balance of small GTPases. This review aimed to update the literature on the effects of histatins in cell migration, with a focus on wound healing. We will also discuss the consequences that this increasing field will have in disease and therapy design.

KEYWORDS
angiogenesis, cell migration, endosome, histatin-1, wound healing

1 | ORAL WOUND HEALING: OVERVIEW

Wound healing is a complex multifactorial process that could be envisioned as a succession of phases, including hemostasis, inflammation, proliferation, and remodeling (Larjava, 2012; Figure 1). It has long been known that wounds in the oral mucosa heal faster and more efficiently than those in the skin, and several factors have been shown to contribute with these differences, including tissue architecture, vascularization, and the presence of saliva, among others (Glim, van Egmond, Niessen, Everts, & Beelen, 2013). However, the cellular and molecular mechanisms whereby such factors contribute to efficient wound healing in the oral cavity remain poorly understood. This is particularly true for the saliva, which aside providing a humid environment, rich in growth factors and chemokines, contains a plethora of molecules with unknown roles in the context of wound healing (Brand, Ligtenberg, & Veerman, 2014). In fact, accumulating evidence has shown that saliva helps to accelerate the re-epithelialization of the oral mucosa (Engeland, Bosch, Cacioppo, & Marucha, 2006; Szpaderska, Zuckerman, & DiPietro, 2003), and this is supported on evidence obtained from loss-of-function in salivary glands and xerostomia models (Azuma et al., 2014; Bodner & Dayan, 1995; Epstein & Scully, 1992; Keswani et al., 2013). Based on studies in animal models, relevant roles have been attributed to the growth factors that reside in saliva, where epidermal growth factor (EGF) promotes cell proliferation and migration, as well as the remodeling the extracellular matrix (Chen et al., 1993; Cohen, 1965; Hutson, Niall, Evans, & Fowler, 1979; Konturek, Dembinski, Warzecha, Brzozowski, & Gregory, 1988). Other factors including nerve growth factor (NGF), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), and insulin-like growth factor (IGF) are involved in the maintenance and integrity of the oral cavity and tissue repair (Zelles, Purushotham, Macauley, Oxford, & Humphreys-Beher, 1995). Nevertheless, it is intriguing that the salivary concentration of many of these growth factors is significantly lower in humans, when compared to animals (Fisher & Lakshmanan,

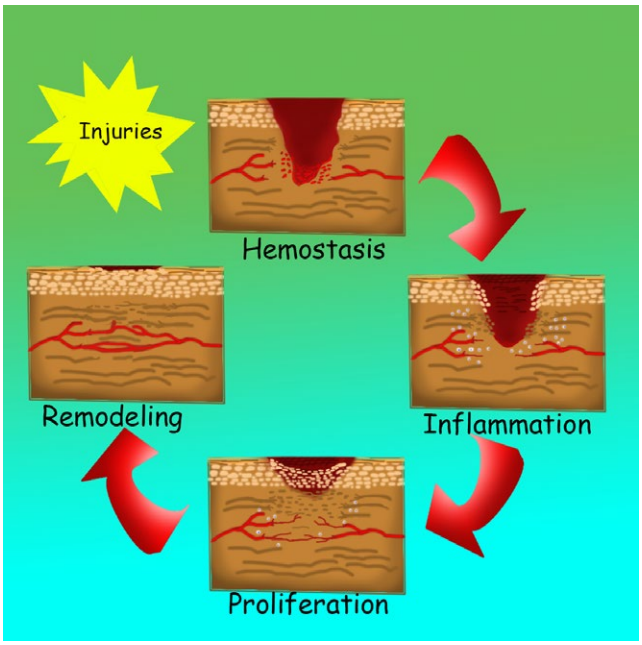

FIGURE 1 Phases of wound healing. A brief representation of the phases involved in oral mucosal wound healing: (i) hemostasis, (ii) inflammation, (iii) proliferation, and (iv) remodeling

1990; Murphy, Watson, Metz, & Forssmann, 1980; Oudhoff et al., 2008). In this respect, the role of a family of cationic peptides—*the histatins*—which are present in saliva of higher primates has arisen as a critical modulator in epithelial wound healing, and more specifically, during the cell migration responses involved in tissue repair (Oudhoff et al., 2008; Oudhoff, Kroeze et al., 2009).

The phenomenon of cell migration is central to restoring the functional integrity of the epithelial barriers, as it is required to achieve the responses of the different cell types involved in tissue repair, including epithelial cells, fibroblasts, and immune and endothelial cells, among others (Schaffer & Nanney, 1996). Cell migration is the basis of the re-epithelialization phase and plays a primary role in angiogenesis, which provides with oxygen and nutrients to the repairing tissue (Tonnesen, Feng, & Clark, 2000). Consequently, inefficient cell migration will result in impaired wound healing (Figure 1).

