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The effect of extracellular prolidase on repair processes in experimental models of inflammation and mechanical damage in HaCaT keratinocytes.

Ph.D. dissertation as a collection of papers
in the field of medical and health sciences
in the discipline of medical science

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Table of Content

1. Funding

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2. Articles Included in the Dissertation

The goal of the papers included in this dissertation was to evaluate the biological effect of extracellular prolidase on repair processes in experimental models of inflammation and mechanical damage in HaCaT keratinocytes.

I presented the research hypothesis in the review:

P1. **Misiura M**., Miltyk W.: *Current Understanding of the Emerging Role of Prolidase in Cellular Metabolism*. International Journal of Molecular Sciences. 2020; 21, 5906. doi: 10.3390/ijms21165906. IF: 5.923, MEiN: 140 points

The results of the performed experiments were presented in two research papers:

P2. **Misiura M**., Baszanowska W., Ościłowska I., Pałka J., Miltyk W.: P*rolidase Stimulates Proliferation and Migration through Activation of the PI3K/Akt/mTOR Signaling Pathway in Human Keratinocytes*. International Journal of Molecular Sciences. 2020; 21, 9243. doi: 10.3390/ijms21239243. IF: 5.923, MEiN: 140 points

P3. **Nizioł M.**, Ościłowska I., Baszanowska W., Pałka J., Besio R., Forlino A., Miltyk W.: *Recombinant Prolidase Activates EGFR-Dependent Cell Growth in an Experimental Model of Inflammation in HaCaT Keratinocytes. Implication for Wound Healing.* Frontiers in Molecular Biosciences. 2022; 9:876348. doi: 10.3389/fmolb.2022.876348. IF: 5.246, MEiN: 140 points

3. List of a Candidate's Publications

Article type	Number	Impact Factor	MEiN points
Articles included in the dissertation	3	17.092	420
Articles not included in the dissertation	24	56.392	1447
Conference abstracts	22	0.000	0
Summary	49	73.484	1867

4. Introduction

Among proteases cleaving proteins into polypeptides, there is one unique enzyme which specificity is limited only to imidodipeptides containing proline (Pro) or hydroxyproline (Hyp) at the carboxyl-terminus. The process of proteolysis is driven by prolidase (PEPD) [EC 3.4.13.9], which hydrolyses the bond of proline-containing di- or tripeptides (1). The reason why only prolidase can cleave those proline-containing peptides is that proline as a building block for various biomolecules (e.g., neuroactive peptides or growth factors) protects the polypeptide structure from hydrolysis. Due to the presence of a pyrrolidine ring in the structure of the amino acid, the biological structure of the proline-containing biomolecule is prevented from unexpected proteolysis (2). Prolidase belongs to the family of metallopeptidases due to the presence of divalent cation in the active site. PEPD is isolated from eukaryotic cells and also was found in other domains such as bacteria (3). Metal requirements in PEPD active sites differ between organisms, nevertheless, in humans, manganese is necessary for maintaining its biochemical activity. The highest activity of PEPD was noted in the renal tissue, intestinal mucosa, and red blood cells, while in plasma and hypothalamus tissue was low (4). The most specific substrate for this enzyme activity is glycyl-proline (Gly-Pro) (5).

Enzymatic properties of PEPD are linked to a disease called prolidase deficiency (PD, OMIM 170100), which manifests itself as massive imidodipeptiduria, difficult wound healing, and mental retardation, and a weakened immune system. To date, no effective treatment for PD has been developed (6). Moreover, there are research papers indicating the clinical importance of prolidase in disorders of collagen metabolism (7-16), metabolic disorders (17-23), and cancers (24-28). In addition to its enzymatic function, prolidase regulates many biological processes. In the cell metabolism, PEPD functions as a ligand of epidermal growth factor receptor (EGFR) and epidermal growth factor receptor 2 (HER2)-dependent signaling pathways (29-33), modulates p53 activity (34, 35), and interferon α/β receptor expression (36). Since downstream EGFR signaling leads to increased DNA synthesis, while prolidase is bound to this receptor, this finding shows promise for the improvement of regenerative therapy aimed at promoting cell proliferation and growth. Through EGFR-dependent stimulation, PEPD can be a beneficial factor in the treatment of diseases manifested by chronic ulceration or inflammation.

4.1. General information on prolidase

Prolidase is a peptidase belonging to the family of metallopeptidases that depend on divalent cations which enable its catalytic activity. PEPD is also named as X-Pro dipeptidase, proline dipeptidase, imidodipeptidase, and peptidase D (2). At the molecular level, the gene coding prolidase is located on the long arm of chromosome 19 at locus 13.11 and it consists of 15 exons (5). Point mutations result in the absence or decrease of PEPD catalytic activity, leading to a genetic disease known as PD. To date, there are 29 identified point mutations in the gene structure causing a decrease or complete loss of PEPD activity. Out of these, 8 point mutations are implicated in phenotypic consequences (37). At the structural level, the human prolidase molecule is a homodimer consisting of two subunits. Each subunit consists of N- and C-terminal domains of 493 amino acids (AA) each (38). The molecular weight of the human PEPD subunit is 58 kDa (39). Due to the presence of the C-terminal domain (185-493 AA), PEPD exhibits the similarity to the 'pita bread' peptidases such as β-aminopeptidase, and methionine aminopeptidase, and creatinase (40). The N-terminal domain (1-185 AA) remains less tightly bound to the substrate. The importance of the C-terminal domain is also reflected by the presence of an active site, where the substrate binds, with the Mn^{2+} ion required for its proper functionality. Each monomer is connected by the disulfide bond occurring between Cys158A and Cys158B (41). Divalent cations such as Zn^{2+}, Mg^{2+}, Ca^{2+}, and Co^{2+} may also act as cofactors required for the enzymatic activity of PEPD. However, prolidase activity, containing non-manganese cation, decreases below 30% (39).

Prolidase as hydrolases requires H_2O for the enzymatic reaction (38). PEPD presents the highest specificity for Gly-Pro in the *trans* conformation (1). Although prolidase has the highest catalytic activity against Gly-Pro [38], it also hydrolyses other C-terminal proline-containing dipeptides such as Ala-Pro, Phe-Pro, Met-Pro, Val-Pro, and Leu-Pro (42) The main source of substrates (Gly-Pro) are proteins rich in amino acid sequences containing C-terminal proline or hydroxyproline, e.g. collagen (43), complement component C1q (44), dietary proteins (45) and other biomolecules such as substance P, plasminogen, oxytocin, vasopressin, and angiotensin (46). Of these, collagen is the most common source of Gly-Pro. Prolidase acting in the cytosol, not as typical enzymes in lysosomes, liberates free proline and glycine from di- and tripeptides [46]. To date, it remains unknown why prolidase is expressed in the cytosolic location. PEPD occurs abundantly in enterocytes probably participating in the cleavage of proline-containing dipeptides from the diet (47). Based on mRNA analysis, the highest level of *PEPD* gene expression is reported in the renal tissue, small intestine, and

duodenum (48), however, it is not similar to cell types with the highest prolidase activity, named, erythrocytes and human skin fibroblasts (42). It was also demonstrated that platelet-rich plasma (PRP) is an abundant PEPD source (49, 50).

4.2. The biological significance of prolidase as a dipeptidase

At the cellular level, prolidase activity significantly contributes to proline supply that acts as an intracellular molecule modulating collagen turnover (51-53), cell growth, proliferation, and differentiation (54) as well as angiogenesis (55), and glucose metabolism (56). The small amino acid plays a considerable role in energy metabolism and redox balance (57). Lately, efforts have been made to great extent in elucidating the role of proline in the energy production of cancer cells (58). Among sources of proline, prolidase-mediated collagen turnover and enzymatic conversion of pyrroline-5-carboxylic acid (P5C) are mentioned. P5C can serve as a precursor and product of proline-related conversions and it originates from glutamate or ornithine with the participation of P5C synthase (P5CS) (59) or ornithine aminotransferase (OAT), respectively (60). P5C can be reduced with P5C reductase (PYCR) and proline is oxidized with the mitochondrial enzyme – proline dehydrogenase/proline oxidase (PRODH/POX) (61). During this process electrons are transported to the electron transport chain producing ATP or they directly reduce oxygen, producing reactive oxygen species (ROS) (62). P5C, as an intermediate molecule, links the proline metabolism with the tricarboxylic acid cycle and urea cycle (57). Recently, it has been found that overexpressed prolidase through higher proline availability and downregulated PRODH/POX expression induced autophagy in MCF-7 breast cancer cells (63).

The biological significance of enzymatic prolidase-dependent products was highlighted as described above. In recent times, it was found that prolidase activity is regulated by activated receptors on the cell membrane such as a β_1-integrin receptor, IGF-1 receptor (IGF-1R), and TGF-β_1 receptor (TGF-β_1R). Stimulated β_1-integrin receptor by type I collagen (64) and, its agonist, thrombin (53) enables activation of downstream FAK kinase and the signal goes through Sos, Ras, and Raf proteins to the ERK1/2 kinases. The signaling reaches the nucleus and influences the transcription of the genes accelerating cell growth, proliferation, and differentiation. An increase in prolidase activity and collagen biosynthesis were also observed. This observation confirms that stimulation of β_1-integrin receptor regulates the availability of proline used as a substrate for collagen synthesis. Prolidase activity is also

regulated by an IGF-1R-dependent pathway (52), which stimulates cell growth, proliferation, and collagen biosynthesis.

Prolidase activity, through supplying proline, plays a role in the regulation of angiogenesis (55) via modulating the function of HIF-1α and HIF-1α-dependent molecules including VEGF, Glut-1, and TGF-β which are involved in angiogenesis (65), glucose metabolism (56) and control of cell proliferation and differentiation (54), respectively. Proline and hydroxyproline, liberated by prolidase, can inhibit the degradation of the HIF-1α transcription factor *via* the VHL-dependent proteasome degradation pathway. A study showed hydroxyproline is even more potent than proline in the protection of HIF-1α from its degradation. Possibly, this phenomenon results from the hindrance in the hydroxylation of proline in the oxygen-dependent domain of HIF-1α and it prevents HIF-1α from interacting with VHL (55). As the study of Surazynski et al. (55) suggests, overexpressed PEPD influences HIF-1α-dependent molecules such as VEGF and Glut-1 through an increase in the level of their expression. Thus, at the cellular level accelerated prolidase activity may boost the VEGF-controlled angiogenesis. Another study (66) showed a TGF-β_1R-dependent mechanism for activation of prolidase and regulation of proline availability. The authors demonstrated that proline released from dipeptides by prolidase influenced the expression of TGF-β_1R. In an *in vitro* model, it was observed that extracellular proline stimulated phosphorylation of Akt and mTOR kinases. The role of the Akt/mTOR pathway is implicated in cell growth, proliferation, and differentiation. mTOR was indicated as an essential component of the relationship between prolidase activity and PI3K/Akt/mTOR pathway-related cell survival. Phosphorylation-activated mTOR kinase as a metabolic sensor accelerates cell capability to cell growth, proliferation, and migration via coordination of the growth factors-dependent signaling, an increase in protein synthesis, and energetic status (67).

At the transcriptional level, prolidase activity products: Pro or Hyp modulates biological effects of NF-κB transcription factor (45). The enzyme activity significantly decreases the expression of this transcription factor, probably, due to the increased level of Pro or Hyp since proline may protect NF-κB from activation by preventing the NF-κB-IκBα complex from degradation (68).

All described processes are presented in Figure 1.

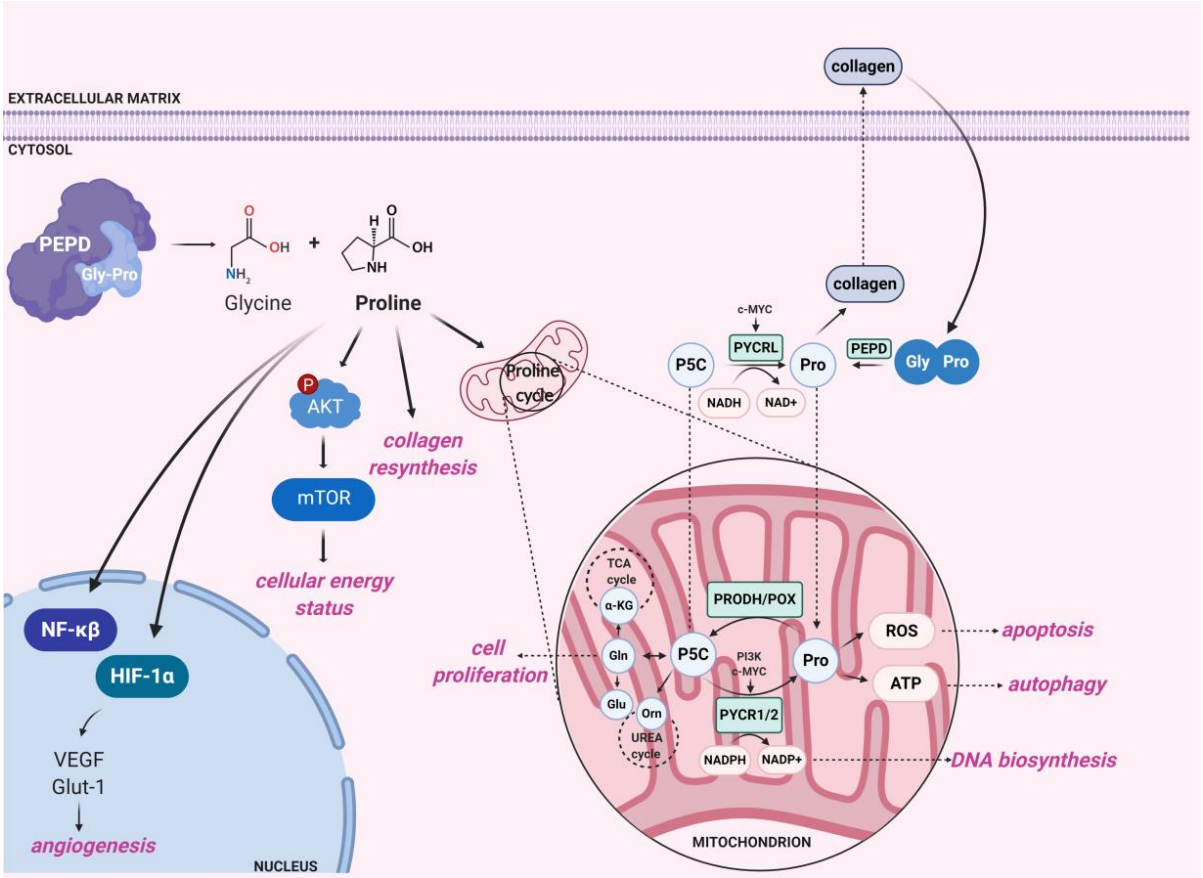

Figure 1. The enzymatic activity of PEPD affects various cellular processes. Prolidase supplies substrates for collagen resynthesis. Proline, the product of PEPD activity, modulates intracellular energetic status via Akt/mTOR pathway, inhibits HIF-1α degradation, and mediates the proline cycle. Created with Biorender.com.

4.3. Clinical significance of prolidase as a dipeptidase

Many researchers implicated the role of prolidase activity in various cancers as well as pathological conditions associated with collagen turnover (7-16). For instance, increased prolidase activity has been observed in melanoma (24), breast cancer (25), lung cancer (26), ovary cancer (27), and endometrial cancer (28). However, the clinical significance of prolidase is reflected in prolidase deficiency accompanied by its low or absent enzymatic activity. It is a rare autosomal recessive disorder characterized by massive imidodipeptiduria, skin lesions, recurrent infections, mental retardation, and elevated proline-containing dipeptides in plasma (69-74). The incidence of the disease is 1-2 cases per million births (75). Patients who suffered from prolidase deficiency struggled with skin lesions particularly on the legs such as diffuse telangiectasia, purpuric rash, crusting erythematous dermatitis, or progressive ulceration (4). Connective tissue disturbances may be explained by the impaired

degradation of di- and tripeptides containing C-terminal proline or hydroxyproline due to a lack of a significant decrease in PEPD activity. Accumulating di- and tripeptides from collagen turnover cannot be reutilized resulting in skin lesions. In PD patients, immunodeficiency is also detected possibly resulting from defective C1q complement assembled of repeated Gly-Pro dipeptide (44). Another possible explanation for impaired immune response in PD patients could be the recently reported role of PEPD in interferon α/β receptor (IFNAR1) function (36). They found that the PEPD molecule is required for the receptor maturation and surface expression as well induction of interferon β (IFN β)-dependent genes. In PD-derived fibroblasts, downregulation of IFNAR1 surface expression and inhibition of IFNβ-downstream signaling were detected. While mental disorders in PD patients may be explained by impaired deactivation of C-terminal proline-containing neuropeptides. The clinical symptoms may happen due to the low level of proline acting as a neurotransmitter (76). On the other hand, Hui et al. (77) reported an increase in PEPD activity in the neuronal tissue. There are reports connecting an accumulation of proline and mental dysfunctions (78, 79) as a result of excessively glutamate-stimulated NMDA receptors causing the death of neurons (78). Based on the novel function of PEPD, it seems to be beneficial to reconsider PD pathomechanism as the underlying enormous release of prolidase molecules from injured cells. PEPD, present in extracellular space, may interact with various receptors, e.g. EGFR (29) and HER2 (32). To date, it is known that both PEPD wild-type and mutated can suppress EGFR/HER2-downstream signaling when they are overexpressed (32). In the view of the new function of PEPD, likely, its plasma concentration is not diminished in PD patients, however, further research is crucial for supporting this hypothesis. It is also worth investigating the status of EGFR/HER2 in patients with prolidase deficiency.

4.4. Prolidase as a cellular regulator

The study of Yang et al. (29) began a new era of research on prolidase since they proved PEPD's role as an EGFR ligand and further broaden the knowledge of regulatory functions of prolidase not as a dipeptidase. Since then, at the cellular level, PEPD was implicated in the activation of EGFR, downregulation of EGFR/HER2 signaling when overexpressed, regulation of p53 activity, and IFNAR1 maturation. All of the findings shed new light on PEPD functions and pave the way for developing novel anticancer treatment strategies and other clinical conditions such as wound healing. The paragraphs below under this section describe novel functions of PEPD as a cellular regulator.

4.4.1. Prolidase as a ligand of EGFR

The first study presenting the novel function of prolidase was published by Yang et al. (29) in 2013. Firstly, the authors found that prolidase located extracellularly can directly bind to the EGFR (29). As an EGFR ligand, homodimeric prolidase deviates structurally from other EGFR ligands (epidermal growth factor (EGF), neuregulin, transforming growth factor, amphiregulin, heparin-binding EGF-like growth factor, and epiregulin) (80). Known so far EGFR ligands share the characteristic EGF motif $CX_7CX_{4-5}CX_{10-13}CXCX_8GXRC$ where X is an amino acid. An additional difference between PEPD and recognized EGFR ligands is the cytoplasmic location of PEPD (81). To be explored remains which PEPD domain or region forms a bond with the extracellular domain of the receptor. In contrast to EGF, prolidase binds to EGFR domain 2 while EGF is bound to EGFR domains 1 and 3. Once prolidase as a homodimer connects to EGFR, tetrameter is formed as an EGFR dimer and PEPD dimer (32). It entails EGFR activation and transduction of intracellular signaling through phosphorylation of the intracellular domain which has kinase activity. Among various EGFR-downstream pathways are the PI3K/Akt/mTOR, Ras/Raf/ERK1/2, and JAK/STAT3 axes. Finally, the signal reaches the nucleus causing genes transcription accelerating cell growth, proliferation, and differentiation (82).