2 | ROLE OF CELL MIGRATION IN ORAL WOUND HEALING

2.1 | Cell migration: cellular and molecular aspects

Cell migration is involved in different phases of multicellular organisms, with roles ranging from embryonic development, immune responses, wound healing, and cancer. Mechanistically, cell migration can be envisioned as the succession of repetitive steps that involve cell polarization, formation of cellular protrusions and new contacts with the extracellular matrix (ECM), maturation of cell-ECM adhesions, cell body contraction, retraction of the cell rear, and disassembly of cell-ECM adhesions (reviewed in (Ridley et al., 2003), Figure 2). Cell polarization leads to the establishment of the so-called "leading edge" and "rear end," allowing to the re-localization of organelles, such as the Golgi, microtubule organizing center, endoplasmic reticulum, and a subset of secretory vesicles, in a process controlled by the Rho small GTPases, Cdc42, and Rac1. Cellular protrusions are relevant to facilitate the formation of cell-ECM adhesions via integrin engagement and the formation of plasma membrane-/actin-enriched structures, such as *lamellipodia* and membrane *ruffles* (Burridge & Wennerberg, 2004; Heasman & Ridley, 2008). Integrin-ECM adhesions mature to form macromolecular complexes, known as focal adhesions (FAs), which contain more than 150 components, including adaptor proteins, kinases, and signaling molecules (Gardel, Schneider, Aratyn-Schaus, & Waterman, 2010). Among these proteins, FAK (focal adhesion kinase) has a central role in controlling focal adhesion (FA) maturation and stability, via autophosphorylation and phosphorylation of downstream effectors (Kleinschmidt & Schlaepfer, 2017). Mature FAs contact each other via actin-based stress fibers, which provide tensile forces that contribute to the contraction of the cell body, in a process that depends on the small GTPase RhoA (Gardel et al., 2010). Finally, cell migration requires coordinated detachment of the rear-end and the disassembly of FAs, by mechanisms that remain poorly understood, although it is known to depend on FAK (Hamadi et al., 2005), calpain-dependent proteolysis (Franco et al., 2004), and components of the endocytic machinery, such as dynamin, Dab2, clathrin, and Rab5 (Chao & Kunz, 2009; Ezratty, Bertaux, Marcantonio, & Gundersen, 2009; Ezratty, Partridge, & Gundersen, 2005; Mendoza et al., 2013).

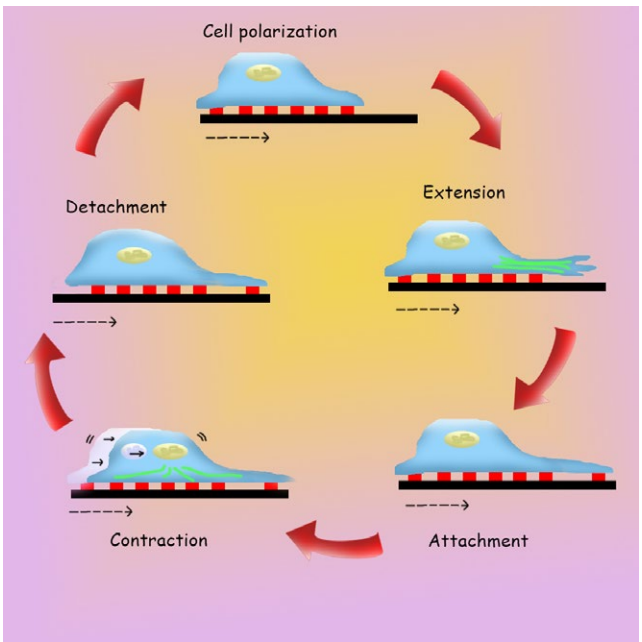

FIGURE 2 Steps involved in cell migration. Cell migration can be defined as a sequence of repetitive steps, as obtained from 2D in vitro models using mesenchymal cells. Cell polarization involves re-localization of organelles, which is followed by cellular protrusion (extension) and formation of new cell-ECM contacts. Subsequently, contraction of the cell body is controlled by the actin cytoskeleton, which is followed by the disassembly of cellular adhesions (detachment) by action of intracellular proteases, kinases (such as FAK), and the endocytic machinery (see text for details)

Particularly, recent studies from our laboratory showed that calpain2 is trafficked via early endosomes in a Rab5-dependent manner, leading to FA disassembly (Mendoza et al., 2017).

Aside the well-documented role of Rho GTPases in the different steps of cell migration, evidence provided by different groups has shown that another family of small GTPases, the Rabs, is also relevant to a subset of phases in the migration process (reviewed in Mendoza, Diaz, & Torres, 2014; Mosesson, Mills, & Yarden, 2008; Zhen & Stenmark, 2015). Particularly, the early endosome regulator, Rab5, has been shown to control spatiotemporal activation of Rac1 via recruitment of the Rac1-GEF Tiam1 to early endosomes, allowing to the activation of Rac1, local actin polymerization, *lamellipodia* formation, and cell migration (Palamidessi et al., 2008; see section 3.3). In fact, Rab5 is activated during endothelial cell migration via RIN2, a Rab5-GEF that accumulates at early endosomes (Sandri et al., 2012). This signaling mechanism is not only relevant in endothelial cells, but it also cooperates with the enhanced capacity of tumor cells to migrate and invade (Diaz et al., 2014; Palamidessi et al., 2008). As aforementioned, most of this knowledge has been obtained in tumor cells or non-oral cell models, and hence, it will be particularly relevant to elucidate its relevance in the context of oral wound healing. Later in this review, we will provide a detailed discussion of this signaling mechanism, as recent studies have shown it to be relevant in the context of oral angiogenesis (see section 3.3).

2.2 | Cell migration in re-epithelialization

Keratinocytes residing in the oral mucosa play central roles in maintaining tissue integrity, because they provide mechanical, chemical, and biological barriers to environmental insults (Kirfel & Herzog, 2004). Specifically, oral keratinocytes contribute to the repair of damaged tissues during the re-epithelialization phase, which is of great relevance in wound healing, as any failure in the re-epithelialization will lead to inefficient tissue repair and the onset of chronic wounds (Richards et al., 1996; Usui, Mansbridge, Carter, Fujita, & Olerud, 2008). Upon tissue damage, keratinocytes that surround the injury site undergo activation, in a process that is modulated by different cues, including locally secreted factors (growth factors, cytokines, and ECM components; Larjava, Salo, Haapasalmi, Kramer, & Heino, 1993; Wang, Wang, Farhangfar, Zimmer, & Zhang, 2012), and the presence of saliva (Brand et al., 2014). Keratinocyte activation is followed by the downregulation of cell adhesion molecules involved in cell-cell adhesion (Beaudry et al., 2010), leading to morphological changes, loss of epithelial cell polarization, and the acquisition of cell asymmetry with an actin-rich leading edge facing toward the wounded area (Odland & Ross, 1968; Sciubba, Waterhouse, & Meyer, 1978). These events depend on the activation of Rac1, because the inhibition of this small GTPase prevents the re-epithelialization phase in murine models and decreases both keratinocyte migration and proliferation (Castilho et al., 2010). The migration of keratinocytes requires anchorage with new ECM components and, hence, changes in the integrin repertoire (Larjava, Koivisto, Hakkinen, & Heino, 2011; Larjava et al., 1993), which is accompanied by local degradation of the ECM via metalloproteinase (MMP) release (Garlick, Parks, Welgus, & Taichman, 1996; Steffensen, Hakkinen, & Larjava, 2001). Moreover, keratinocytes are able to produce their own provisional ECM, in order to facilitate their migration. Failure in ECM secretion impairs the re-epithelialization process, as shown in patients with mutations in the glycoprotein laminin 332, which delays wound healing (Schneider, Muhle, & Pacho, 2007). Epithelial cell migration ceases upon contact of both epithelial fronts and the wound is re-populated, via contact inhibition (Larjava et al., 1993). At the last stage of the re-epithelialization phase, keratinocytes continue with their proliferation and differentiation events (Garlick et al., 1996).

2.3 | Cell migration in angiogenesis

Angiogenesis is a central event during the proliferative phase of wound healing, because it contributes to the formation of new blood vessels from pre-existing vasculature, and provides nutrients and oxygen into the injured site (Risau, 1997). Upon wounding, endothelial cells migrate and invade the connective tissue, leading to the early phase of angiogenesis, in a process that is controlled by several extracellular molecules, including the vascular endothelial growth factor (VEGF, secreted by keratinocytes and macrophages) and FGF (secreted by macrophages and damaged endothelial cells), allowing for the proliferation and migration of endothelial cells, and vascular morphogenesis (Rousseau, Houle, & Huot, 2000). VEGF is a potent angiogenic factor, which has been shown to depend on nitric oxide release (Williams et al., 2000), and is able to induce chemotactic endothelial cell migration via its cognate receptors VEGFR1 and VEGFR2 (Olsson, Dimberg, Kreuger, & Claesson-Welsh, 2006). Endothelial cell migration involves the formation of *lamellipodia* at the leading edge, which is associated with decreased RhoA activity, and consequently diminished ROCK activity and myosin II-dependent contractility (Guo & Giancotti, 2004).

Alternatively, the ECM plays an important role in angiogenesis, as endothelial cells respond to the ECM composition, structure, and mechanical properties, by modulating their adhesiveness and migratory potential. For instance, the density of collagen fibers has been shown to control vessel size and density (Critser, Kreger, Voytik-Harbin, & Yoder, 2010). Also, transient ECM components during wound healing, such as type I collagen and fibrin, serve as cues that modulate endothelial cell migration (Dejana et al., 1985), whereas the expression and activation of matrix metalloproteinases, such as the membrane type MT1-MMP, contribute to the migration and invasion of endothelial cells at two levels, which includes a direct degradation of the immediate ECM and releasing ECM-tied growth factors, such as FGF, VEGF, and IGF (Stratman et al., 2009). Most of these events have also been associated with integrins, which not only provide the cognate receptor to the underlying ECM, but also contribute to activating MMPs, allowing local ECM degradation (Stupack & Cheresh, 2002).

A role of saliva in the angiogenesis of the oral mucosa has been proposed, as animal models undergoing sialoadenectomy of the submandibular gland (the primary source of VEGF) have impaired angiogenesis and mucosal wound healing, as shown in a murine palatal wound model. In fact, by gain-of-function experiments, these studies

showed that VEGF is a critical factor contained in murine saliva, which contributes to the angiogenic response in palatal mucosa (Keswani et al., 2013). Notwithstanding, it should be kept in mind that the salivary concentration of growth factors is not necessarily equivalent in humans and animal models (i.e., canine and rodent models (Brand et al., 2014)), raising the question as to whether these conclusions can be extended to humans. Thus, regardless of the acknowledged relevance of saliva in angiogenesis, the precise factors contained in saliva that contribute to endothelial cell responses remain largely known. In this respect, the histatins have been recently suggested as novel pro-angiogenic factors, by controlling endothelial cell adhesion and migration (Torres et al., 2017; van Dijk, Ferrando et al., 2017; see upcoming sections).