As the study (29) showed, in comparison to the strongest EGFR ligand, EGF, prolidase exhibits lower affinity to EGFR and is 350 times weaker. Nevertheless, the induction of PEPD-activated EGFR signaling lasts longer, and PEPD activity is not required. A comparison of EGFR-activated signaling by wild-type PEPD and its enzymatically inactive mutant ($PEPD^{G278D}$) confirmed that catalytic activity is not necessary. What is more, $PEPD^{G278D}$ effectively abolishes EGFR-downstream signaling pathways (Akt, ERK1/2, and STAT3 pathways) triggering the inhibition of cancer cell proliferation in the mice model (32).

As intracellular PEPD can not activate EGFR as the ligand, it needs to be outside of the cell. Therefore, it is essential to find an answer for the source of extracellular prolidase stimulating EGFR. The first source of prolidase that comes to mind are cells releasing intracellular content after injury. The *in vitro* study showed that 2.7 nM PEPD is sufficient to activate EGFR (29), while in the *in vivo* experiments, PEPD concentration reached 3 nM in the bloodstream after chemical damage to liver cells. This finding entails a new question: does prolidase undergo extracellular degradation preventing it from unexpected EGFR stimulation? A recent study by Yang et al. (83) revealed that serine proteases, present in

plasma, inactivates PEPD through a coagulation cascade involving intrinsic and extrinsic pathways. This report showed that once prolidase is present in the bloodstream, it leads to rapid binding of PEPD with a proline-rich domain of factor XII followed by intrinsic pathway activation. An activated cascade of coagulation triggers the activation of factor VII (extrinsic pathway). Activated factor VII inactivates prolidase circulating in the bloodstream. The mechanism of the observed effects remains unexplained. Another question regarding prolidase exported out of the cell remains unanswered, namely, how prolidase can be secreted to extracellular space.

4.4.2. Prolidase as a ligand of HER2

Following the finding of PEPD-dependent EGFR activation, the authors made a discovery that PEPD functions as a ligand of HER2 (30), another receptor from the family of EGFR. Homodimeric PEPD binds to HER2 domain 3 in the extracellular domain. The mechanism of overexpressed HER2-downstream signaling depends on the state of the receptor. If HER2 is a monomer form, PEPD activates HER2 through dimerization and phosphorylation but it happens slowly. In turn, if HER2 is a dimer, the receptor activation by PEPD occurs more rapidly. Overexpressed HER2-downstream singling is abolished by disruption of the HER2-Src complex. As a result, the invasive phenotype of cancer cells with overexpressed HER2 upon binding with PEPD was significantly inhibited. Similar to EGFR activation, PEPD activity is not involved in HER2 regulation.

Yang et al. (31) demonstrated that enzymatically inactive mutant PEPD ($PEPD^{G278D}$) triggers stronger inhibitory activity in cancer growth suppression as compared to wild-type PEPD. The mechanism underlying this phenomenon may result from the stimulation of HIF-1α by PEPD activity-derived products as pro-survival factors in cancer cells (55). The effect of PEPD-dependent on overexpressed HER2-downstream signaling included the inhibition of pro-proliferative phenotype through receptor dephosphorylation and dephosphorylation of its downstream kinases such as Src, Akt, ERK1/2, and STAT3. The inhibitory function of PEPD as HER2 ligand covered also activation of the apoptosis protein markers such as downregulation of Bcl-2, upregulation of Bax, and caspase activation (caspases-3, -8, and -9). Due to the anticancer function of PEPD, cancer cell sensitivity to drug treatment was improved.

Figure 2 demonstrates the summary of PEPD-dependent EGFR- and HER2-downstream signaling evoking intracellular responses.

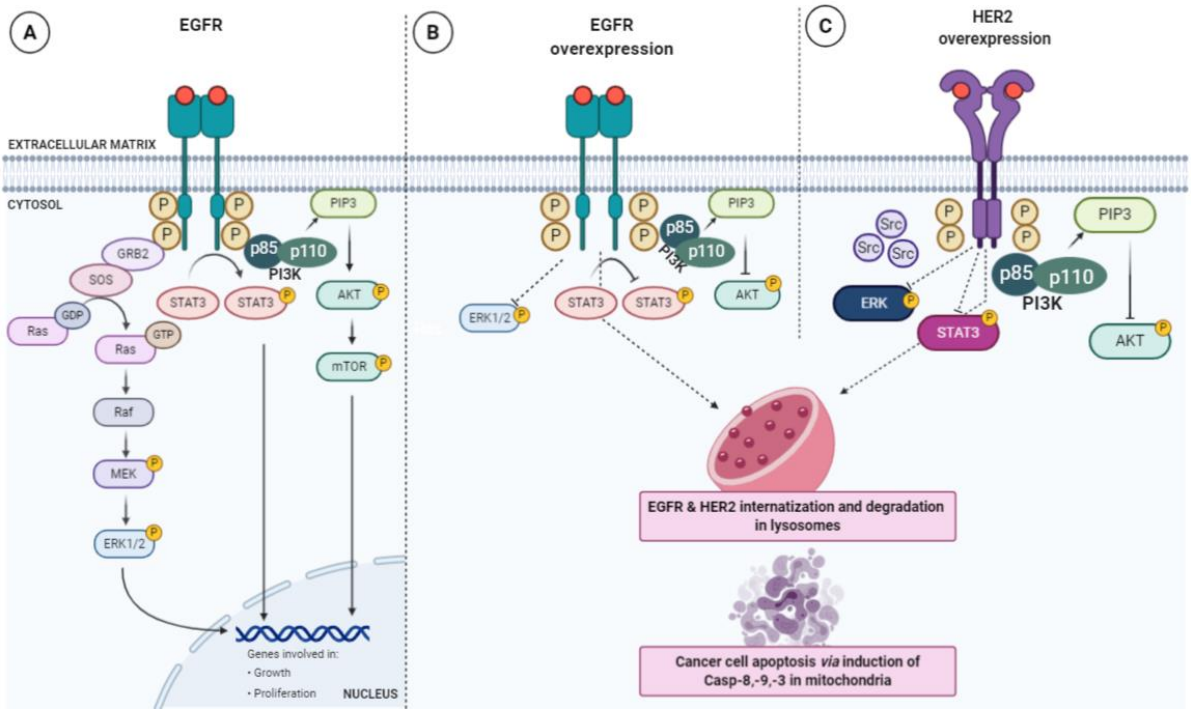

Figure 2. PEPD-dependent EGFR- and HER2-downstream signaling evoking intracellular responses. Created with Biorender.com.

4.4.3. Prolidase as a regulator p53 function

Apart from the role of PEPD as an EGFR/HER2 ligand, a novel function of prolidase is implicated in the regulation of tumor suppressor protein as an essential component in the proper function of p53 (34). Among several upstream and downstream regulating mechanisms of 53 function mechanisms (84), prolidase also exhibits regulatory function on mutated p53 through 1) limiting p53 subcellular translocation and 2) inhibiting p53 phosphorylation (34). Recently, the understanding of the complex PEPD-p53 mutants was progressed (35) indicating that disruption of this complex leads to regaining the normal function of p53. These findings were not limited to indicating the new function of PEPD in p53 regulation through direct binding but also expanded the knowledge about PEPD subcellular localization. Previously, it was believed that PEPD is the cytosolic molecule, however, the authors found PEPD in the cytosol, nucleus, and mitochondria.

In vitro knockdown of PEPD significantly limited cell survival via activation apoptosis pathways (intrinsic and extrinsic) while *in vivo* tumors significantly regressed. The molecular mechanism for the observed effects relies on the prevention of p53 from its subcellular translocation from cytosol to mitochondria by PEPD. p53 transcriptional activity requires

phosphorylation at Ser6 and Ser15 positions in its transactivation domain and this process is inhibited by prolidase.

Forming the PEPD-p53 complex occurs due to the binding of the PEPD catalytic domain to the proline-rich domain in the p53 structure. Similarly to EGFR and HER2 binding, the PEPD motif linked to p53 has not been detected yet. Another similarity to EGFR and HER2 activation by PEPD is that catalytic activity is not required.

Another recent study on the relation between PEPD and p53 (35) indicates that if mutated p53 liberates from the PEPD-p53 complex, it is modified at the post-translational level. As research showed p53 mutant undergoes acetylation at the K373 position enabling its refolding and reactivation. In other words, the knockdown of PEPD contributes to turning oncogenic p53 mutants into tumor suppressor proteins.

4.4.4. Prolidase as a regulator of interferon α/β receptor function

A new function of PEPD in an inflammatory system, independent of its enzymatic activity, was reported by Lubick et al. (36). The proper function of IFNAR1 relies on the post-translational modification in which PEPD is involved. IFNAR1 signaling plays a critical role in the immune response against viruses. The authors showed that the N-terminal domain of prolidase is bound to non-structural protein 5 (virus protein), and IFNAR1 antagonist. As a result, IFNAR1 is downregulated and its surface expression was decreased due to the impaired glycosylation process after the translation step of protein synthesis. In addition to this, the PEPD molecule played a remarkable role in the induction of IFN β-dependent genes and IFNβ-dependent signaling in antiviral response.

4.5. Summary remarks

Taken together, prolidase is involved in numerous biological processes at the cellular level acting as a “friend” or “foe”. Figure 3 presents the biological activity of prolidase as an enzyme as well as a regulatory protein in cellular metabolism.

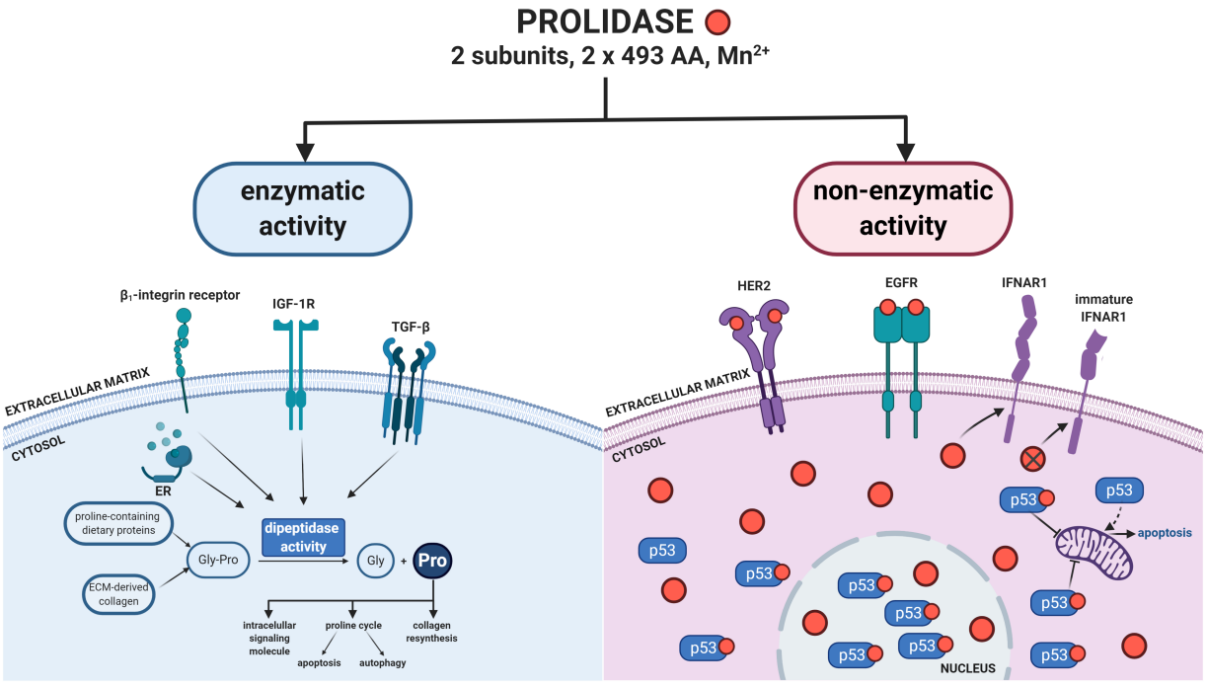

Figure 3. Dual functions of prolidase at the cellular level as an enzyme and a ligand. Created with Biorender.com.

“Friend” functions of PEPD include activation of EGFR- and HER2-downstream singling pathways, suppression of overexpressed EGFR- and HER2-downstream singling pathways accompanied by a tumor regression, prevention of p53 from unexpected activation, recovery of p53 suppressor functions, and IFNAR maturation at the cellular level. Prolidase a ligand of EGFR serves as an interface in the regenerative processes ongoing in damaged tissues and chronic inflammation through stimulation of cell growth, proliferation, and differentiation. While in developing anticancer treatment, prolidase seems to be a pivotal factor in understanding the mechanism involved in cancer control.

"Foe" functions of PEPD cover delivery of proline and hydroxyproline as a consequence of its enzymatic activity that modulates intracellular signaling, with special emphasis on the mechanism for switching cancer cell phenotype under the conditions of ATP (pro-survival mode) or ROS (pro-apoptotic mode) generation. PEPD activity-derived products may also trigger HIF-1α activity contributing to the pro-survival milieu in cancer cells.

5. Study Aims

Given the fact that prolidase is an EGFR ligand and activates the EGFR-downstream signaling, I hypothesized that extracellular PEPD may remarkably contribute to cell proliferation and migration facilitating wound repair.

The aim of the research performed for the doctoral dissertation was to evaluate the effect of extracellular prolidase on repair processes in experimental models of inflammation and mechanical damage in HaCaT keratinocytes.

To achieve the research objectives, a series of scientific activities were undertaken as follows:

1. Evaluation of the effect of porcine prolidase on cell vitality, proline and collagen biosynthesis, prolidase activity, and expression of the selected EGFR-downstream proteins in HaCaT cells.

2. Evaluation of the effect of porcine prolidase on cell viability, proliferation, migration, proline and collagen biosynthesis, prolidase activity, and expression of the selected receptors (EGFR, β_1-integrin, IGF-1R, and TGF-β_1R) and their downstream signaling proteins under the conditions of mechanical damage in HaCaT cells.

3. Evaluation of the effect of recombinant human prolidase wild-type on cell viability, proliferation, migration, cell cycle, activation of downstream signaling pathways induced by prolidase-dependent stimulation of the EGFR and NF-κB pathway under the conditions of IL-1β-induced inflammation in HaCaT cells.

4. Evaluation of the effect of recombinant human prolidase mutants (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) on cell proliferation, cell cycle, and activation of signaling pathways induced by prolidase-dependent stimulation of the EGFR under the conditions of IL-1β-induced inflammation in HaCaT cells.

6. Materials and Methods

6.1. HaCaT cell culture

HaCaT cells purchased from CLS Cell Lines Service (300493; Eppelheim, Germany) were cultured in a DMEM cell culture medium (PanBiotech, Aidenbach, Bayern, Germany) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 1% antibiotic (penicillin/streptomycin; Gibco, Carlsbad, CA, USA) in a cell incubator at 37 °C, and 5% CO_2. The cell culture medium was changed every 3 days until cells reached 80% of confluency.

6.2. Production of recombinant human prolidase in *E. coli* expression system

The constructs for wild-type recombinant human prolidase (rhPEPD) and mutant forms (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) were prepared as previously described (39, 85). *E. Coli* BL21(DE3) competent cells (Thermo Fisher Scientific, Waltham, MA, USA) were transformed with the rhPEPD-containing vector using the heat shock method. After stimulation with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Bioshop, Canada), cells were centrifuged and resuspended in lysis buffer followed by two-step purification. Firstly, the HisTrap column (BioRad Laboratories, Hercules, CA, USA) with Ni-NTA affinity resin (IMAC) equilibrated with 0.1 M $NiSO_4$ was used for polyhistidine-tagged proteins. Then, the eluted mixture was concentrated (Amicon-Ultra 10; Merck Millipore, Burlington, MA, USA) and loaded onto a gel filtration column (Superdex 200; Pharmacia, New Jersey, NJ, USA). Activation of the recombinant proteins was performed with 1 mM Mn^{2+} at 37°C for 1 hour. Purified proteins were dialyzed against PBS (12 h, 4°C). Aliquoted proteins in 200 µl strip tubes were stored at -80 °C. Before treatment, the concentration of purified proteins was determined using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

6.3. HaCaT treatment

Under conditions of mechanical damage, confluent cells (5–8th passages) were scratched using a sterile 200 µl pipette tip, washed twice with PBS, and incubated with prolidase from the porcine kidney (Sigma Aldrich, Saint Louis, MO, USA) at the concentrations of 1-100 nM. Under experimental conditions of inflammation, HaCaT cells were incubated with human recombinant IL-1β (10 ng/ml; Sigma Aldrich, Saint Louis, MO, USA) and human

recombinant prolidase (rhPEPD^{WT}, rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) at concentrations of 10-250 nM.

6.4. *In vitro* wound-healing assay

Scratched HaCaT cells were photographed every 24 h cells using an inverted optical microscope (objective 40x; Nikon; Minato, Tokyo, Japan) to monitor the wound closure area. The open gap was measured by ImageJ software (https://imagej.nih.gov/ij/) and its rate was calculated as indicated in the formula below. The results were presented as a percent of the control value.

$$\text{wound healing rate} = \frac{\text{original wound area} - \text{unhealed wound area}}{\text{original wound area}}$$

6.5. Cell viability assay

Cell viability of HaCaT cells was measured using Cell Titer Blue assay as described in the manufacturer's instructions (Promega, Madison, WI, USA). After treatment, the resazurin-containing solution was added to each well with cultured cells and incubated (37 °C, 2 h). Absorbance was read on TECAN Infinite® M200 PRO (Tecan Group Ltd., Männedorf, Switzerland) at 570 nm. 600 nm served as a reference wavelength. The results were presented as a percent of the control value.

6.6. Cell vitality assay

The cell vitality was assessed by measurement of the level of intracellular thiols. After treatment, cells were washed twice with warm PBS (pH 7.4) and trypsinized (c=0.25%). Then, cells were centrifuged (5 min, 500 x g), the pellet was washed with PBS and stained with commercially available Solution 5 (ChemoMetec, Denmark) containing VitaBright-48, acridine orange, and propidium iodide. Cell Vitality Assay was evaluated on an image cytometer Nucleo Counter NC-3000 (ChemoMetec, Denmark). The results were presented as percent of the control value.

6.7. Cell proliferation assay

The proliferation of HaCaT cells was determined with the use of CyQUANT® Cell Proliferation Assay (Thermo Fisher Scientific, Waltham, MA, USA). After incubation, cells were rinsed twice with PBS (pH 7.4) and frozen at −80 °C until analysis. Before analysis,

samples were quickly thawed at room temperature (RT) and mixed with a 200 µL solution consisting of the CyQUANT® GR dye and cell-lysis buffer. The plate was incubated for 5 min at RT protected from light. Fluorescence was read on TECAN Infinite® M200 PRO (Tecan Group Ltd., Männedorf, Switzerland) at 480 nm and 520 nm as excitation and emission wavelengths, respectively. The results were presented as the percent of the control value.