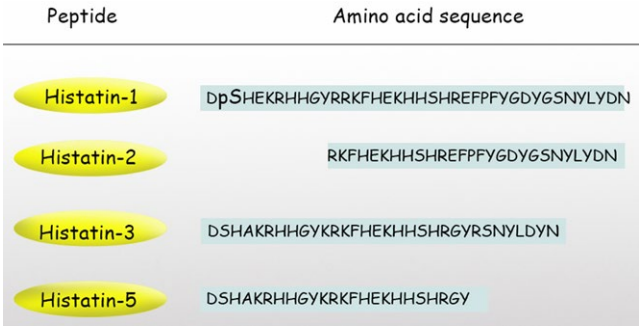

FIGURE 3 The histatins. Peptide sequence representation for histatin-1, histatin-2, histatin-3, and histatin-5, which are the most studied in the context of cell migration and their anti-microbial activity

3 | HISTATINS, CELL MIGRATION, AND ORAL WOUND HEALING

3.1 | Histatins, an overview

3.1.1 | The histatins

Histatins are a family of histidine-rich low molecular weight proteins (Azen, 1972; Baum, Bird, Millar, & Longton, 1976; Hay, 1975; Oppenheim et al., 1986, 1988), which are expressed in the saliva of humans and higher primates (Padovan et al., 2010), and have been widely studied for their anti-fungal activity (Helmerhorst et al., 1999; Oppenheim et al., 1986, 1988). Within this family of proteins, histatin-1 and histatin-3 are full-length products encoded by the genes HIS1 and HIS2, respectively (Figure 3), while the smaller members, histatin-2, -4, -5, -6, -7, -8, -9, -10, -11, and -12, result from proteolytic cleavage of histatin-1 and histatin-3 (reviewed in (Melino, Santone, Di Nardo, & Sarkar, 2014)). These proteins are synthesized and secreted by the serous acini of the parotid, submandibular, sublingual, and von Ebner glands (Ahmad, Piludu, Oppenheim, Helmerhorst, & Hand, 2004; Sabatini & Azen, 1989; Schenkels, Veerman, & Nieuw Amerongen, 1995; vanderSpek et al., 1989; Takano, Malamud, Bennick, Oppenheim, & Hand, 1993). Intriguingly, recent reports have detected the expression of these peptides in other tissues, for instance, histatin-1 in human lacrimal gland (Ali et al., 2017; Shah et al., 2016), melanoma cell lines, and metastatic lesions of patients diagnosed with melanoma (Yaguchi & Kawakami, 2012), and histatin-5 in human ocular surface (Steele, Jumblatt, Smith, & Pierce, 2002).

3.1.2 | Proteolytic cleavage and salivary fluctuations

Proteolytic cleavage of histatins occurs both intracellularly (Messana et al., 2008, 2015; Troxler, Offner, Xu, Vanderspek, & Oppenheim, 1990) and extracellularly (Xu, Lal, & Pollock, 1992). Specifically, histatin-3 undergoes intracellular cleavage, prior to the storage in the secretory granules, yielding histatin-5 and histatin-6, as products (Messana et al., 2008). Other histatin-3-derived products have been suggested to be cleaved intracellularly, although this remains unclear (Castagnola et al., 2004; Troxler et al., 1990). On the other hand, histatin-2 is suggested to derive from histatin-1 by proteolytic cleavage via salivary enzymes (Xu et al., 1992). Once secreted into the oral cavity, histatins accumulate in whole saliva, reaching concentrations that fluctuate according to the circadian cycle and salivary flow, with a peak of concentration around 17:00 and 18:00 hr (Castagnola et al., 2002). Aging is another factor affecting histatin production, and an inverse relationship has been established between aging and gland concentration of histatins (Johnson, Yeh, & Dodds, 2000). A third factor that modulates histatin concentration is the acquired enamel pellicle, which has been shown to contain intact histatins (Siqueira, Margolis, Helmerhorst, Mendes, & Oppenheim, 2010), hence buffering their amounts in whole saliva (Campese, Sun, Bosch, Oppenheim, & Helmerhorst, 2009). Finally, it is relevant to consider the presence of salivary proteolytic enzymes, which have a direct impact on proteolytic cleavage and the consequent reduction in histatin concentrations (Campese et al., 2009; Sun, Salih, Oppenheim, & Helmerhorst, 2009). Intriguingly, it has been shown that adsorption of histatin-1 to hydroxyapatite protects it against proteolytic cleavage (McDonald, Goldberg, Tabbara, Mendes, & Siqueira, 2011).

3.1.3 | Anti-microbial properties

Histatins have been extensively studied for their anti-microbial action, and within this family, histatin-5 possess the highest anti-fungal activity (Oppenheim et al., 1988). In vitro studies have shown that histatin-5 has anti-fungal effects on *Candida albicans* (Oppenheim et al., 1988), *Candida krusei*, *Candida glabrata*, *Cryptococcus neoformans*, and *Aspergillus fumigatus* (Helmerhorst et al., 1999). In this context, the relevance ofhistatin-5 in disease was highlighted in the study by Khan et al., which showed that lower levels of histatin-5 in HIV-positive patients correlate with a higher prevalence of *Candida albicans* (Khan et al., 2013). Histatins have also been suggested to have anti-bacterial activity, that is, histatin-5 and derivative peptides are potent molecules against *Staphylococcus aureus* (Welling, Brouwer, van 't Hof, Veerman, & Amerongen, 2007). Additionally, in vitro studies indicate that histatin-5 can inhibit both host proteases (MMP-2 and MMP-9) and proteases derived from *Porphyromonas gingivalis* (Arg-gingipaine and Lys-gingipaine) and *Clostridium histolyticum* (clostripain), further

supporting the anti-microbial role for these peptides via protease inhibition (Gusman, Grogan, Kagan, Troxler, & Oppenheim, 2001). These and several other studies have pointed out to the relevance of histatins as important anti-microbial peptides in the oral cavity (Krzysciak et al., 2015).

3.1.4 | Roles in the acquired enamel pellicle and hydroxyapatite

In this context, the role of histatin-1 has been more deeply explored, because histatin-1 phosphorylation on Ser2 is a prerequisite for efficient binding to hydroxyapatite crystals (Driscoll et al., 1995). This is important, because it allows histatin-1 to become part of the acquired enamel pellicle (Lee et al., 2013; Siqueira et al., 2010), a structure involved in enamel mineral homeostasis, dental lubrication, and microbial colonization (Siqueira, Custodio, & McDonald, 2012). Another documented feature of histatin-1 is the ability to inhibit spontaneous growth of hydroxyapatite crystals (Oppenheim et al., 1986). This phenomenon is shared by other salivary phosphoproteins and proline-rich proteins, which act as stabilizers of human saliva supersaturation, facilitating the re-mineralization of enamel lesions (Margolis, Kwak, & Yamazaki, 2014). This conclusion is supported by studies showing that salivary samples obtained from patients with high risk of caries, contained lower concentrations of histatin-1, when compared to caries-free patients (Vitorino et al., 2005), and other studies showing that histatin-1 levels increase in whole saliva upon treatment against caries in early childhood (Sun et al., 2016). Overall, histatin-1 is crucial in different aspects involved in the maintenance of oral structures, namely enamel's mineral homeostasis, anti-fungal and anti-bacterial activities, as well as the potential relationship with caries onset and progression. Aside from their canonical roles as anti-microbial agents and in enamel homeostasis, additional functions for histatins have been documented, in the maintenance and repair of soft tissues, including the oral mucosa. This topic will be discussed in the upcoming paragraphs.

3.2 | Histatins and oral epithelial cell migration

During the last decade, it was commonly accepted that saliva promotes wound healing in the oral mucosa by improving the re-epithelialization phase, mainly via increased keratinocyte migration and proliferation (Brand et al., 2014; Glim et al., 2013). Most of that evidence was supported by animal model-based studies. However, back in 2008, Oudhoff and colleagues demonstrated that human saliva promotes the migration of cultured human oral keratinocytes in vitro (Oudhoff et al., 2008). Their study aimed to identify human salivary factors that accounted for increased wound healing efficacy in vitro. To this end, using a screening approach of salivary fractions and proteomic analyses, the authors showed for the first time that histatins are the main factors contained in human saliva, which promoted the migration of oral keratinocytes in vitro (Oudhoff et al., 2008; Oudhoff, Kroeze et al., 2009). This was intriguing, because growth factors known to promote cell migration (i.e., motogenic factors), and previously shown to be contained in the saliva of rodents, such as EGF and NGF, were substantially lower in humans, further indicating that the migration-promoting effects of human saliva were not associated to these factors, but due to histatins instead (Brand et al., 2014; Fisher & Lakshmanan, 1990; Murphy et al., 1980; Oudhoff et al., 2008). This landmark work paved the way for subsequent studies that showed additional functions of histatins that go beyond those considered as canonical, namely in the context of cell adhesion and migration.

Not all histatins promote cell migration. So far, evidence has shown that histatin-1, and its cleavage product histatin-2, as well as histatin-3, but not histatin-5, promote the migration of oral keratinocytes in vitro (Oudhoff et al., 2008). Also, early studies showed that the D-enantiomer of histatin-2 fails to stimulate cell migration, which is opposite to its anti-microbial effects, suggesting the involvement of a specific, yet unknown cognate receptor (Oudhoff et al., 2008; Oudhoff, Kroeze et al., 2009). By sequence analysis, it was proposed that the C-terminal amino acid sequence SNYLYDN, which is common for histatin-1, -2, and -3 (Figure 3), is the minimal region necessary for promoting cell migration, and it was referred to as the wound-healing domain (WH) (Sun et al., 2009). However, a stepwise truncation analysis performed on histatin-2 (also known as histatin-1[12–38]) showed that the sequence SHREFPFYGDYGS encompassing the amino acids 20–32 (and hence excluding the C-terminal sequence NYLYDN) contains the minimal elements necessary to promote oral keratinocyte migration (Oudhoff, Kroeze et al., 2009). The latter finding is intriguing in the context of the pro-migratory effects of histatins, as several conclusions can be drawn: First, phosphorylation on Serine2 (a post-translational modification specific for histatin-1 (Oppenheim et al., 1988)) is not required for cell migration; second, the WH domain does not necessarily overlap with the suggested anti-fungal domain (Melino et al., 2014); third, the requirement of a putative receptor. In this respect, it has been suggested that G-protein coupled receptors are involved in histatin-dependent epithelial cell migration, as treatment with pertussis toxin prevented the migration of oral keratinocytes induced by histatin-2 (Oudhoff, Kroeze et al., 2009). However, the precise identity of cognate receptors implicated in histatin-dependent migration of epithelial cells remains elusive. The scenario becomes more complex, when extending these analyses to other cell types, such as endothelial cells, because the latter have been suggested to require VEGFR during histatin-induced migration (Torres et al., 2017; see text below).