6.8. Cell cycle analysis

After incubation, cells were trypsinized (c=0.25%), centrifuged (5 min, 500 x g), and washed twice with PBS. The pellet was suspended in 0.5 ml of PBS, fixed in 4.5 ml of ice-cold 70% ethanol, and stored at 4 °C until analysis. On the day of analysis, after centrifugation (5 min, 500 x g) and rinsing with PBS, the pellet was resuspended in DAPI-containing Solution 3 (Chemometec, Denmark). After incubation (37 °C, 5 min), the analysis of the ethanol-fixed cell cycle was conducted using an image cytometer (NC-3000, Chemometec, Denmark).

6.9. Preparation of lysates

The cells were harvested with RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) containing protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Roche, Basel, Switzerland), phosphatase inhibitor cocktail (PhosSTOP, Roche, Basel, Switzerland), and viscolase (A&A Biotechnology, Gdańsk, Poland). Then, lysates were incubated on ice for 10 min and sonicated 3 times (15 sec on and 5 sec off), and centrifuged (4 °C, 10 min, 12,000 × g). The supernatant was aliquoted in 200 µl strip tubes and frozen at −80 °C until protein analysis. The Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was employed for the quantification of protein concentration.

6.10. Western immunoblotting

Equal amounts of protein were diluted in RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) and mixed with Laemmli buffer (120 mM Tris-HCl, 20% glycerol, 0.4% SDS, 0.02% bromophenol blue, pH 6.8) with freshly added 5% β-mercaptoethanol (Sigma Aldrich, Saint Louis, MO, USA). After denaturation, the samples were loaded onto SDS-PAGE gels followed by blotting onto polyvinylidene difluoride (PVDF; BioRad Laboratories, Hercules, CA, USA) membranes. The blocking step was performed with either 5% non-fat dried milk (Santa Cruz Biotechnology, Dallas, TX, USA) or BSA (Sigma Aldrich, Saint Louis, MO, USA) in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) for

1h at RT with gentle rotation. The membranes were incubated (16 h, 4 °C) with primary antibodies listed below, followed by incubation with alkaline phosphatase-linked goat anti-rabbit or anti-mouse antibodies (Sigma Aldrich, Saint Louis, MO, USA) for 1 h at RT. The membranes were washed thrice in TBS-T for 5 min. Protein bands were detected with 1-Step™ NBT/BCIP Substrate Solution (Thermo Fisher Scientific, Waltham, MA, USA) and protein band intensities were semi-quantitatively measured with ImageJ software (https://imagej.nih.gov/ij/). All experiments were run in triplicates.

6.11. List of antibodies

The PVDF membranes were incubated with the selected primary antibodies diluted in 5% BSA as indicated in brackets. Cell Signaling Technology (Danvers, MA, USA) delivered following primary antibodies: Akt Rabbit mAb (1:2000), Cyclin D Rabbit mAb (1:1000), E-Cadherin Rabbit mAb (1:1000), EGF Receptor Rabbit mAb (1:1000), GAPDH Rabbit mAb (1:1000), HIF-1α Rabbit mAb (1:1000), IKKα Mouse mAb (1:1000), IKKβ Rabbit mAb (1:1000), IκBα Rabbit mAb (1:1000), Lamin A/C Mouse mAB (1:1000), mTOR Rabbit mAb (1:1000), N-Cadherin Rabbit mAb (1:1000), NF-κB p65 Rabbit Antibody (1:1000), p44/42 MAPK (ERK1/2) Rabbit mAb (1:1000), IGF-1 Receptor β Rabbit mAb (1:1000), phospho-mTOR (Ser2448) Rabbit mAb (1:1000), FAK Rabbit mAb (1:1000), phospho-FAK (Tyr397) Rabbit mAb (1:1000), Integrin β1 Receptor Rabbit mAb (1:2000), PI3 Kinase p85 Rabbit mAb (1:1000), phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) Antibody (1:1000), PCNA Rabbit mAb (1:1000), phospho-Akt (Ser473) Rabbit mAb (1:2000), phospho-EGF Receptor (Tyr1068) Rabbit mAb (1:1000), phospho-IKKα/β (Ser176/180) Rabbit mAb (1:1000), phospho-IκBα (Ser32) Rabbit mAb (1:1000), phospho-NF-κB p65 (Ser536) Rabbit mAb (1:1000), phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) Rabbit mAb (1:1000), phospho-Stat3 (Tyr705) Rabbit Ab (1:1000), Stat3 Rabbit mAb (1:1000), Cox2 Rabbit mAb (1:1000), TGF-β Receptor I Rabbit Antibody (1:1000), Thymidine Kinase 1 Rabbit mAb (1:1000). Mouse anti-Grb2 (1:500) and mouse anti-Sos1 (1:1000) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Secondary alkaline phosphatase-conjugated anti-mouse or anti-rabbit antibodies were diluted 10^4 times (Sigma Aldrich; Saint Louis, MO, USA).

6.12. Immunofluorescence staining and confocal microscopy

After incubation, cells were washed with PBS and fixed with 3.7% paraformaldehyde. Depending on protein localization, permeabilization with 0.1% Triton for 5 min was performed or omitted. The blocking step with 3% fetal horse serum (1 h, RT) was followed by incubation (4°C, ON)with the selected primary antibodies (EGF Receptor Rabbit mAb, FAK Rabbit mAb, NF-κB p65 Rabbit Antibody) diluted 500x, 100x, and 400x, respectively. Anti-rabbit FITC-linked antibody (Becton Dickinson, Franklin Lakes, NJ, USA) was used as a secondary antibody at a concentration of 5 μg/ml for (1 h, RT protected from light). The cell nuclei were stained with Hoechst (1 ng/ml). A confocal laser scanning microscope (BD Pathway 855 Bioimager, Becton Dickinson, Franklin Lakes, NJ, USA) with AttoVision software was employed for immunofluorescence staining visualization.

6.13. Evaluation of collagen biosynthesis

The principle of Peterkofsky's method (86) for the determination of collagen biosynthesis is the incorporation of radioactive 5-[3H]-proline (5 μCi/ml; Hartmann Analytic, Germany) into proteins able to be digested by *C. histolyticum* collagenase (Sigma Aldrich, Saint Louis, MO, USA). After treatment, cells were washed with PBS (pH 7.4), harvested with proline-containing PBS, and frozen at -80 °C until the day of analysis. The radiometric read was performed with Liquid Scintillation Analyzer Tri-Carb 2810 TR (PerkinElmer, Waltham, MA, USA). The results were normalized to total protein biosynthesis and were presented as a percent of the control value.

6.14. Determination of prolidase activity

The activity of prolidase was determined according to the method published by Besio et al. (87). Protein concentration was measured using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). An equal amount (50 μg) of proteins was mixed with 50 mM Tris–HCl (pH 7.8) containing 1 mM $MnCl_2$ and 0.75 mM glutathione and incubated (1 h, 50°C). The enzymatic reaction with 100 mM glycyl-proline lasted 30 min at 50°C and was stopped by the addition of 0.45 M ice-cold TCA. After centrifugation (15 min, 12 000 x g), the supernatant fraction was mixed with Chinard's reagent (12 min, 90 °C) in a proportion of 1:9, and then incubated on ice (15 min). Absorbance was read at 515 nm on TECAN Infinite® M200 PRO (Männedorf, Switzerland). The results were expressed as a percent of the control value.

6.15. LC-MS-based quantitative analysis

The measurement of proline concentration in HaCaT cells was conducted according to the method of Klupczynska et al. (88). Briefly, cells were scraped in ice-cold methanol with an internal standard (25 µM d3-proline; Sigma Aldrich, Saint Louis, MO, USA) and then stored at -80 °C until analysis. Samples were analyzed using Agilent 1260 Infinity HPLC system coupled to Agilent 6530 Q-TOF mass spectrometry detector with electrospray ionization (Agilent Technologies, Santa Clara, CA, USA) as an ion source in positive ionization mode. Methanol-extracted cell lysates were injected onto a HILIC column (Luna HILIC, 2 x 100 mm, 3 µm, Phenomenex, Torrance, CA, USA) thermostated at 30 °C. All samples were randomized before analysis. The results were normalized to total protein concentration and expressed as a percent of the control value.

6.16. Gelatin zymography assay

Gelatin zymography protease assay (89) was used to determine the activities of metalloproteinases (MMP)-2 and -9 released from the cells to the medium. After treatment, 5 ml of cell culture media were collected and concentrated using Vivaspin® 2 Centrifugal Concentrator (Vivaproducts, Inc. Littleton, MA, USA). Protein concentration was measured using the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). 20 µg of protein per lane was loaded onto 10% SDS-PAGE gels containing freshly prepared 1 mg/mL gelatin (Sigma Aldrich, Saint Louis, MO, USA). After electrophoresis, the gels were agitated with gelatinase renaturation buffer and incubated with the gelatinase reaction buffer (37°C, 18 h). The MMP bands were stained with the Coomassie method and scanned.

6.17. Statistical analysis

All experiments were performed at least in triplicates and the experiments were repeated three times. Data were presented as a mean ± standard deviation (SD). For statistical calculations, a one-way analysis of variance (ANOVA) with Dunnett's correction and *t*-test was used. GraphPad Prism 5.01 (GraphPad Software, San Diego, USA) was employed for statistical analysis. Statistically significant differences were marked as *, ^, # $p < 0.05$, **, ^^, ## $p < 0.01$, ***, ^^^, ### $p < 0.001$ and ****, ^^^^, #### $p < 0.0001$.

7. Results

7.1. Experiment design of the model for experimental wound healing under the conditions of mechanical damage and IL-1β-induced inflammation

As there are numerous diseases manifested by hard-to-heal wounds or delays in a wound healing process, the main goal was to propose a new strategy for improved wound healing. Thus, I tested the hypothesis: does prolidase, as an EGFR ligand, stimulate experimental wound healing?

Since skin is frequently injured and 95% of the total cell mass in the epidermis are keratinocytes, they were chosen for further experiments. Among cell lines available on market, immortalized HaCaT keratinocyte cell line was selected based on the simplicity of cell culture which enabled non-biased result interpretation.

Prolidase treatment has been established based on literature reports and own experiments. Prolidase concentration ranging from 1 – 250 nM was subjected to test the cell viability. None of these concentrations had a toxic effect on cells and for further experiments, prolidase at the concentration of 1 – 100 nM was employed.

Recovery of an injured tissue includes cell migration, proliferation, and differentiation as well interaction with various biomolecules, and finally remodeling of matrix components. To assess experimental wound re-epithelialization scratch assay was applied, while the inflammation stage was induced by a pro-inflammatory factor, human recombinant IL-1β. Figure 4 presents the study design of the model for experimental wound healing under the conditions of mechanical damage and IL-1β-induced inflammation.

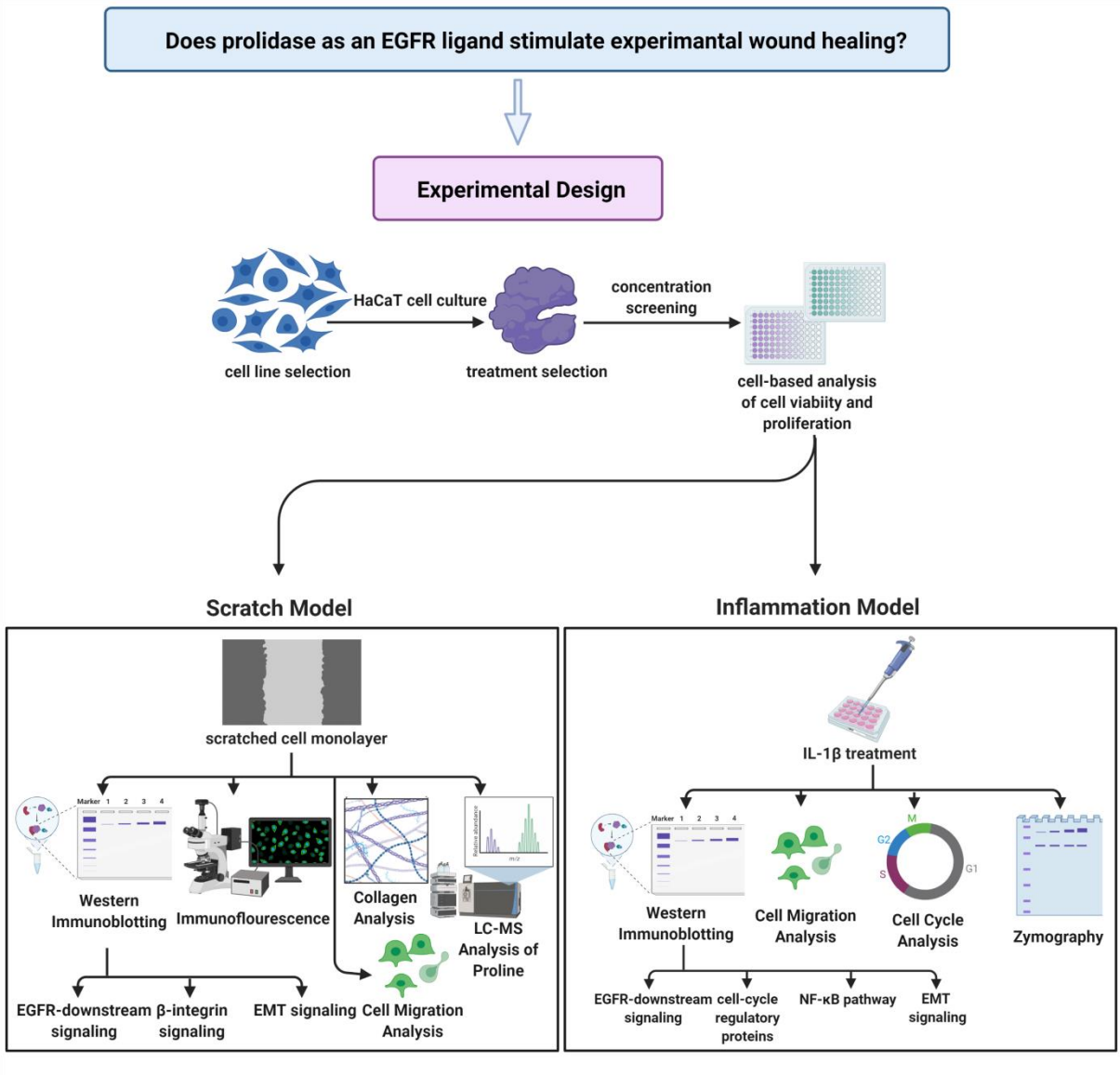

Figure 4. Experiment design of the model for an experimental wound healing under the conditions of mechanical damage and IL-1β-induced inflammation. Created with Biorender.com.

The experimental model of mechanical damage was obtained by scratching the confluent cell monolayer with a sterile 200 µl pipette tip forming a gap. For induction of inflammation, LPS and IL-1β were tested in the range of 0.1-10 µg/ml and 0.5-100 ng/ml, respectively, for 12, 24, and 48 h as a pretreatment, posttreatment and concomitant treatment. Based on the results from cell viability and IL-6 production, for further study, the experimental conditions of inflammation in HaCaT cells were mimicked by an addition to the cell culture medium IL-1β (10 ng/ml) as concomitant treatment with prolidase.

The effects of extracellular prolidase were evaluated on cell proliferation, vitality and migration, PEPD activity, collagen biosynthesis, intracellular proline concentration, and

expression of the selected proteins related to EGFR, IGFR, β-integrin, and protein markers of epithelial-to-mesenchymal transition (EMT) under the conditions of mechanical damage in HaCaT cells. For confirmation of prolidase-dependent induction of EGFR-downstream signaling and experimental wound healing, HaCaT cells were pretreated with an EGFR inhibitor, gefitinib (2 μM, 2 h). In turn, a selective PI3K inhibitor, LY294002 (50 μM, 2 h) was applied to prove the role of the PI3K/Akt/mTOR axis in PEPD-mediated cell proliferation and experimental wound healing. The concentration of the inhibitors was selected based on literature reports.

The effects of extracellular prolidase were investigated on cell viability, proliferation, cell cycle, metalloproteinase activity, and expression of the selected proteins related to EGFR, NF-κB, cell-cycle regulatory proteins, and protein markers of EMT under the conditions of IL-1β-induced inflammation in HaCaT cells. To confirm the prolidase-mediated induction of EGFR-downstream signaling in IL-1β-induced inflammation, HaCaT cells were subjected to pretreatment with gefitinib (2 μM, 2 h). Under inflammatory conditions, the evaluation of the effects of prolidase mutants on cell proliferation, cell cycle, and expression of the selected proteins related to EGFR was performed.

7.2. The evaluation of the effect of extracellular prolidase on cell proliferation, vitality, and migration, PEPD activity, collagen biosynthesis, intracellular proline concentration, and expression of the selected proteins related to EGFR, IGFR, β_1-Integrin, and epithelial-to-mesenchymal transition under the conditions of mechanical damage in HaCaT cells

After scratching the cell monolayer, it was found that the proliferation of keratinocytes was noticeably increased in a time-dependent manner (Figure 5A). The investigation of the migratory activity of HaCaT cells demonstrated that prolidase contributed to progressive wound closure in a concentration- and time-dependent manner. Prolidase treatment of the cells for 48 h resulted in almost complete closure of the wound, whereas the wounded area of control cells (without prolidase) remained unchanged (Figure 5B). The effect of prolidase on cell vitality and intracellular prolidase activity was not changed in HaCaT cells in both controls and scratched cell models.

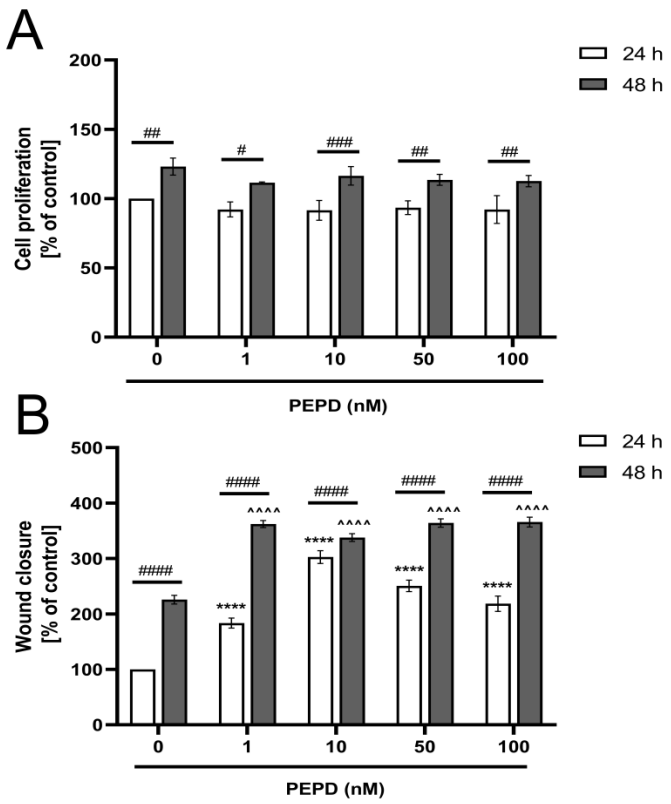

Figure 5. The effect of prolidase on (A) HaCaT cell proliferation; (B) HaCaT cell migration after 24 and 48 h treatment. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group. Statistical significances are expressed as *, ^, # $p < 0.05$, **, ^^, ## $p < 0.01$, ***, ^^^, ### $p < 0.001$ and ****, ^^^^, #### $p < 0.0001$.