In oral epithelial cells, histatins have been shown to trigger a subset of signaling pathways associated with cell migration, specifically by activating ERK1/2, but not p38MAPK, nor EGFR-signaling, as shown by pharmacological inhibition experiments in cells exposed to histatin-2 (Oudhoff et al., 2008; Oudhoff, Kroeze et al., 2009). The involvement of ERK1/2 in cell migration was recently extended to endothelial cells treated with histatin-1 (Torres et al., 2017). Although studies have pursued the nature of histatin-cell surface binding and subsequent internalization (Oudhoff et al., 2008), mechanistic details about their mode of internalization (i.e., clathrin-dependent or independent pathways) and subsequent endocytic trafficking remain obscure, and it will be relevant to

elucidate the underlying signaling pathways triggered, in light of the recent findings suggesting the involvement of the so-called "signaling endosomes" during histatin-driven cell migration (Torres et al., 2017; see section 3.3).

3.3 | Histatins in fibroblast and non-oral epithelial cell migration

Biological effects of histatins were initially thought to be restricted to epithelial cells derived from the oral cavity, which is intuitively expected, because until then, these peptides were detected almost exclusively in human saliva. However, subsequent studies showed that histatin-1 and histatin-2 are also able to trigger biological responses in other cell types. While the pro-migratory effects of histatins were initially described in human oral epithelial cell lines, TR146 and HO-1-N-1, as shown in wound-closure and Boyden Chamber Assays, respectively (Oudhoff et al., 2008), subsequent studies extended these effects to gingival fibroblasts and non-oral epithelial cells (Oudhoff, Kroeze et al., 2009; Oudhoff, van den Keijbus et al., 2009). Specifically, histatin-2 promoted the migration of human primary gingival fibroblasts in wound-closure assays (Oudhoff, van den Keijbus et al., 2009), whereas it promoted the migration of primary skin keratinocytes and re-epithelialization in vitro, using a full skin equivalent wound model (Oudhoff, Kroeze et al., 2009). Also, histatin-2 promoted the migration of primary dermal fibroblasts and MCF-7 breast carcinoma cells (Oudhoff, van den Keijbus et al., 2009). In this respect, recalling the sequence of steps required for cell migration (Figure 2), both cell-ECM attachment and spreading are critical events, where histatins have been shown to elicit their effects, based on different oral and non-oral cell models. Specifically, histatin-1 promoted the spreading of ARPE human retinal pigment epithelial cells, Caco-2 human colorectal adenocarcinoma cells, as well as primary gingival and dermal fibroblasts (van Dijk, Nazmi, Bolscher, Veerman, & Stap, 2015). Similar observations have been made in primary human endothelial cells and endothelial-based cell lines, where histatin-1 increased cell spreading on ECM matrices (van Dijk, Ferrando et al., 2017; Torres et al., 2017).

In general, the extended effects that histatins, and particularly histatin-1, have on a plethora of cells lines with different origins are intriguing and include cells ranging from oral keratinocytes, gingival fibroblasts, skin and corneal epithelial cells, as well as dermal fibroblasts. In fact, recent studies have extended the effects of this particular histatin toward another cell type relevant in wound healing, that is, the endothelial cell (Torres et al., 2017).

3.4 | Histatin-1 and endothelial cell adhesion and migration: endothelial barrier and angiogenesis

Endothelial function is a relevant aspect in the maintenance of tissue integrity and this is particularly central to the wound-healing process. Given the effects of histatins in epithelial cell migration and in the re-epithelialization process during wound healing, the question that arises is whether histatins might be required to other phases of wound healing, for instance, angiogenesis. In fact, two recent works have shown independently that histatin-1 is required for endothelial barrier function and angiogenesis (van Dijk, Ferrando et al., 2017; Torres et al., 2017). In one case, histatin-1 promoted the spreading of primary human umbilical vein endothelial cells (HUVECs), as shown in trans-endothelial resistance experiments (van Dijk, Ferrando et al., 2017), which is reminiscent to the effects of this histatin on human intestinal epithelial cell lines (van Dijk et al., 2015). At the same time, histatin-1 was also shown to promote endothelial cell adhesion and spreading onto fibronectin-coated matrices and to promote the migration of these cells on this substratum, as observed with HUVECs and the EA.hy926 cell line (Torres et al., 2017). As aforementioned, cell-ECM attachment and spreading are central steps in cell migration, and these are known to require the activation of Rho GTPases (Ridley et al., 2003). In the latter study, histatin-1 was shown to activate Rac1 (i.e., increases Rac1-GTP levels) in endothelial cells via the so-called "RIN2/Rab5/Rac1 axis" (Torres et al., 2017; Figure 4), a signaling pathway that is relevant for vascular morphogenesis and angiogenesis (Sandri et al., 2012). Specifically, histatin-1 promoted the recruitment of RIN2, a Rab5-GEF within early endosomes, leading to increased Rab5-GTP levels, and hence, augmented Rac1 activity and endothelial cell spreading. Most importantly, this signaling axis was relevant for vascular morphogenesis in vitro and angiogenesis in vivo, using cultured human endothelial cells and the chick chorioallantoic membrane assay, respectively (Torres et al., 2017). Also, alike the studies performed in epithelial cells (Oudhoff et al., 2008), histatin-1 activated ERK1/2 in endothelial cells, suggesting that similar mechanisms are involved (Torres et al., 2017). Several questions may be drawn from these studies, such as the identity of the cognate receptor that turns on the RIN2/Rab5/Rac1 axis in endothelial cells (Figure 4), because this pathway has been previously reported in the context integrin-ECM engagement (Sandri et al., 2012). Intriguingly, our unpublished data suggest the participation of VEGFR, as pharmacological inhibition of this receptor prevents histatin-1-dependent cell migration, raising the possibility of a cross-talk between convergent pathways downstream of integrins and growth factor receptors (Ivaska & Heino, 2011). Future studies will be required to address these possibilities.

3.5 | Histatins and cancer

Based on the migration-promoting effects of histatins, and because enhanced cell migration is a hallmark commonly described in cancer cells, it might be tempting to speculate on a potential role of histatins in tumorigenesis and tumor progression. Indeed, a study has shown that histatin-1 is highly expressed in melanoma cell lines and clinical samples, implicating it as a potential prognostic marker for this malignancy (Yaguchi & Kawakami, 2012). Likewise, a subsequent study correlated the amount of salivary histatin-3 with the progression of oral squamous cell carcinoma in patients, using MALDI-TOF-MS combined with magnetic beads (Jiang, Wang, Xu, Peng, & Chen, 2015). These studies are relevant in the context of the searching for new biomarkers that allow an early and reliable diagnosis of different types of cancer. Aside from their potential as biomarkers, deciphering the biological significance of a putative gain-of-function (or even

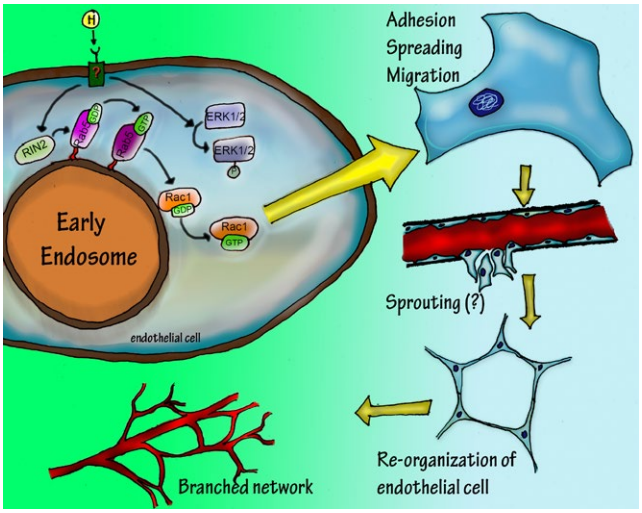

FIGURE 4 Proposed model for histatin-1-dependent endothelial cell migration. Histatin-1 ("H" in the figure) was recently shown to stimulate endothelial cell migration via activation of the RIN2-Rab5-Rac1 axis at the so-called "signaling endosomes." Specifically, histatin-1 promotes the recruitment of RIN2, a Rab5-GEF, to early endosomes, leading to Rab5-GTP loading and hence increased Rac1-GTP levels. These signaling events are relevant to endothelial cell adhesion, migration, and spreading, as well as for vascular morphogenesis in vitro and angiogenesis in vivo. Alternatively, histatin-1 triggers ERK1/2 activation, which is reminiscent of those observations made in oral epithelial cells. Putative receptor(s) involved in such responses (labeled with the symbol "?" in the figure) remain largely unclear

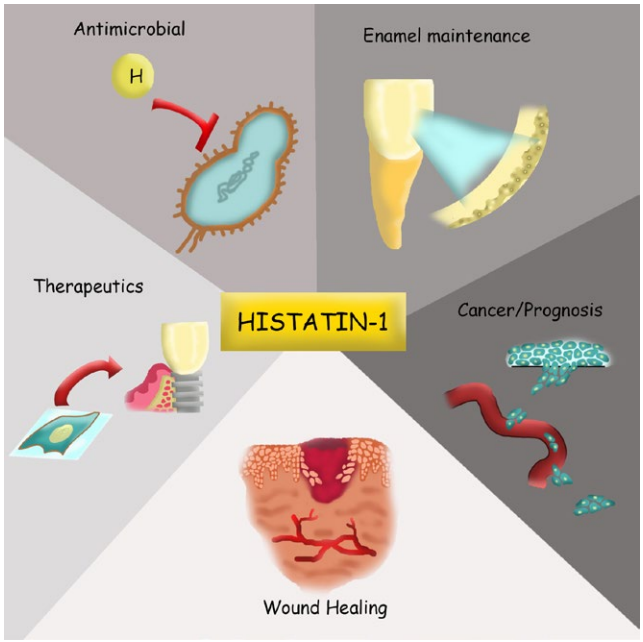

FIGURE 5 Histatin-1, biological activities and potential applications. A summary of different biological functions and potential applications of histatin-1 is shown. These include canonical roles, such as anti-microbial activity and enamel/acquired pellicle homeostasis. In addition, histatin-1 has been widely documented as a pro-migratory factor in a plethora of oral and non-oral cells, while more recent studies have suggested a potential role in cancer. Importantly, increasing studies have pointed to possible applications in biomaterials and therapeutics (see text for details)