As shown in Figure 6A, in prolidase-treated cells the expressions of the total forms of EGFR, PI3K (p85), Akt, and mTOR proteins were increased in a concentration-dependent manner. Prolidase-treated HaCaT cells augmented phosphorylation of EGFR (Tyr1068) and downstream proteins including PI3K p85 (Tyr458)/p55 (Tyr199), Akt (Ser473), and mTOR (Ser2448). EGFR activation was accompanied by the upregulation of the β_1-integrin receptor and IGF-1R expressions and an increase in the downstream protein expression of FAK (total and phosphorylated), Grb2, and Sos1 (Figure 6B). The increase in EGFR and FAK protein expression was confirmed using immunocytochemical staining and visualized with confocal microscopy (Figures 6C, D).

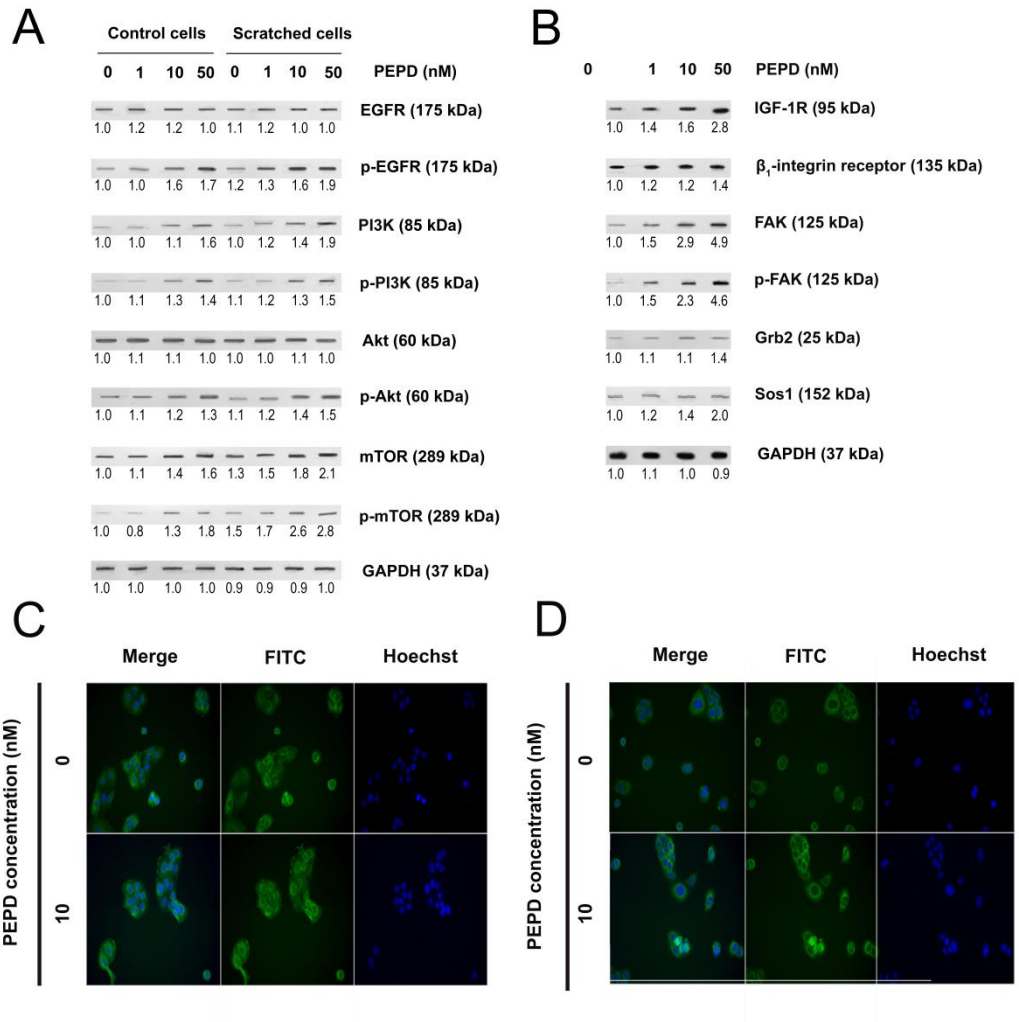

Figure 6. The effect of prolidase on the expression of (A) EGFR-downstream signaling; (B) β_1-integrin and IGF-1R-dependent signaling; (C) EGFR; (D) FAK in HaCaT cells. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group.

A specific EGFR inhibitor, gefitinib, abolished PEPD-related EGFR and Akt phosphorylation and downregulated expressions of the total EGFR and Akt forms (Figure 7A). Gefitinib-pretreated and then stimulated with prolidase HaCaT cells lost the ability to migrate (Figure 7B).

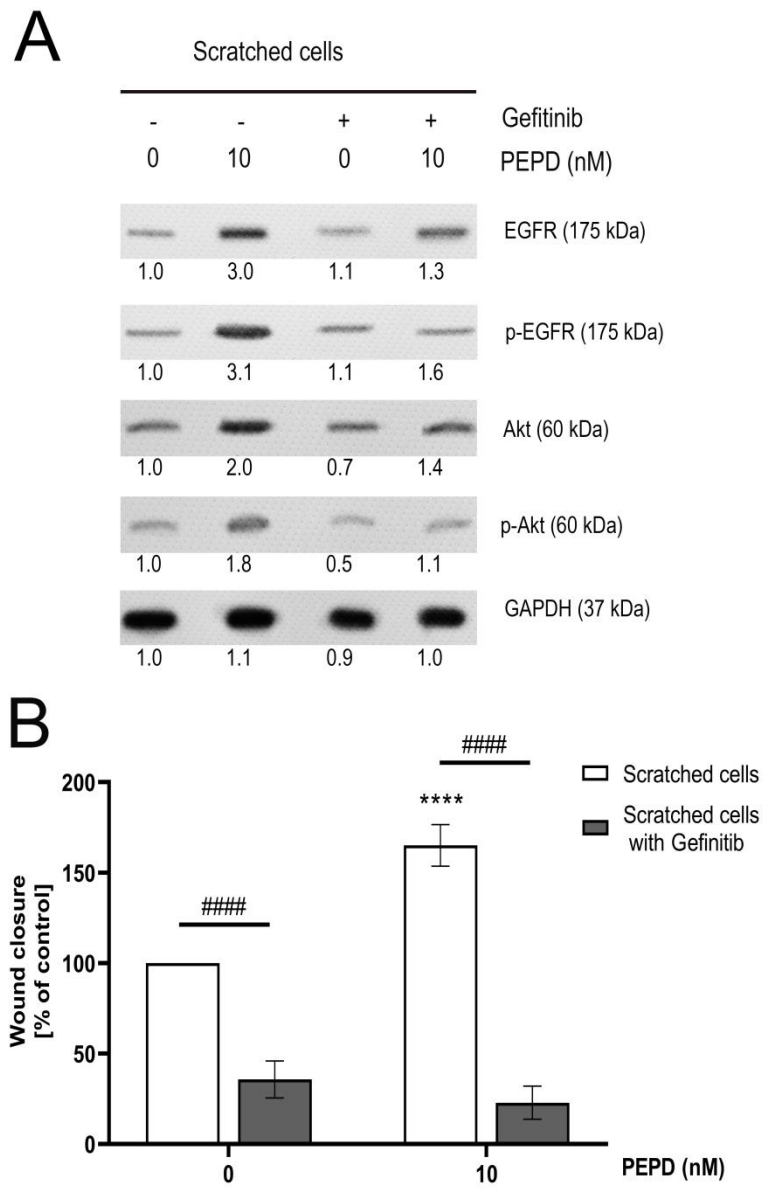

Figure 7. The effect of prolidase in gefitinib-pretreated HaCaT cells on (A) EGFR activation; (B) EGFR-mediated cell migration. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group. Statistical significances are expressed as *, ^,$^{\#}$ $p < 0.05$, **, ^^, $^{\#\#}$ $p < 0.01$, ***, ^^^, $^{\#\#\#}$ $p < 0.001$ and ****, ^^^^, $^{\#\#\#\#}$ $p < 0.0001$.

In PEPD-dependent EGFR activation PI3K/Akt/mTOR was involved. HaCaT cells were pretreated with the specific PI3K inhibitor, LY294002 which downregulated the expression of prolidase-induced signaling proteins such as PI3K, Akt, and mTOR (Figure 8A). The functional effect of the PI3K/Akt/mTOR block was tested in the context of cell proliferation and migration. In prolidase-treated keratinocytes, cell proliferation (Figure 8B) and migration (Figure 8C) were slowed down when treated with the inhibitor.

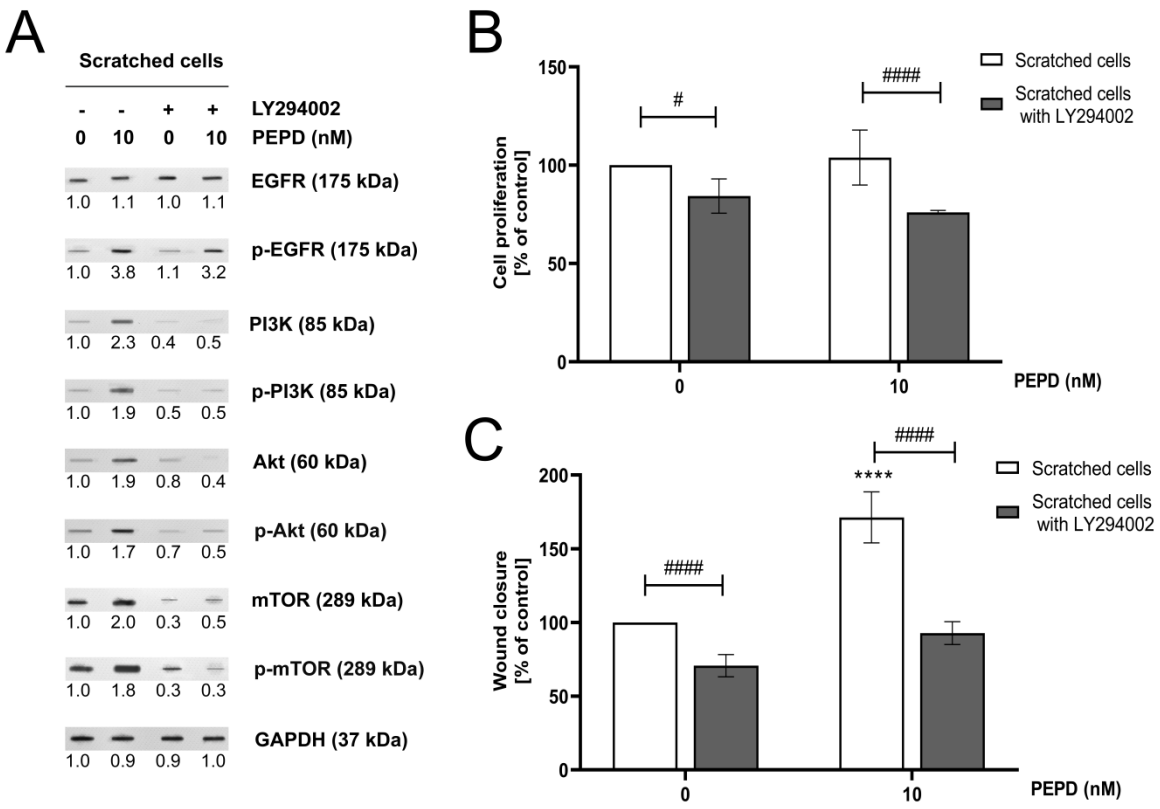

Figure 8. The effect of prolidase in LY294002-pretreated HaCaT cells on (A) EGFR activation; (B) HaCaT cell proliferation; (C) HaCaT cell migration. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group. Statistical significances are expressed as *, ^,$^{\#}$ $p < 0.05$, **, ^^, $^{\#\#}$ $p < 0.01$, ***, ^^^, $^{\#\#\#}$ $p < 0.001$ and ****, ^^^^, $^{\#\#\#\#}$ $p < 0.0001$.

As shown in Figure 9A, prolidase stimulated collagen biosynthesis in a concentration-dependent manner in normal cells. In turn, in wounded HaCaT cells after 24 h incubation with prolidase, the rate of collagen biosynthesis was 2-fold higher than in normal keratinocytes. Prolidase inhibited the expression of NF-κB, a known collagen inhibitor, suggesting the mechanism for prolidase-dependent collagen biosynthesis (Figure 9B). The LC–MS-based analysis showed an increase in proline concentration in a dose-dependent manner both in prolidase-treated normal and scratched cells (Figure 9C).

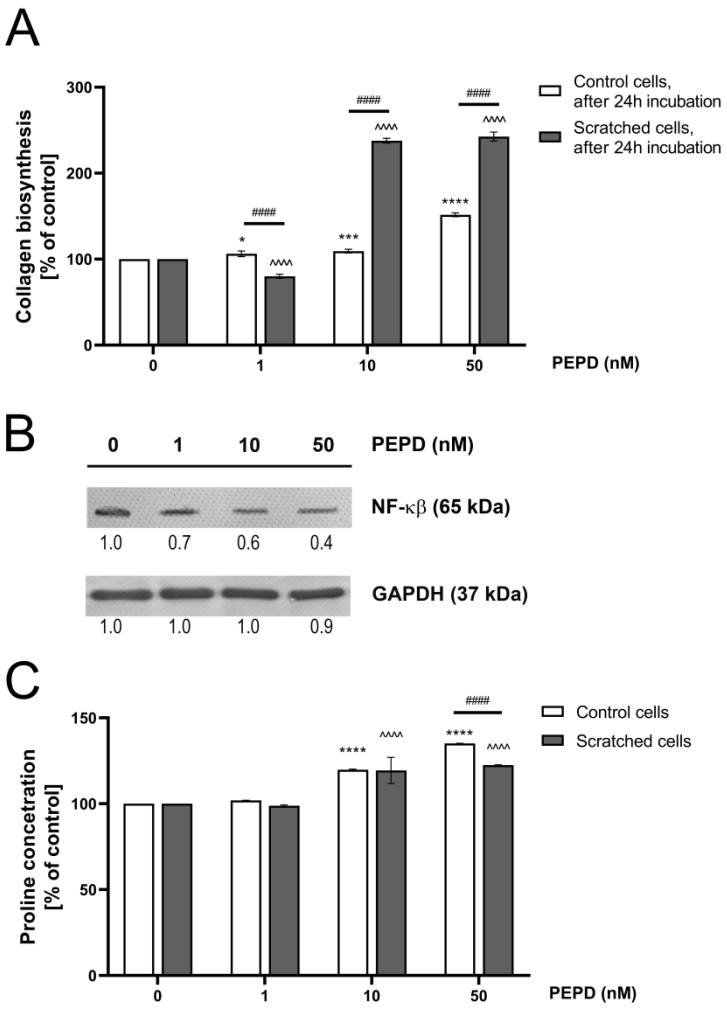

Figure 9. The effect of prolidase on (A) collagen biosynthesis after 24 h treatment; (B) NF-κB expression; (C) proline concentration in scratched and normal HaCaT cells. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group. Statistical significances are expressed as *, ^,$^{\#}$ $p < 0.05$, **, ^^, $^{\#\#}$ $p < 0.01$, ***, ^^^, $^{\#\#\#}$ $p < 0.001$ and ****, ^^^^, $^{\#\#\#\#}$ $p < 0.0001$.

In the scratching experiment, prolidase enhanced the migratory capacity of keratinocytes. The levels of expression of key epithelial-to-mesenchymal (EMT) protein markers such as E-cadherin and N-cadherin were slightly downregulated and upregulated, respectively (Figure 10). The levels of expression of TGF-β_1R and ERK1/2 were also upregulated, compared to control cells indicating that EMT occurred through the non-canonical pathway.

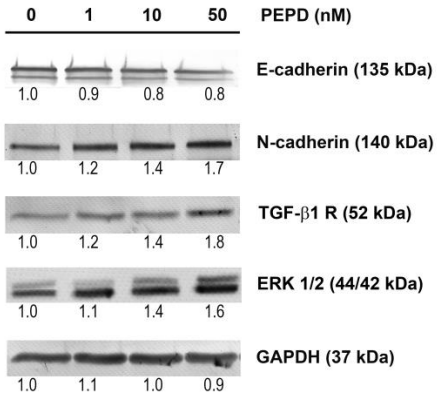

Figure 10. The effect of prolidase on EMT protein markers in the model of mechanically damaged HaCaT cells. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group.

The results of the conducted studies were published in the original paper:

P2. **Misiura M**., Baszanowska W., Ościłowska I., Pałka J., Miltyk W.: P*rolidase Stimulates Proliferation and Migration through Activation of the PI3K/Akt/mTOR Signaling Pathway in Human Keratinocytes*. International Journal of Molecular Sciences. 2020; 21, 9243. doi: 10.3390/ijms21239243. IF: 5.923, MEiN: 140 points

7.3. The evaluation of the effect of extracellular prolidase on cell proliferation and viability, cell cycle, metalloproteinase activity, and expression of the selected proteins related to EGFR, NF-κB, cell-cycle, and epithelial-to-mesenchymal transition under the conditions of IL-1β-induced inflammation in HaCaT cells

Under normal conditions, human recombinant wild-type prolidase (rhPEPD^{WT}) did not affect HaCaT cell proliferation. However, in the presence of IL-1β, rhPEPD^{WT} increased the proliferation of the studied cells in a concentration-dependent manner (Figure 11A). In IL-1β- and rhPEPD^{WT}-treated keratinocytes, a significant decrease in the percentage of cells in the G_1 phase (growth) and an increase in the percentage of cells in the G_2/M phase (mitosis) were observed in comparison to control cells (Figures 11B, C). Protein expression analysis showed upregulation of the expression of cyclin D, thymidine kinase 1, and PCNA in rhPEPD^{WT} - and IL-1β-stimulated HaCaT cells (Figure 11D).

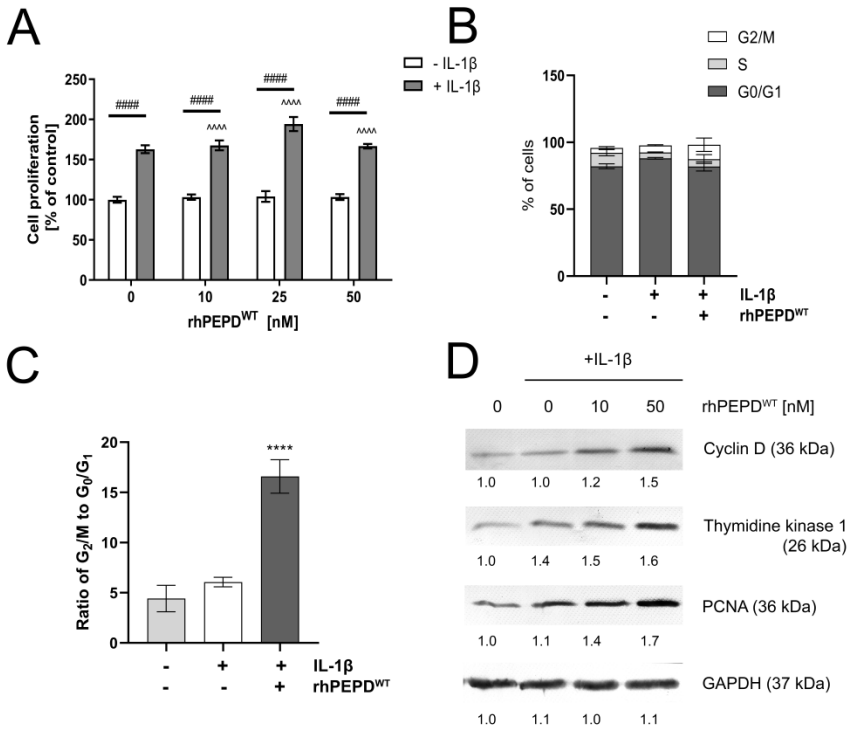

Figure 11. The effect of rhPEPD^{WT} on (A) IL-1β-induced HaCaT cell proliferation: (B); cell percentage in G_0/G_1, S, and G_2/M phases (C) cell cycle presented as the ratio of G_2/M to G_0/G_1 phase; (D) expression of the selected cell-cycle regulatory proteins. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group. Statistical significances are expressed as *, ^,$^{\#}$ $p < 0.05$, **, ^^, $^{\#\#}$ $p < 0.01$, ***, ^^^, $^{\#\#\#}$ $p < 0.001$ and ****, ^^^^, $^{\#\#\#\#}$ $p < 0.0001$.