loss-of-function) for histatins remains obscure. This is relevant, because a few studies have evaluated the effects of histatins in non-oral epithelial tumor cells, including MCF7 breast carcinoma (Oudhoff, van den Keijbus et al., 2009), Caco-2 epithelial colorectal carcinoma cells (van Dijk et al., 2015; van Dijk, Ferrando et al., 2017), and melanoma cell lines (C32mel, SKmel23, and A375 (Yaguchi & Kawakami, 2012)), although conclusions are somewhat controversial. Specifically, treatment of MCF-7 cells and C32mel cells with exogenous histatin-2 and histatin-1, respectively, promoted cell migration in both cases (Oudhoff, van den Keijbus et al., 2009; Yaguchi & Kawakami, 2012). Accordingly, knockdown of endogenous histatin-1 in SKmel23 cells or its overexpression in A375 cells prevented or promoted cell migration, respectively (Yaguchi & Kawakami, 2012). However, another study showed that histatin-1 promotes cell-cell adhesion in Caco-2 epithelial cancer cells, leading to increased transepithelial resistance and a potential role in the maintenance of the epithelial layer (van Dijk et al., 2015). In fact, a more recent study showed that histatin-1 increases cell-cell adhesion markers in Caco-2 cells, including E-cadherin and ZO-1, whereas it counteracts the effects of EMT (epithelial to mesenchymal transition) inducing agents, including EGF and TGF-β, as shown in spheroid assays derived from TR146 epithelial cells (van Dijk, Ferrando et al., 2017). However, additional studies will be required to address the precise effects of histatin-1 and other histatins in the EMT process, with focus on their relevance in cancer. This is particularly important, considering earlier studies that propose histatin-1 as a prognostic marker in melanoma (Yaguchi & Kawakami, 2012), where it could be tempting to speculate that histatin-1 promotes an EMT reversal (MET, mesenchymal to epithelial transition) in metastatic tumor cells upon homing. Future studies will be needed to determine the possible role of this protein in tumorigenesis and cancer progression. So far, studies showing a possible role for histatins in cancer have been performed in non-oral epithelial cells, however, and importantly, whether these roles for histatins could be extended to malignant cells derived from the oral cavity have remained largely unexplored.

4 | PERSPECTIVES

Although histatins have been commonly recognized as agents involved in mineral homeostasis and anti-microbial action (Figure 5), compelling evidence obtained in the last few years has shown that these salivary peptides, and particularly histatin-1, act as critical agents that promote oral epithelial cell migration (Oudhoff et al., 2008), which has a substantial impact in the process of epithelialization (Oudhoff, Kroeze et al., 2009). In addition, histatin's pro-migratory effects are not limited to epithelial cells, but also extend to fibroblasts (Oudhoff, van den Keijbus et al., 2009), osteoblasts (van Dijk, Beker et al., 2017), fat cells (Boink, van den Broek et al., 2016), and endothelial cells (van Dijk, Ferrando et al., 2017; Torres et al., 2017). In the latter, histatin-1

not only increases migration, but also promotes the integrity of the endothelial barrier (van Dijk, Ferrando et al., 2017), stimulates vascular morphogenesis in vitro, and increases angiogenesis in vivo (Torres et al., 2017; Figure 5). These findings are somewhat intriguing, because it remains unclear how could a human peptide (Padovan et al., 2010) exerts its biological effects on other species, such as chicken. In fact, other studies have extended the effects of histatin-1 to other animal models, including MDCK canine epithelial cells and MC3T3-E1 mouse preosteoblast cells (van Dijk, Beker et al., 2017). One possible answer is that this peptide uses a receptor that is conserved between species. Another possible answer is that the histidine/arginine-rich composition in this peptide allows the interaction with negatively charged proteins in the plasma membrane, permitting downstream signaling and endocytosis, as do arginine-rich peptides (Nakase et al., 2007).

This increasing field opens possibilities, including the potential use of histatin-1 as an adjuvant in wound repair, due to its effects in promoting the re-epithelialization of wounds (Oudhoff, Kroeze et al., 2009) and its angiogenic responses (Torres et al., 2017). Furthermore, histatin-1 is apparently innocuous, and it is stable in inflammatory environments, which is particularly observed in the synthetically produced form (Boink, Roffel et al., 2016). Finally, another possible clinical application of histatin-1 is in implantology, due to the ability to promote cell adhesion to titanium surfaces by this peptide (Figure 5; van Dijk, Beker et al., 2017). All the above would facilitate osseointegration of the implant and improve its prognosis.

In summary, histatins are versatile peptides with a great potential in tissue engineering and regenerative medicine (Figure 5). However, there is still a lack of research to elucidate the mechanisms by which these peptides exert their function, in such a way that their potential can be better exploited.

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AUTHOR CONTRIBUTIONS

Pedro Torres, Martín Castro, Montserrat Reyes, and Vicente A. Torres wrote the review.

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