It was found that rhPEPD^{WT} in the presence of IL-1β activated EGFR-downstream signaling kinases (Figure 12A). Interestingly, rhPEPD^{WT} induced the expression of both total and phosphorylated forms. Phosphorylation of EGFR (Tyr1068) occurred after treatment with rhPEPD^{WT} in a concentration-dependent manner. The phosphorylation of p-Akt (Ser473) was stimulated in prolidase-treated HaCaT cells in comparison to control non-treated cells. Similarly, the activation of ERK1/2 (Thr202/Tyr204) and STAT3 (Tyr705) was more pronounced in the cells cultured in the presence of prolidase and IL-1β than in non-treated cells. These results were confirmed by a blockade of EGFR with the specific inhibitor, gefitinib, which effectively suppressed rhPEPD^{WT}-dependent phosphorylation of EGFR, Akt, STAT3, and ERK1/2 (Figure 12B).

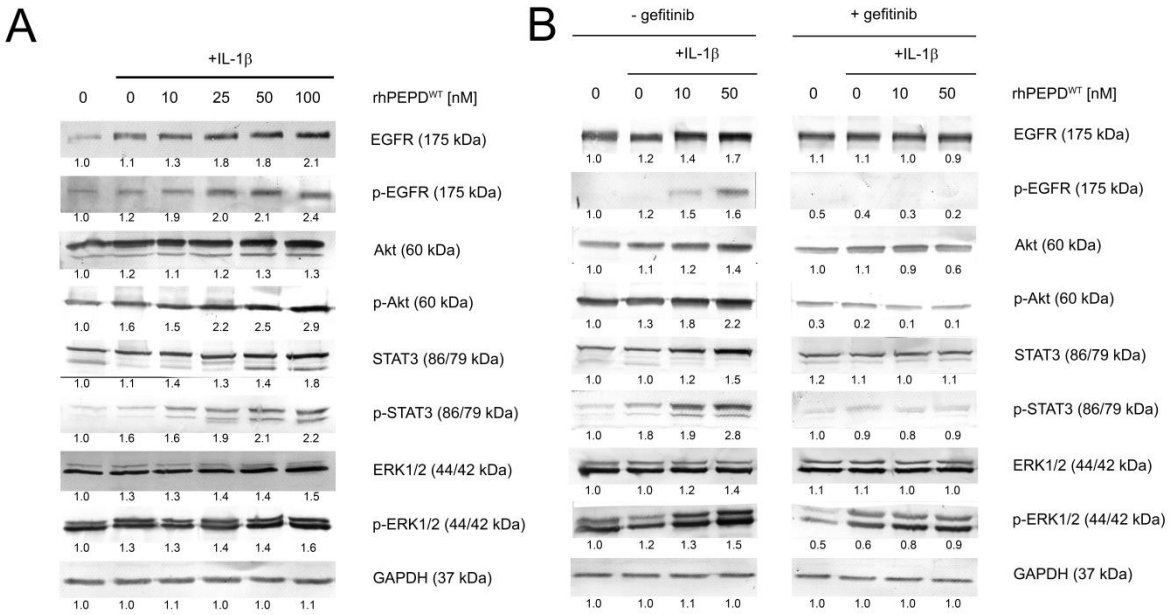

Figure. 12. The effect of rhPEPD^{WT} on (A) EGFR-downstream signaling pathway in HaCaT cells; (B) EGFR-downstream signaling pathway in HaCaT cells pretreated with gefitinib. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group.

Proliferating HaCaT cells entailed the ability to migrate via EMT phenotype. In PEPD-treated cells E-cadherin was downregulated while HIF-1α, TGF-β_1R, Cox-2, and N-cadherin were upregulated in the response of IL-1β, suggesting a rhPEPD^{WT}-dependent mechanism for increased cell motility undergoing via TGF-β_1R and Cox-2 pathway (Figure 13A). The cell migration assay supported the statement on cell motility which was improved in the presence of IL-1β-treatment and interestingly rhPEPD^{WT} augmented HaCaT cell migration (Figure 13B).

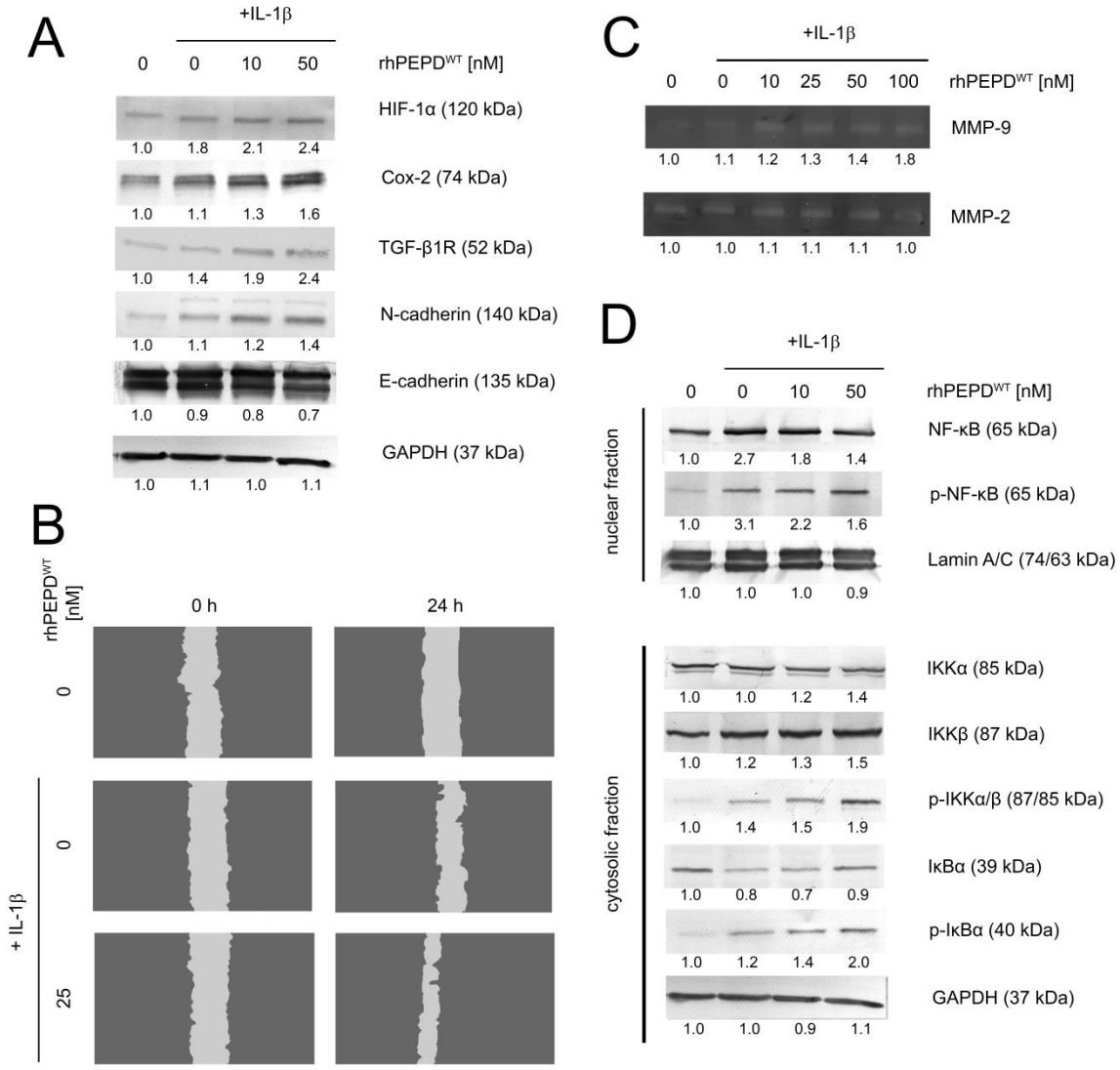

Figure 13. The effect of rhPEPD^{WT} on (A) EMT protein markers; (B) cell migration; (C) MMP-2 and -9 activities; (D) NF-κB pathway in the model of IL-1β-induced inflammation in HaCaT cells. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group.

EMT was accompanied by matrix remodeling since rhPEPD^{WT} in the presence of IL-1β induced MMP-9 activity in a concentration-dependent manner, while MMP-2 remained unchanged in both stimulated and non-stimulated cells (Figure 13C). It is likely that, in the induction of MMP-9 activity, NF-κB was involved. Upon rhPEPD^{WT} stimulation occurred phosphorylation of IκB kinases, IKKα and β, at Ser176/180. Then, NF-κB was activated by degradation of IκBα releasing the p65 subunit from the IκBα/NF-κB complex. An increase in the level of p-IκBα and p-IKKα/β was accompanied by cytosolic IκBα degradation (Figure 13D).

The results of the conducted studies were published in the original paper:

P3. **Nizioł M.**, Ościłowska I., Baszanowska W., Pałka J., Besio R., Forlino A., Miltyk W.: *Recombinant Prolidase Activates EGFR-Dependent Cell Growth in an Experimental Model of Inflammation in HaCaT Keratinocytes. Implication for Wound Healing.* Frontiers in Molecular Biosciences. 2022; 9:876348. doi: 10.3389/fmolb.2022.876348. IF: 5.246, MEiN: 140 points

7.4 The evaluation of the effect of prolidase mutants (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) on cell proliferation, cell cycle, and expression of the selected proteins related to EGFR under the conditions of IL-1β-induced inflammation in HaCaT cells

Variant rhPEPD-G448R did not induce the proliferation of HaCaT cells both in the presence and absence of IL-1β (Figure 14A). However, rhPEPD-E412K and rhPEPD-231delY mutants in the presence of IL-1β significantly increased the cell proliferation, while in the absence of IL-1β only rhPEPD-E412K affected the process (Figures 14B, C). The cells cultured with wild-type PEPD in the presence of IL-1β showed a drastic increase in the ratio, namely significantly decreased the percentage of cells in the G_0/G_1 phase and increased the percentage of cells in the G_2/M phase, compared to control cells cultured in the absence of IL-1β (Figure 14D). However, treatment of the cells with PEPD mutants in the presence of IL-1β decreased significantly the ratio of G_2/M to G_1/G_0 with a more pronounced effect in the case of rhPEPD-G448R.

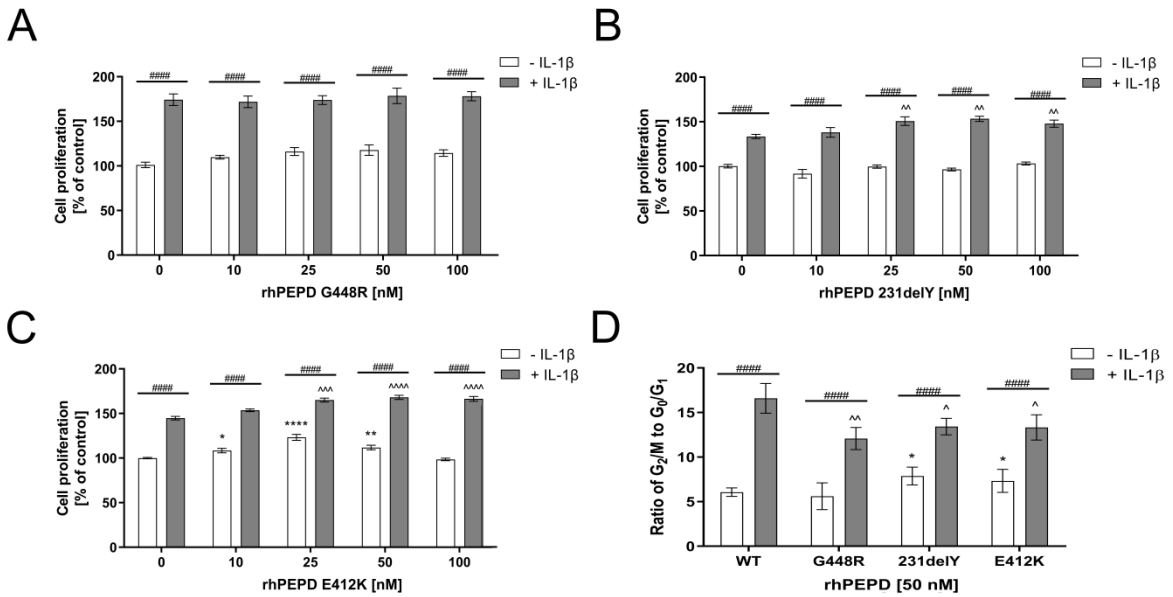

Figure 14. The effect of mutated variants of PEPD on cell proliferation (A) rhPEPD-G448R; (B) rhPEPD-231delY; (C) rhPEPD-E412K; (D) cell cycle presented as the ratio of the cell percentage in G_2/M to G_0/G_1 phase in HaCaT cells in the presence or absence of IL-1β. Statistical significances are expressed as *, ^, # $p < 0.05$, **, ^^, ## $p < 0.01$, ***, ^^^, ### $p < 0.001$ and ****, ^^^^, #### $p < 0.0001$.

As shown in Figure 15, all studied PEPD mutants in the presence of IL-1β induced phosphorylation of EGFR and some downstream signaling proteins (Akt, ERK1/2, STAT3) as detected by Western immunoblot. rhPEPD-G448R as a ligand of EGFR was able to stimulate downstream signaling proteins, however, cell cycle analysis showed that the response was weaker compared to other PEPD mutants (rhPEPD-231delY and rhPEPD-E412K).

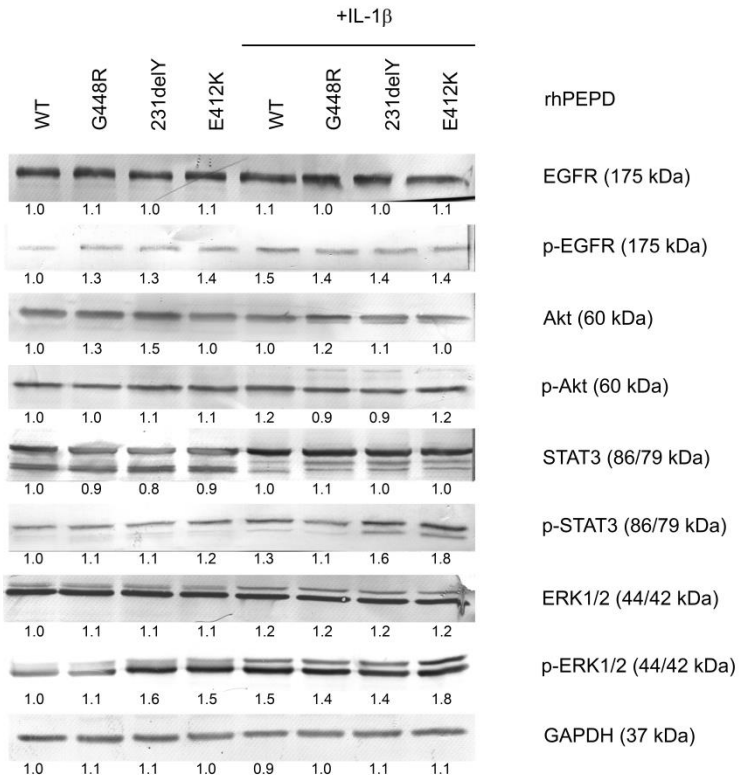

Figure 15. The effect of rhPEPD^{WT} and PEPD mutants (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) on EGFR-downstream signaling proteins in IL-1β-treated HaCaT cells. The data are presented as the mean ± SD, n = 3 in each group and compared to the control group.

The results of the conducted studies were published in the original paper:

P3. **Nizioł M.**, Ościłowska I., Baszanowska W., Pałka J., Besio R., Forlino A., Miltyk W.: *Recombinant Prolidase Activates EGFR-Dependent Cell Growth in an Experimental Model of Inflammation in HaCaT Keratinocytes. Implication for Wound Healing.* Frontiers in Molecular Biosciences. 2022; 9:876348. doi: 10.3389/fmolb.2022.876348. IF: 5.246, MEiN: 140 points

8. Discussion

Impaired wound healing is observed in numerous conditions such as acute and chronic diseases, aging, or post-surgery (90), thus understanding the complex regulatory mechanism of the repair process and searching for new boosters for tissue regeneration are of great relevance. Since prolidase is the EGFR ligand (29) and EGFR signaling is involved in the regulation of cell growth, proliferation, and migration (91), I hypothesized that PEPD can be considered a stimulating factor for experimental wound healing. The series of research were conducted aiming to evaluate the effect of extracellular prolidase and its mutant forms on repair processes in experimental models of inflammation induced by interleukin-1β (IL-1β) and mechanical damage to skin keratinocytes. In the first step of the research, I conducted a literature review to gather the latest knowledge of the PEPD functions and designed two *in vitro* models to study the effects of extracellular PEPD on IL-1β-induced inflammation and scratched keratinocytes.

The review included in this dissertation covers the latest knowledge of the molecular mechanisms and functions of prolidase in the regulation of key biomolecules in cellular metabolism and focuses on the contribution of PEPD-mediated signaling in physiological and pathological conditions. Based on experimental evidence the paper describes in detail several mechanisms regulating PEPD activity, including the activation of the β_1-integrin receptor, IGF-1 receptor, and TGF-β_1 receptor (51, 66). Regulation of proline availability in the mitochondrial proline cycle via PEPD seems to be a limiting factor for collagen resynthesis or intracellular signal transduction. However, its biological activity as a cellular regular is of emerging research interest. So far, it is known that prolidase serves as a regulator of p53 function (34, 35), affects interferon-α/β receptor maturation (36), and is a ligand of EGFR (29) and epidermal growth factor receptor 2 (HER2) (30). These previously unknown findings regarding the novel function of PEPD emphasize the importance of the protein at the level of cell functionality and at the same time stress how the currently available knowledge is limited in terms of the complex protein signaling system involved in the regulation of cell metabolism.

One of the study aims was to evaluate the effect of extracellular prolidase, as an EGFR ligand, on HaCaT cell proliferation and migration in the experimental model of wound healing to test the re-epithelialization capacity of keratinocytes. Own study revealed that prolidase in a concentration- and time-dependent manner induced proliferation and migration of "wounded" cells, where the effects were more pronounced. PEPD significantly improved

cell migration to close the scratch area in a model of wound healing *in vitro*. Under normal conditions, during re-epithelialization, keratinocytes are attracted to the injury site to restore tissue continuity (92). Here, it was found that PEPD promoted the migration of HaCaT cells via EMT since the changes in expression of key EMT protein markers such as upregulation of N-cadherin and downregulation of E-cadherin were observed. The amount of TGF-β_1R and ERK1/2 were also increased which suggests the EMT process in HaCaT cells facilitates wound healing. Further study showed that when prolidase was present in the culture medium activation of EGFR downstream signaling through PI3K/Akt/mTOR axis occurred. To prove that prolidase binds to EGFR on the surface of keratinocytes an EGFR inhibitor, gefitinib was employed. Inhibition of PEPD-mediated EGFR signaling by gefitinib resulted in a significant decrease in active and total EGFR and Akt. It was demonstrated that prolidase-mediated wound closure rate was also abolished in the presence of gefitinib supporting the observation that PEPD-driven activation of EGFR is a crucial event in HaCaT cell migration. Subsequently, to establish whether PI3K/Akt/mTOR is functionally linked to the proliferation and migration of HaCaT cells, an inhibitor of PI3K, LY294002 was applied for testing whether this axis was involved. Blockade of this signaling pathway resulted in noticeable inhibition of the amount of phospho- and total forms of EGFR-downstream proteins. Functionally, decreased cell proliferation and migration were observed after pretreatment with the inhibitor.

The study suggested that cooperation between EGFR and adhesion receptors (e.g. β_1-integrin) is required to reach complete biological responses in tissue regeneration. Moreover, prolidase was found to activate the expressions of the β_1-integrin receptor and insulin-like growth factor 1 receptor (IGF-1R). The cross-talk of these receptors is of great importance in wound healing since the role of both receptors in anabolic processes is well established (49). Researchers indicated their essential role in collagen biosynthesis. Both, the β_1-integrin and IGF-1 receptors transmit signals that induce collagen biosynthesis (45, 93). This process is of critical importance in the last step of wound healing and scar formation (49). The study demonstrated that prolidase-stimulated HaCaT cells enhanced collagen biosynthesis as a result of upregulation of β_1-integrin and IGF-1R receptors inducing autophosphorylation of FAK. Then, the signal is transmitted to Grb2, through Src and Shc proteins and then Sos, Ras, and Raf proteins are induced to finally reach ERK1/2 kinases (94, 95). Moreover, we demonstrated that NF-κB expression was decreased under prolidase treatment in keratinocytes. Since NF-κB is known as an inhibitor of the expression of α1 and α2 subunits

of type I collagen (96-98), the results suggest that a drop in NF-κB expression facilitated collagen biosynthesis. Moreover, proline content increased in the cells treated with prolidase, providing a substrate for the synthesis of collagen (99). The functional significance of prolidase is reflected in the delivery of proline from imidodipeptides (mostly collagen-derived degradation products) for collagen resynthesis and other proteins containing proline. Since the intracellular activity of prolidase did not change in prolidase-treated cells, it is likely that in the experimental conditions proline came from the enzymatic reduction of pyrroline-5-carboxylic acid derived from glutamate or ornithine (62).

As I observed PEPD-augmented keratinocyte proliferation and migration upon scratch, I raised the question of whether mediators of inflammation, released from mechanically damaged cells, contribute to the significant promotion of cell proliferation, growth, and migration. Thus, further study was conducted to investigate the biological effects of human recombinant PEPD (rhPEPD) on keratinocytes in an *in vitro* model of IL-1β-induced inflammation. I found that simultaneous application of PEPD and IL-1β contributed to the significant increase in keratinocyte proliferation, a process that could be of great importance in wound healing. Indeed, PEPD significantly augmented this process through EGFR signaling. The key finding of my study was that PEPD activates EGFR-downstream signaling proteins including Akt, ERK1/2, and STAT3, which are implicated in keratinocyte migration, proliferation, and epithelialization during the inflammatory phase of the wound healing process. Inhibition of PEPD-dependent EGFR activation by gefitinib led to a decrease in the amount of phosphorylated and total forms of EGFR and Akt, ERK1/2, and STAT3 confirming that PEPD is a ligand of this receptor. The mechanism underlying the molecular basis for PEPD-mediated cell cycle progression in keratinocytes undergoes upregulating of several cell-cycle regulating proteins including cyclin D, thymidine kinase 1, and PCNA. It was found that during stimulation of EGFR in the presence of IL-1β the EMT occurred, as detected by changes in the expression of EMT markers such as downregulation of E-cadherin and upregulation of N-cadherin, To support the changes in EMT protein markers, cell migration was analyzed. The results revealed that rhPEPD^{WT} and IL-1β contributed to an increase in cell motility. Moreover, HIF-1α, Cox-2, and TGF-β_1R were significantly pronounced upon rhPEPD^{WT} treatment. The phenomenon was accompanied by an increase in the activity of MMP-9. Experimental evidence indicated that total NF-κB expression was remarkably increased due to IL-1β treatment and gradually decreased under PEPD treatment of keratinocytes, however, NF-κB phosphorylation was remarkably high, similarly to

phosphorylated forms of IκB kinases (IKKα/β) and IκBα. Since NF-κB signaling modulates MMP-9 activation (100), the results suggest that in rhPEPD^{WT}-mediated MMP-9 activation, the NF-κB pathway may be involved. Taken together, the study suggests that EGFR and IL-1β signaling synergistically promote keratinocyte proliferation and migration through cell cycle progression and the EMT event. Additionally, the activated MMPs support the migratory phenotype of cells by digestion of the surrounding ECM.

On the whole, the observations from the conducted research point to raise the question of why under conditions of scratch and IL-1β stimuli the effects of prolidase are more pronounced than in normal cells where cell proliferation and migration remain mostly unchanged upon prolidase supplementation. Does IL-1β stimulate the release of PEPD from HaCaT cells? It is well established that keratinocytes are the source and target for cytokines. A vast range of inflammatory mediators is expressed and secreted by keratinocytes that have multiple consequences not only for inflammatory cells through the promotion of leukocytes migration, and amplification of inflammatory responses but also on keratinocytes to promote their proliferation and differentiation processes (101). Thus, under experimental scratch conditions, while the cell membrane is disrupted, it is probable that a variety of inflammatory mediators are released from keratinocytes. The interplay between prolidase and secreted mediators of inflammation contributes to the induction of cell proliferation, growth, and migration. The research on the effect of prolidase in IL-1β-induced inflammation supports the hypothesis that prolidase in the presence of IL-1β strengthens the proliferative and migratory capacity of keratinocytes. The molecular mechanism underlying PEPD-induced cell proliferation and growth undergoes through EGFR signaling, cell cycle progression, EMT, as well as matrix remodeling. It cannot be excluded that IL-1β stimulates the release of PEPD from keratinocytes, however, it needs further experiments supporting this hypothesis. Based on my own experience, I found that prolidase is expressed by keratinocytes, although PEPD activity is low. To date, the system for prolidase transport outside the cell remains unknown unless the cell membrane is discontinued. Under chemically-induced cell disruption PEPD concentration significantly increases (29). Another possible source of prolidase can be platelets in the bloodstream (49, 50). Platelets are essential players in the initial stage of inflammation as they carry various inflammatory mediators. Upon activation and degranulation of platelets, growth factors and prolidase-containing load is released close to the wounded area. It is known that PRP is used in regenerative medicine facilitating the recovery from tissue injuries (102-105).

Since wild-type PEPD was shown to induce EGFR signaling and accelerate cell proliferation in IL-1β-induced HaCaT cells, it has been considered whether some mutated PEPD (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) would evoke an opposite effect on the process. All these variants occur were identified in patients with prolidase deficiency (PD, OMIM 170100) (85). PD is a rare autosomal recessive disorder characterized by massive imidodipeptiduria, skin lesions, and elevated proline-containing dipeptides in plasma (6). Currently, it is believed that several mutated alleles in the *PEPD* gene (6) are responsible for PD. However, no clues about the molecular mechanisms are so far available since clinical phenotype does not always respond to genotype (106). Currently, PD is diagnosed by low or a lack of PEPD activity, however, the clinical outcome may be related to deprivation of extracellular function of PEPD. This hypothesis would be confirmed since PD therapy was unsuccessful with the application of proline or proline-convertible amino acids (3). As PD remains incurable and the mechanism unknown, we sought to explore the effect of the selected mutated variants of PEPD on EGFR-downstream proteins under IL-1β-induced inflammation. Interestingly, EGFR-downstream protein analysis showed that some mutated variants of PEPD (rhPEPD-231delY and rhPEPD-E412K) were able to activate EGFR-dependent intracellular signal and induce HaCaT cell proliferation stronger than another mutated variant (rhPEPD-G448R). The intensity of the intracellular responses and cell proliferation rate were weaker upon rhPEPD mutant treatment compared to wild-type rhPEPD. The observed differences seem to match the clinical outcomes as PD patients manifest a wide range of symptoms (85). My study suggests that not only PEPD activity but an extracellular function of PEPD may be involved in the mechanism underlying prolidase deficiency.

The results of my research revealed for the first time that PEPD activates EGFR-dependent cell growth and proliferation in experimental models of inflammation and mechanical damage in HaCaT keratinocytes. This indicates that PEPD as the EGFR ligand may play a role as a stimulating factor in wound healing. Both enzymatically active and inactive rhPEPD may modulate with different intensities of EGFR signaling. The knowledge may be useful for further approaches to therapy of wound healing disorders. As the study demonstrated promising effects of prolidase in cell proliferation, migration, and collagen biosynthesis, further investigations are necessary to explore its role in PD. It would be beneficial to confirm these results in an *in vivo* model as the *in vitro* model has some limitations.

9. Conclusion

1. Prolidase as an EGFR ligand stimulates keratinocytes to proliferation and migration via Akt/PI3K/mTOR pathway under the conditions of experimental wound healing.
2. Enzymatically active and inactive prolidase may modulate EGFR signaling with different intensities.
3. Extracellular prolidase acting through EGFR induces growth, migration, collagen biosynthesis, and ECM remodeling in cultured keratinocytes.
4. Prolidase augments the proliferation and migration of keratinocytes under inflammatory conditions.
5. Endogenous prolidase may represent a therapeutic agent for treating skin wounds.

10. Articles Included in the Dissertation

P1. **Misiura M.,** Miltyk W.: *Current Understanding of the Emerging Role of Prolidase in Cellular Metabolism.*

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Review

Current Understanding of the Emerging Role of Prolidase in Cellular Metabolism

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Abstract: Prolidase [EC 3.4.13.9], known as PEPD, cleaves di- and tripeptides containing carboxyl-terminal proline or hydroxyproline. For decades, prolidase has been thoroughly investigated, and several mechanisms regulating its activity are known, including the activation of the β_1-integrin receptor, insulin-like growth factor 1 receptor (IGF-1) receptor, and transforming growth factor (TGF)-β_1 receptor. This process may result in increased availability of proline in the mitochondrial proline cycle, thus making proline serve as a substrate for the resynthesis of collagen, an intracellular signaling molecule. However, as a ligand, PEPD can bind directly to the epidermal growth factor receptor (EGFR, epidermal growth factor receptor 2 (HER2)) and regulate cellular metabolism. Recent reports have indicated that PEPD protects p53 from uncontrolled p53 subcellular activation and its translocation between cellular compartments. PEPD also participates in the maturation of the interferon α/β receptor by regulating its expression. In addition to the biological effects, prolidase demonstrates clinical significance reflected in the disease known as prolidase deficiency. It is also known that prolidase activity is affected in collagen metabolism disorders, metabolic, and oncological conditions. In this article, we review the latest knowledge about prolidase and highlight its biological function, and thus provide an in-depth understanding of prolidase as a dipeptidase and protein regulating the function of key biomolecules in cellular metabolism.

Keywords: prolidase; PEPD; EGFR; cellular metabolism

1. Introduction

Proline has a unique pyrrolidine ring that protects the polypeptide structure from hydrolysis. The presence of proline in numerous biomolecules (e.g., neuroactive peptides or growth factors) prevents unexpected proteolysis in order to maintain their biological activity [1]. However, there are factors responsible for the degradation of peptides containing proline at the C-terminus. One of the enzymes involved in this process is prolidase [EC 3.4.13.9], which catalyzes the hydrolysis of X-Pro or X-Hyp to proline or hydroxyproline and X amino acid [2]. Prolidase (PEPD) belongs to the group of dipeptidases and cleaves di- and tripeptide containing carboxyl-terminal proline or hydroxyproline. The most specific substrate for this enzyme activity is glycyl-proline (Gly-Pro) [2]. Its enzymatic properties link to the disease known as prolidase deficiency (PD), which is manifested by massive imidodipeptiduria, hard-to-heal wounds, mental retardation, and impaired immune system. To date, no effective PD treatment has been developed [3]. Moreover, there are reports indicating the clinical relevance of prolidase in collagen metabolism malfunctions [4–13], metabolic [14–20], and oncological disorders [21–25]. In addition to its catalytic activity, prolidase regulates numerous biological processes. At the cellular level, PEPD acts as a regulator of epidermal growth factor receptor (EGFR) and epidermal growth factor receptor 2 (HER2)-dependent signaling pathways [26–30], p53 activity [31], and expression of the interferon α/β receptor [32].

The purpose of this review is to present the latest knowledge about prolidase as well as its biological significance at the cellular level in the aspect of its catalytic-dependent and -independent biological activity. The enzymatic-dependent function of prolidase concerning its clinical importance in PD, collagen turnover, metabolic conditions, and cancers is discussed. We focus on in-depth understanding of the biological properties of prolidase as a dipeptidase and a molecule regulating the function of key biofactors in the cellular metabolism.

2. Regulatory Functions of Prolidase

In the 1950s, Adams et al. [33] published the first report on prolidase. For the next decades, researchers focused solely on the enzymatic function of this enzyme. Most publications concern prolidase deficiency—a genetic disease resulted from a decrease in or lack of PEPD activity. Disturbed prolidase activity has been reported in various pathological conditions associated with collagen metabolism and tumors. A breakthrough in the research on the biological role of prolidase was the study conducted by Yang et al. [26], presenting a new unknown function of PEPD as an epidermal growth factor receptor ligand. Since then, knowledge about the biological role of PEPD catalytic-independent activity has been expanded. Apart from the said finding, the role of prolidase in regulating p53 and interferon α/β receptor has also been discovered. The paragraphs below in this section describe novel functions of PEPD as a cellular regulator. Figure 1 presents biological activity of prolidase as an enzyme as well as a regulatory protein in cellular metabolism.

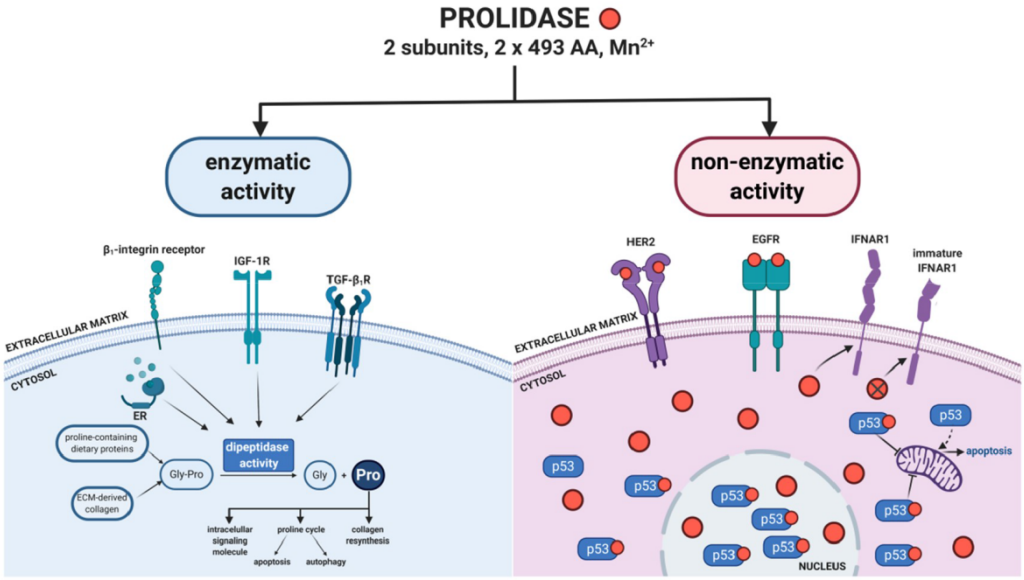

Figure 1. Enzymatic and non-enzymatic prolidase activity. Prolidase exhibits dual mechanism of biological activity. As an enzyme, prolidase provides proline for collagen resynthesis. The amino acid acts as a signaling molecule and a mediator in mitochondrial proline cycle. Extracellularly, prolidase binds directly to EGFR and HER2, while intracellularly it regulates the function of p53 and IFNAR1. Red dots indicate prolidase. ER—estrogen receptor, IGF-1R—insulin-like growth factor 1 receptor, HER2—epidermal growth factor receptor 2, EGFR—epidermal growth factor receptor, IFNAR1—interferon α/β receptor, Pro—proline, Gly—glycine, Gly-Pro—glycyl-proline, TGF-β_1R—transforming growth factor β_1 receptor. Created with BioRender.com.

Enzyme-independent biological activity of PEPD includes its role in regulating the functions of other molecules. Recent scientific reports have expanded the knowledge of prolidase and its interactions with biomolecules at the cellular level. Researchers have demonstrated the role of prolidase as an EGFR

and HER2 ligand regulating signaling pathways dependent on these receptors. PEPD also regulates the function of p53 and plasma serine proteases as well as expression of interferon α/β receptor.

2.1. Prolidase as an Epidermal Growth Factor Receptor (ErbB1/EGFR) Ligand

Yang et al. shed new light on the function of prolidase by publishing several papers [26–30] in which the authors demonstrated that PEPD is a ligand of receptors belonging to the family of epidermal growth factor receptors (ErbB1/EGFR and ErbB2/HER2). They showed that the affinity of prolidase to these receptors is lower than EGF, but the effects of EGFR-dependent signal induction last longer. The activation of these signaling pathways does not require any enzymatic activity of prolidase, which suggests the new role of PEPD in cellular metabolism. Structurally, EGFR is a transmembrane receptor comprised of: the intracellular region at the carboxyl terminus, exhibiting protein kinase activity and the extracellular region that binds to a ligand. In addition to PEPD, several EGFR ligands have been identified, e.g., heparin-binding EGF-like growth factor (HB-EGF), transforming growth factor (TGF), amphiregulin, epiregulin, and neuregulin [34]. However, prolidase, as a homodimeric molecule, differs structurally from the group of EGFR ligands. PEPD does not share the characteristic EGF motif with other ligands ($CX_7CX_{4–5}CX_{10–13}CXCX_8GXRC$—X represents an amino acid) and its cytoplasmic location differs from that of typical EGFR ligands [35]. Still, it is not known which domain or region of PEPD binds to the extracellular domain of EGFR. Binding of prolidase to the EGFR extracellular domain causes its dimerization. As a result, the intracellular domain with protein kinase activity conducts a signal to downstream proteins in the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), Ras/Raf/extracellular signal-regulated kinase (ERK), and Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathways. Activated proteins stimulate transcription of genes associated with cell growth, differentiation, and proliferation [36].

The first Yang's discovery [26] proved that prolidase binds directly to EGFR and activates the receptor in a dose-dependent manner. Comparing the affinity of EGF and PEPD to the EGFR extracellular domain, EGF is a more potent ligand than prolidase. In the study of the aforementioned author, the dissociation constant (K_d) was around 15nM for EGF, while K_d for PEPD established at 5.3 μM, which indicated that EGF is an about 350 times stronger ligand for EGFR compared to PEPD. Further studies confirmed the affinity of PEPD to EGFR with K_d equaling 17.7 nM [29]. It is probable that these discrepancies in K_d resulted from differences in the experimental model. In the first research [26], the model used for the study was the EGFR-Fc immunoglobulin G (IgG) 1 chimera, while the second model [29] to assess K_d of PEPD-EGFR binding represented the full-length EGF receptor. It is known that the extracellular fragment of EGFR comprises four domains. Prolidase binds to domain 2 as opposed to EGF, which binds to domains 1 and 3 of EGFR. Prolidase is associated with EGFR only on the cell surface as a homodimer, eventually forming a tetrameter (EGFR dimer + PEPD dimer) [29]. An EGF, as a stronger EGFR ligand, displaces PEPD from its bond with EGFR. Confocal images demonstrated that PEPD and EGFR colocalize the cell membrane, which supports the hypothesis that PEPD binds to EGFR in the form of a ligand-receptor relationship. At low concentration, prolidase (2.7 nM) can activate EGFR by phosphorylation of tyrosine at positions 1068 and 1173 followed by EGFR-downstream protein induction (Akt, STAT3, and ERK1/2). Figure 2A demonstrates PEPD-dependent EGFR-downstream signaling pathways. This finding shows that PEPD stimulates three different downstream signaling pathways of EGFR. The effects of equal EGF and PEPD concentrations on the aforementioned pathways are similar. EGF activates EGFR-dependent signaling faster than PEPD, while prolidase-dependent stimulation lasts longer. The most likely explanation for this phenomenon is that EGFR is internalized and degraded more slowly under PEPD treatment. Applying EGFR inhibitor resulted in a nearly complete blockade of ERK1/2 phosphorylation upon PEPD treatment. EGFR-downstream signaling pathways lead to increased DNA synthesis in a dose-dependent manner upon prolidase stimulation. These findings appear promising for the improvement of regenerative therapy, aiming to promote cell proliferation and growth. Through

EGFR-dependent stimulation, PEPD may be a beneficial factor in the treatment of diseases manifested by ulceration or chronic inflammation [37].

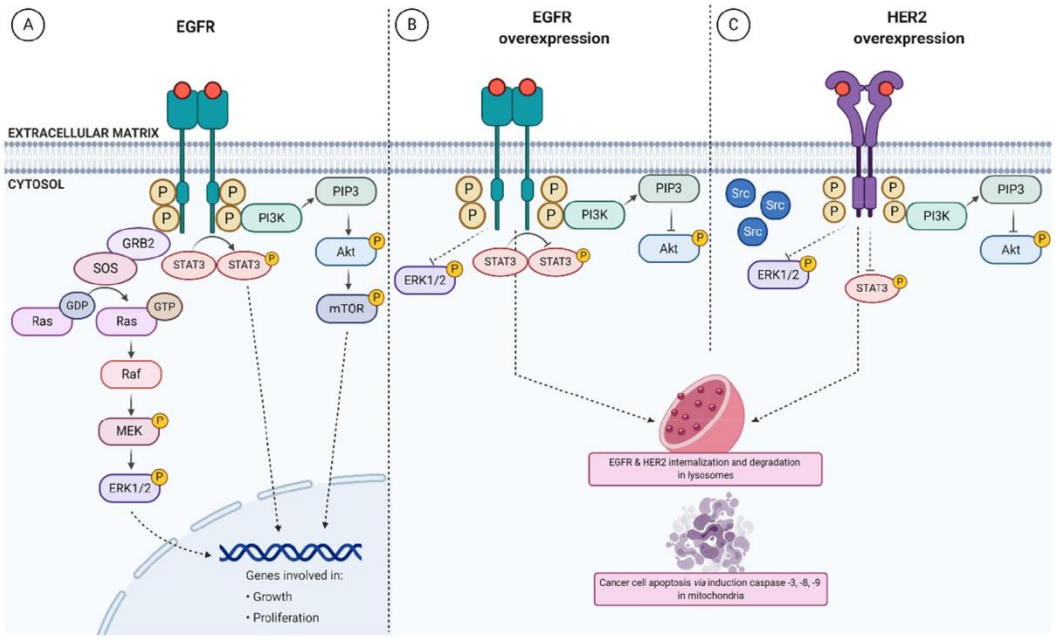

Figure 2. Prolidase-dependent EGFR- and HER2-downstream signaling. Prolidase binds to EGFR and HER2, evoking intracellular responses. (**A**) Under physiological conditions, direct binding of PEPD to EGF receptor results in the induction of pro-growth and pro-proliferation pathways such as phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), extracellular signal-regulated kinase (ERK)1/2, and signal transducer and activator of transcription 3 (STAT3). (**B**) Under overexpression of EGFR, prolidase silences Akt, ERK1/2, and STAT3 pathways followed by internalization and degradation of the receptor. (**C**) PEPD affects upregulated HER2 via dissociation of HER2-Src complex, inhibition of Akt, ERK1/2 and STAT3 pathways, and induction of apoptosis. Red dots indicate prolidase and circled 'P' presents phosphorylation event. HER2—epidermal growth factor receptor 2, EGFR—epidermal growth factor receptor. Created with BioRender.com.

Yang et al. [26] discovered that the prolidase enzymatic activity is not required to activate the EGF receptor and its downstream signaling proteins. The effect of active and enzymatically inactive forms of prolidase confirms this statement. The phosphorylation level of EGFR and downstream kinases is comparable between wild-type PEPD and its enzymatically inactive mutant ($PEPD^{G278D}$). However, intracellular PEPD does not activate EGFR—the molecule has to be present in the extracellular space. Therefore, a question arises of the source of prolidase stimulating this receptor. The most likely sources of PEPD are damaged cells that release cellular content, including cytosolic prolidase. The in vivo experiments showed significantly increased PEPD concentration in the bloodstream after chemical damage to liver cells. PEPD concentration reached 3 nM compared to the control group in which it did not exceed 1 nM. It is known that 2.7 nM of PEPD is sufficient to stimulate EGFR [26]. Under pathological conditions, PEPD level is higher than that concentration, which leads to another question: does prolidase undergo extracellular degradation preventing it from unexpected EGFR stimulation? The recent paper has revealed that serine proteases can inactivate prolidase in plasma via intrinsic and extrinsic cascades of coagulation. The study has demonstrated that factor XII initiates PEPD proteolysis by activating factor X and factor II, which stimulate factor VII. Activated factor VII (FVIIa) degrades prolidase *in vivo*. FVIIa exhibits trypsin-like serine protease activity by cleaving

peptides containing C-terminal arginine or lysine. The authors showed that FVIIa inactivated prolidase. However, the mechanism of PEPD proteolysis remains unexplained [38].

The study reporting PEPD-dependent stimulation of EGFR-downstream signaling pathways contributed to further studies on PEPD-related effects under pathological conditions. EGF receptor overexpression is observed in numerous cancers, including breast, lung, colon cancer, and squamous cell carcinoma [39]; therefore, an attempt was made to assess the effect of prolidase on cell metabolism in conditions of EGFR overexpression. The authors discovered that the enzymatically inactive $PEPD^{G278D}$ silences EGFR-downstream signaling pathways, inhibiting tumor cell proliferation and growth in vivo. Tumor cells in EGFR overexpression were more prone to prolidase, which resulted in inhibition of EGFR phosphorylation and downstream proteins such as Akt and ERK1/2, as well as STAT3 signaling [29]. Figure 2B presents PEPD-related inhibitory effect on overexpressed EGF receptor and EGFR-dependent pathways. The mechanism of EGFR activation by PEPD has not yet been thoroughly studied. It is unknown whether the PEPD-EGFR bond affects cell metabolism in an autocrine or paracrine manner. PEPD is likely to act as an autocrine factor on cells, but there is no evidence for its paracrine effect. Another unclear matter that requires explanation is: which PEPD domain binds to EGFR. The mechanism of prolidase deficiency (PD) is also noteworthy. So far, the most probable cause for PD is diminished enzymatic activity of prolidase. However, considering the newly discovered function of PEPD, a probable PD mechanism might be the underlying excessive release of PEPD from damaged cells and its interactions with other biomolecules including EGFR. It is possible that prolidase concentration in the bloodstream is not reduced in patients with PD. However, there are no papers supporting this hypothesis, and further research explaining the mechanism of PD is crucial. Until now, PD remains incurable, and understanding the causes of the disease may contribute to the development of an effective therapeutic strategy.

PEPD binding to EGFR may be a potential target in treatment of cancers with EGFR overexpression. Since the enzymatically inactive prolidase ($PEPD^{G278D}$) inhibits pro-proliferative signals, it offers a new promising strategy for oncological therapy.

2.2. Prolidase as an ErbB2/HER2 Ligand

Yang et al. [28] found that PEPD also binds to HER2 which, unlike EGFR, is comprised of four domains in the extracellular region. PEPD binds to domain 3, thus contributing to the dimerization of this receptor. It has been demonstrated that PEPD does not bind to the HER2 transmembrane domain or intracellular region. The affinity of prolidase for this receptor was estimated at K_d = 7.3 nM at which prolidase specifically binds to the receptor. HER2 phosphorylation is induced by PEPD, gradually saturating the receptor and leading to its dimerization. HER2, similarly to EGFR, is activated independently of the enzymatic function of prolidase. By using enzymatically inactive prolidase, it has been proven that stimulation of this receptor remains unchanged. Furthermore, intracellular prolidase does not affect HER2-downstream pathways. Overexpressed PEPD entails a significant elevation of its concentration, so PEPD appears to be secreted from the cells. According to the presented results, PEPD concentration in the medium did not exceed 0.3 nM; hence, it could not affect the function of the receptor. No mechanism has yet been found to explain how the enzyme is released from normal cells. PEPD silences HER2-downstream signals under HER2 overexpression, which is another similarity with EGF receptor. The mechanism underlying the inhibition of HER2-downstream signaling under PEPD treatment is based on disruption of HER2-Src association. As a result, DNA synthesis and cell proliferation, invasion, and migration are strongly hindered. This finding indicates a new therapeutic strategy in the treatment of HER-positive carcinomas. However, some doubts occur because HER2 function depends on other receptors from this family. For example, ErbB3 can stimulate HER2 dimerization [27]. To explain this phenomenon, the effect of prolidase on tumor progression with coexisting HER2 overexpression was assessed [28]. Intraperitoneal administration of PEPD at a dose of 0.2 mg/kg has been shown to inhibit tumor growth in vivo only with the concomitant overexpression of HER2. The authors proposed the use of a combination of prolidase and enoxaparin (EP) as a therapeutic

option. EP (as low-molecular-weight heparin) increases plasma prolidase levels by inhibiting PEPD proteolysis [38]. EP itself does not inhibit tumor growth; however, it lowers the dose of prolidase while its plasma concentration is sufficient. As a result, prolidase suppresses HER2-dependent intracellular signals by inhibiting the phosphorylation of Src, Akt, STAT3, and ERK1/2. The effect of PEPD-HER2 interaction is internalization and subsequent HER2 degradation as presented in Figure 2C. Although PEPD does not bind to ErbB3, prolidase prevents this receptor from phosphorylation by inhibiting Akt expression. It is known that HER2 overexpression is accompanied by phosphorylated Akt, which affects ErbB3 dimerization. In addition, PEPD promotes apoptosis in tumor tissues through decreased expression of B-cell lymphoma 2 (BCL-2) and Bcl-2-associated X (BAX) as well as upregulated expression of caspase-3, -8, -9. The effect of PEPD on HER2-overexpressed signaling in tumors is the suppression of pro-proliferative signaling pathways, induction of apoptosis, and inhibition of tumor progression. In vivo model indicates no signs of toxicity, and body weight of mice remains unchanged [28]. After PEPD administration, it has been observed that the enzymatically inactive $PEPD^{G278D}$ mutant has a stronger inhibitory effect on tumor weight than its enzymatically active form. The reason for PEPD being a limiting factor for tumor mass weight remains to be explored. Based on these observations, further experiments were performed to evaluate the therapeutic effect of $PEPD^{G278D}$ on drug-resistant HER2-positive breast cancer [30]. The enzymatically inactive mutant exhibits cancer in two-step process: in the first phase, it disturbs the interaction of HER2 with other receptors in this family (EGFR, ErbB3) as well as other tyrosine kinase receptors (MET, IGF-1R). It is likely that PEPD disconnects the bond between HER2 and mucin 4 (marker present in 60% of HER2-positive breast cancers, probably related to drug resistance). $PEPD^{G278D}$ also interferes with the HER2-downstream signaling. In the second phase, the receptor undergoes slow internalization followed by degradation in lysosomes. Another beneficial anti-tumor effect of PEPD is related to increased cancer cell sensitivity to drug treatment. Moreover, it was observed that drug-resistant HER2-positive breast cancer cells are more sensitive to the enzymatically inactive $PEPD^{G278D}$ mutant, giving basis for the development of new therapeutic options for this group of cancers.

2.3. Prolidase as a p53 Activity Regulator

In recent years, a new function of prolidase in p53 function has been discovered. PEPD is a key regulator of the key tumor suppressor protein [31]. The report reveals an important role in controlling cellular functions associated with the cell cycle, DNA repair, apoptosis, and cellular metabolism. p53, as the guardian of the genome, protects cells from uncontrolled cell division that may lead to proliferation of mutated cells and promote tumor progression [40]. There are several upstream and downstream regulating mechanisms of p53 function such as its post-translational modifications mediated by mutated in ataxia telangiectasia (ATM), ATM and RAD3-related (ATR), p38 MAP kinase (MAPK), ERK1/2, Checkpoint kinase 1 (CHK1), Checkpoint kinase 2 (CHK2), interactions with murine double minute 2 (MDM2), murine double minute 4 (MDM4), wild-type p53-induced phosphatase (WIP1), p21 as well as chromatin. Apart from these, p53 dynamics itself affects its biological status [41]. The regulation of the guardian of the genome is complex and still needs in-depth investigation. Among those mechanisms, prolidase also exhibits regulatory function on p53 through (1) limiting p53 subcellular transport and (2) inhibiting p53 phosphorylation [31].

The absence of prolidase limits cell survival, as confirmed by *PEPD* gene silencing. It has been observed that p53 activation is associated with PEPD silencing. Further analysis indicates that p53 is located in the cytosol, nucleus, and mitochondria, whereas prolidase occurs only in the cytosol and nucleus. It is the very first evidence to support the assumption that prolidase can translocate to the nucleus [42]. PEPD prevents p53 from its translocation into mitochondria where apoptosis is initiated. Prolidase can also inhibit p53 transcriptional activity by inhibiting the protein phosphorylation in its transactivation domain. Under silenced PEPD conditions, p53 phosphorylation at the Ser6 and Ser15 positions is promoted. The aforementioned findings prove that prolidase regulates both transcription-dependent and -independent functions of p53 [31].

Yang et al. [31] demonstrated that PEPD regulates the activity of p53 through direct binding to this protein. They showed that PEPD catalytic domain is bound to the proline-rich domain of p53; however, PEPD motif binding to p53 is unknown. Prolidase enzymatic activity is not required for either regulating p53 function or binding to this transcription factor. About 6% of prolidase molecules have been found to bind to p53, while more than half of p53 molecules bind to PEPD, which indicated that prolidase protects p53 from uncontrolled activation. Under cellular stress, the PEPD-p53 complex dissociates, releasing and activating p53. An example of stress conditions at the cellular level is oxidative stress caused by reactive oxygen species (ROS). Restoration of redox balance prevents PEPD-p53 from dissociation as well as subsequent inhibition of cell growth and induction of apoptosis. Experimentally, oxidative balance was restored by N-acetylcysteine as a 'scavenger' of ROS. As a result, p53 activity was diminished via complexing by PEPD [31]. Since oxidative stress is generated in patients undergoing chemotherapy, PEPD may be a useful factor in combination with chemotherapeutics, although further research is required in this field. So far, opinions of experts on the administration of anti-cancer drugs accompanied by antioxidants are varied [43,44].

In summary, the report illustrates the important biological function of PEPD, independently of its catalytic activity, in regulating p53 activity. The PEPD-p53 complex has been observed in the nucleus and cytosol. The dual mechanism of p53 regulation by prolidase includes inhibition of subcellular translocation and p53 phosphorylation. Disrupted redox balance leads to dissociation of the complex and stimulation of p53 activity. Since p53 determines the cell fate, this finding may contribute to further research on the pathomechanisms of numerous diseases accompanied by oxidative stress.

2.4. Prolidase as a Regulator of Interferon α/β Receptor

Lubick et al. [32] presented a new physiological function of prolidase in which PEPD modulates the functionality of interferon α/β receptor (IFNAR1). Interferon α/β-dependent signaling is a key pathway involved in the immune response against viruses, i.e., tick-borne encephalitis virus and West Nile virus. The research results indicate that IFNAR1 expression is diminished during a flavivirus-induced infection. A common feature of the flaviviruses is the use of non-structural protein 5 (NS5) as an IFNAR1 antagonist [45,46], leading to the suppression of the immune response. The research shows that the N-terminal PEPD domain binds to NS5, decreasing interferon α/β receptor expression [32]. To clarify the role of prolidase in regulating IFNAR1 expression, the authors silenced PEPD and observed that post-translational modification of the receptor is impaired. The glycosylation is required for IFNAR1 to perform receptor functions. The conclusion is that prolidase is involved in the maturation of interferon α/β receptor. Similarly, to other non-enzymatic properties of prolidase, PEPD regulates IFNAR1 function independently of its catalytic activity [32]. To sum up, prolidase—as a cytosolic molecule—is blocked by viral protein, which leads to inhibition of IFNAR1-dependent immune response and more precisely: the role of PEPD in the immune response against flaviviruses could initiate the development of PEPD-based antiviral therapies.

3. Enzyme-Dependent Activity of Prolidase

At the cellular level, the biological processes dependent on the enzymatic activity of prolidase result from the biological activity of enzymatic reaction products: proline or hydroxyproline (Pro or Hyp, respectively). Most scientific reports focus on biological properties of proline. Enzymatic activity of PEPD is necessary for the collagen turnover as the main component of the extracellular matrix, participating in the proteolysis of di- and tripeptide derived from degradation of collagen and proline-containing proteins.

3.1. Prolidase as a Dipeptidase: General Structure, Physical Properties, and Substrate Specificity

Prolidase belongs to the family of metallopeptidases dependent on divalent cations that enable its catalytic activity. PEPD is known as X-Pro dipeptidase, proline dipeptidase, imidodipeptidase, and peptidase D [1].

From a molecular point of view, prolidase is encoded by the *PEPD* gene located on the long arm of chromosome 19 at locus 13.11. It has been observed that the gene structure has 15 exons [2]. Point mutations in this gene are responsible for the lack or reduction of the enzymatic activity, and thus causing prolidase deficiency. It is known that the *PEPD* gene has 29 point mutations that result in a reduction or complete loss of the enzymatic activity. Out of these, eight point mutations are of clinical significance [47]. Prolidase can exist in three isoforms depending on the transcriptomic variant. Isoform 1 is the product of the longest transcript, while prolidase isoform 2 is shortened by an internal segment from 184 to 224 nucleotide. Because of alternative splicing, isoform 3 is deprived of a nucleotide fragment from 68 to 131 nucleotides [48]. It is known that in eukaryotes, prolidase undergoes post-translational modifications such as glycosylation and phosphorylation. The analysis of the carbohydrate content in the prolidase structure showed that it constitutes 0.5% of the total protein mass [49]. This report presents prolidase as a glycoprotein; however, further research is needed to assess the binding sites of carbohydrate groups and evaluate whether glycosylation affects biological properties of prolidase. Indirectly, it has been demonstrated that glycosylation does not influence the catalytic activity of the enzyme [42]. Another study showed that N-glycosylation can occur at N13 and N172 sites, and O-glycosylation—at position T458 in the amino acid chain [50]. In terms of PEPD phosphorylation, there have been several reports [51,52] confirming that this post-translational modification increases PEPD enzymatic activity. The sites of phosphorylation include Ser109, Ser134, Ser198, Ser236, Thr86, Tyr117, and Tyr124. The authors showed that these amino acids are phosphorylated as a response to stimulation via NO/cGMP/MAPK pathways, which means that nitric oxide regulates prolidase activity. Ysrayl et al. provided further evidence supporting the phenomenon of prolidase phosphorylation [53]. They observed that cocaine stimulates prolidase phosphorylation and increases its enzymatic activity. They also demonstrated that phosphorylation of prolidase depends on the iNOS pathway, which inhibits the level of phosphorylated protein, which is consistent with the previous study [51].

Structurally, human prolidase is a homodimer consisting of two subunits, 493 amino acids (AA) each [54]. The molecular weight of one subunit is 58 kDa [42]. Both subunits are comprised of the N- and C-terminal domains. The carboxyl-terminal domain (185–493 AA) shares the structure with peptidases from the 'pita-bread' family (e.g., aminopeptidase P, methionine aminopeptidase, and creatinase) [55]. In this domain, there is an active center in which the Mn^{2+} ion is necessary for its enzymatic activity. The substrate (Gly-Pro) binds to the active center in the C-terminal domain, while the N-terminal domain (1–185 AA) remains less closely linked to the substrate. The disulphide bond between Cys158A and Cys158B links the monomers. Notably, this disulphide bridge is only present in the inactive enzyme-substrate complex [56]. The divalent cations: Zn^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+} can be also present as cofactors required for PEPD enzymatic activity. However, prolidase activity is decreased if one of these cations is located in the active center. Under these conditions, the activity of prolidase drops below 30% [20]. Interestingly, in *Saccharomyces cerevisiae*, Cu^{2+} and Zn^{2+} strongly inhibit prolidase activity [57]. Lupi et al. [42] compared the catalytic activity of endogenous and recombinant prolidase and discovered that optimal conditions for their maximum catalytic activity are: pH 7.8 and 37 °C or 50 °C, respectively.

Prolidase belongs to the group of hydrolases; therefore, the enzymatic reaction catalyzed by PEPD requires H_2O. Wilk et al. [54] established the mechanism of the reaction catalyzed by recombinant human prolidase. First, water binds to Mn^{2+} ions in the active center of prolidase, which leads to the formation of a hydroxyl ion. Then, Gly-Pro binds to the active center, resulting in a change of the enzyme conformation. Approaching His255, it coordinates the -COOH group in the substrate. The =O and $-NH_2$ groups in Gly-Pro are stabilized by manganese ions, providing a positive charge at the carbon atom. A nucleophilic hydroxyl ion attack is followed by a break in the peptide bond, and the products (Gly, Pro) leave the active center. First, Gly is released followed by proline, and then the enzyme returns to its original conformation. The last step involves replenishing water and restoring the enzymatic activity of prolidase.

PEPD exhibits the highest specificity for Gly-Pro in the *trans* conformation [58]. Although prolidase has the highest catalytic activity against Gly-Pro [42], it also hydrolyses other C-terminal proline-containing dipeptides such as Ala-Pro, Phe-Pro, Met-Pro, Val-Pro, and Leu-Pro [50]. Unlike many proteases, prolidase is present in the cytosol [42]. However, it is not known why prolidase occurs in that subcellular location. PEPD is abundantly expressed in enterocytes, where it is probably involved in the hydrolysis of dietary proline-containing dipeptides [59]. Regarding the tissue specificity of prolidase, the highest level of prolidase mRNA expression is observed in the kidneys, small intestine, and duodenum [60], while high prolidase activity has been reported in erythrocytes and human skin fibroblasts [50]. Guszczyn et al. [61] showed that platelet-rich plasma is an important source of prolidase. Apart from mammalian tissues, prolidase also occurs in numerous bacterial species (*Pyrococcus furiosus, Pyrococcus horikoshii, Alteromonas sp., Lactobacillus casei, Lactococcus lactis*) [62].

The key source of substrate (Gly-Pro) are proteins rich in amino acid sequences containing C-terminal proline or hydroxyproline, e.g., collagen [63], complement component C1q [64], dietary proteins [65], and many biomolecules such as substance P, plasminogen, oxytocin, vasopressin, and angiotensin [28]. Out of these, collagen is the most abundant source of Gly-Pro. Its molecule comprises three polypeptide chains in which the Gly-X-Y triplet is commonly repeated. Mostly X and Y are occupied by proline and hydroxyproline, respectively [66]. Thus, a significant role of PEPD is reflected in extracellular matrix (ECM) remodeling as collagen constitutes its structural protein [67,68]. Collagen degradation is initiated by metalloproteinases [69]. Then, the proteolysis of collagen breakdown products by cathepsins and peptidases takes place in lysosomes. However, they cannot degrade di- and tripeptides containing C-terminal proline or hydroxyproline. Prolidase acting in the cytosol releases free amino acids from dipeptides [70]. This process is impaired in prolidase deficiency, manifested by skin lesions resulting from disturbed collagen metabolism. Another symptom of PD is immunodeficiency, which is probably a consequence of impairment in the C1q complement component built from Gly-Pro repeats [64]. Prolidase also plays a role in the deactivation of C-terminal proline-containing neuropeptides, which could be related to mental disorders in patients with PD. Hui et al. [71] observed increased enzymatic activity in the brain tissue. In the central nervous system (CNS), prolidase is responsible for proline delivery. Out of symptoms of prolidase deficiency, mental retardation may occur because of a low level of proline as a neurotransmitter. However, there have been reports in which an increase of proline in CNS results in increased glutamate concentration, leading to neuronal death due to excessive stimulation of N-methyl-D-aspartate (NMDA) receptors. This mechanism could explain the relationship between increased prolidase activity and the pathogenesis of some neurological disorders [72].

3.2. Biological Significance of Prolidase as a Dipeptidase

At the cellular level, prolidase activity is regulated by several mechanisms. Stimulation of the β_1-integrin receptor by type I collagen [30], leading to autophosphorylation of FAK kinase capable of interacting with Grb2 and Src, induces prolidase activity. The signal is further transmitted through SoS, Ras, and Raf pathway to the ERK1/2 kinases. The signaling results in transcription of genes involved in cell growth regulation as well as proliferation [73]. An increase in prolidase activity was also observed while assessing the effect of thrombin as a β_1-integrin receptor agonist. The interaction of thrombin with this receptor stimulates MAP kinase pathway. In relation to intensified PEPD activity, an increase in collagen biosynthesis has been observed, which confirms that stimulation of β_1-integrin receptor regulates the availability of proline used as a substrate for collagen synthesis [74]. Prolidase activity is also regulated by an IGF-1 receptor (IGF-1R)-dependent pathway [75], which induces pathways stimulating cell growth, proliferation, and collagen biosynthesis. Thus, prolidase can directly limit collagen biosynthesis at both the transcriptional [68,76] and post-transcriptional level [77]. Prokop et al. [77] suggested that regulation of prolidase activity is the effect of the crosstalk between IGF-1R and β_1-integrin receptors through stimulation of ERK1/2 and PI3K/Akt/mTOR pathways by both receptors.

In addition to the aforementioned biological processes involving prolidase activity, the enzyme plays a role in the regulation of angiogenesis. Products of prolidase activity, i.e., proline and hydroxyproline, inhibit the degradation of the Hypoxia-inducible factor 1 alpha (HIF-1α) transcription factor via the Von Hippel–Lindau tumor suppressor (VHL)-dependent proteasome pathway. Hydroxyproline acts more potently than proline on the HIF-1α degradation. A possible mechanism explaining this phenomenon is direct or indirect inhibition of proline hydroxylation at positions 402/564 in the oxygen-dependent domain (ODD) of HIF-1α since its hydroxylation is necessary for interaction with VHL [67]. Prolidase is known to affect HIF-1α expression while estrogen receptors are activated [78]. The presence of Pro or Hyp upregulates HIF-1α expression in vitro if cells are cultured with estradiol. The authors observed increased expression of this factor even when estrogen receptors were not stimulated; however, the effect of Pro or Hyp was diminished. It is suggested that α estrogen receptor may connect estrogen-dependent pathways and prolidase activity. HIF-1α-dependent molecules include vascular endothelial growth factor (VEGF), glucose transporter 1 (Glut-1), and TGF-β. They are, respectively, involved in angiogenesis [79], glucose metabolism [80], and control of cell proliferation and differentiation [81]. The study conducted by Surażyński et al. [67] indicated that the level of VEGF and Glut-1 expression is significantly increased under PEPD overexpression. There is evidence that VEGF and Glut-1 overexpression results from the activation of HIF-1α-dependent transcription [67]. VEGF is known to have strong properties for stimulating the expression of $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrin that act as type I collagen receptors in endothelial cells [82], leading to PEPD activity stimulation [73]. Excessive products of prolidase activity stimulate HIF-1α-dependent signaling pathway, boosting the angiogenesis process controlled by VEGF. However, the level of TGF-β expression does not change under PEPD overexpression [67]. Another study indicates that TGF-β and the TGF-β_1 receptors regulate prolidase activity through proline-dependent signaling. Prolidase activity affects the expression of TGF-β_1 receptor (TGF-β_1R) by releasing proline. Prolidase inhibitors (Cbz-Pro, PEP) decrease the expression of this receptor, while, under proline treatment, the expression of TGF-β_1R is increased. The authors showed that the addition of Pro induces the phosphorylation of kinases from Akt/mTOR pathway, which is associated with cell proliferation and growth [76]. Their results suggest that proline regulates signaling pathways in the cytosol dependent on the TGF-β_1 receptor. The authors suggest that mTOR is a key element explaining the relationship between prolidase activity and the status of the PI3K/Akt/mTOR pathway. They hypothesize that this kinase acts as a metabolic sensor coordinating the signaling of growth factors, amino acid availability, and cellular energy status. Stimulation of mTOR by phosphorylation promotes pro-proliferative mode of cells, leading to increased protein synthesis with subsequent cell proliferation, growth, and migration [83]. Therefore, proline acts as an intermediary between prolidase activity and regulation of cell survival [76]. Figure 3 shows enzyme-dependent biological processes at the cellular level.

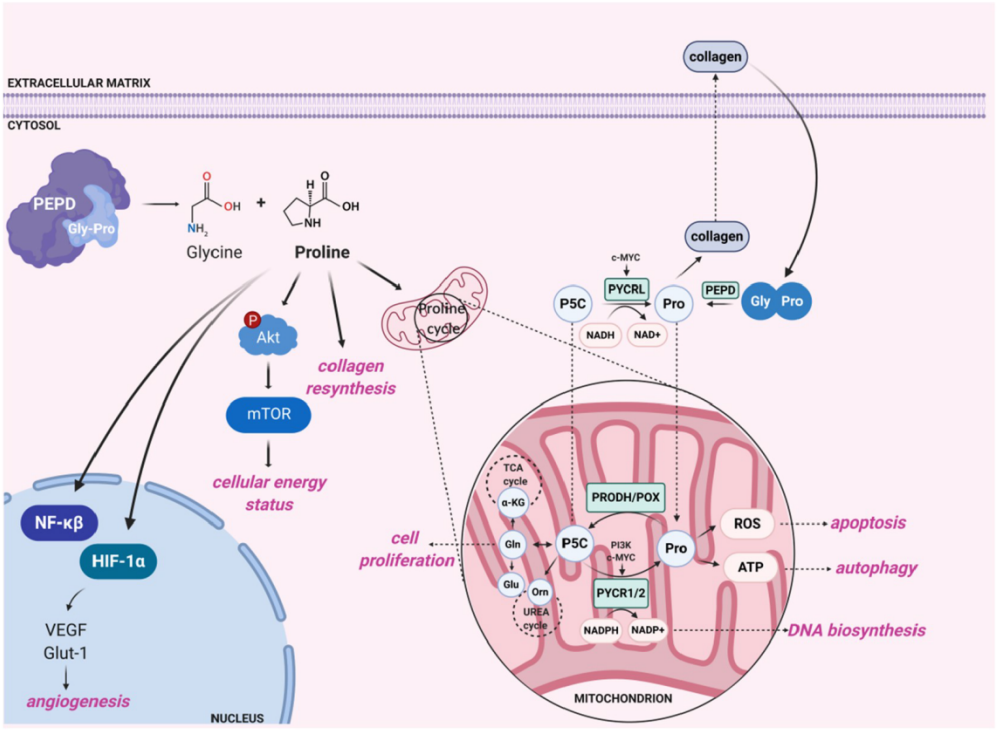

Figure 3. Enzymatic activity of prolidase affects various cellular processes. Prolidase supplies substrates for collagen resynthesis. Proline, the product of prolidase activity, modulates intracellular energetic status via Akt/mTOR pathway, inhibits HIF-1α degradation, and mediates in proline cycle, regulating mitochondrial metabolism. Circled 'P' presents phosphorylation event. PEPD—prolidase, Gly-Pro—glycyl-proline, mTOR—mammalian target of rapamycin, Akt—protein kinase B, PI3K—phosphoinositide 3-kinase, HIF-1α—hypoxia-inducible factor 1α, VEGF—vascular endothelial growth factor, Glut-1—glucose transporter 1, NF-κβ—nuclear factor κβ, α-KG—α-ketoglutarate, P5C—pyrroline-5-carboxylic acid, Pro—proline, Gly—glycine, PYCR1/2/L—pyrroline-5-carboxylic acid reductases, Orn—ornithine, Glu—glutamate, Gln—glutamine, PRODH/POX—proline dehydrogenase/proline oxidase, TCA cycle—tricarboxylic acid cycle, ROS—reactive oxygen species, ATP—adenosine triphosphate, NADPH—reduced nicotinamide adenine dinucleotide phosphate, NADP+—nicotinamide adenine dinucleotide phosphate, NADH—reduced nicotinamide adenine dinucleotide, NAD+—nicotinamide adenine dinucleotide. Created with BioRender.com.

It has also been observed that prolidase activity regulation modulates biological effects of nuclear factor κβ (NF-κβ) transcription factor [65]. Its elevated activity significantly decreases the expression of this transcription factor, which may be related to the increased level of prolidase activity products: Pro or Hyp. It has been evidenced that Pro may protect NF-κβ from activation when the transcription factor is released from the complex with IκBα [84]. Thus, it is likely that high level of Pro or Hyp may prevent IκBα from degradation. NF-κβ is known to strongly inhibit the expression of α_1 and α_2 type I collagen subunits [85].

Prolidase activity can also be regulated by non-steroidal anti-inflammatory drugs (NSAIDs). Fibroblasts treated with NSAIDs showed diminished collagen biosynthesis and prolidase activity. It is likely that NSAIDs exhibit inhibitory effect on collagen metabolism by hindering prolidase activity [86]. Similarly to NSAIDs, collagen degradation products inhibit the activity of prolidase [87].

The biological significance of prolidase is to provide free proline as building blocks for collagen resynthesis. Proline may also serve as a signaling molecule and an energy source, or mediate in maintaining redox balance [88]. In recent years, scientists have made more and more efforts to

explain the role of proline in reprogrammed energetic metabolism of cancer cells [89]. Proline may come from collagen turnover, but also enzymatic conversion of pyrroline-5-carboxylic acid (P5C), which can be either a precursor or product of proline metabolism. P5C is formed by the enzymatic conversion of glutamate or ornithine by P5C synthase (P5CS) [90] or ornithine aminotransferase (OAT), respectively [91]. P5C reduction occurs due to the activity of P5C reductase (PYCR), while the reaction in the opposite direction is catalyzed by the mitochondrial enzyme—proline dehydrogenase/proline oxidase (PRODH/POX) [92]. The proline cycle plays a crucial role in maintaining the redox balance between the cytosol and mitochondria. P5C acts as a central mediator between the tricarboxylic acid cycle, urea cycle, and proline metabolism [77]. De Ingeniis et al. [93] provided evidence of functional differences between 3 isoenzymes of PYCR (PYCR1, PYCR2, PYCRL). The enzymatic properties of PYCR1 and PYCR2 are similar, while PYCRL exhibits a distinct mode of activity. Because P5C can origin from glutamate or ornithine, these reactions are catalyzed by different P5C reductases. PYCR1 and PYCR2 provide proline from glutamate-derived P5C, while PYCRL reduces P5C from ornithine. The enzymatic conversations are accompanied by distinct cofactors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) or reduced nicotinamide adenine dinucleotide (NADH). The authors reported that PYCR1 and PYCR2 are mitochondrial enzymes, while PYCRL is present in the cytosol. Elia et al. [94] came to the opposite conclusion, stating that PYCR1 is responsible for the conversion of P5C to proline in the cytosol, which was also suggested by Phang et al. [95]. Proline biosynthesis has been shown to drive the production of the protein necessary for cell proliferation. Pro-proliferative signaling pathways c-MYC and PI3K stimulate the expression of genes associated with proline synthesis (PYCR1, PYCR2, and PYCRL). In addition, c-MYC is known to enhance the conversion of proline from glutamine by stimulating growth and proliferation of cancer cells [96]. The finding that c-MYC increases the expression of enzymes involved in proline synthesis from P5C has shed new light on the understanding of the proline cycle in cancer cells. Silencing PYCRs inhibits tumor growth by reducing the generation of NADP+ and NADPH. As a consequence, their reduced level reduces the supply of nucleotides required for DNA biosynthesis [97]. Intensified proline conversion into glutamine, glutamate, and aspartate promotes cell proliferation. Proline also impacts pentose phosphate pathway that delivers nucleotides for the synthesis of nucleic acids [97,98]. As can be seen in Figure 3, prolidase and proline are responsible for numerous cellular processes. Prolidase activity significantly contributes to proline supply that acts as an intracellular signaling molecule regulating cellular metabolism in many biochemical pathways.

3.3. Clinical Significance of Prolidase as a Dipeptidase

The clinical significance of prolidase is reflected in various cancers as well as pathological conditions associated with collagen turnover [4–13]. For instance, increased prolidase activity has been observed in melanoma [21], breast cancer [22], lung cancer [23], ovary cancer [24], and endometrial cancer [25]. Over the last few years, the structure of PEPD has been studied due to availability of high-throughput techniques. They enabled the detection of several genetic variants of *PEPD* that may be associated with the development of metabolic diseases. No information is available on the pathogenicity of these variants. However, in the literature, there are papers referring to prolidase activity in type 2 diabetes (T2D) and various of its complications (neuropathy, nephropathy, microalbuminuria, diabetic foot). For instance, the genetic variant of *PEPD* (rs3786897) has been identified in the Japanese population to be associated with increased risk of T2D [14]. The same genetic variant was detected in the Chinese population [90]—n-3 fatty acids interacting with this genetic variant modulate the risk of type 2 diabetes. The analysis of a single nucleotide polymorphism (SNP) identified another potentially pathogenic genetic variant. The rs731839 prolidase variant has been associated with affected adiponectin level responsible for insulin resistance and T2D [15]. Another study linked the same genetic variant of prolidase to lipid metabolism [16]. Wu et al. found prolidase variant (rs889140) related to adiponectin metabolism [17], which affects the tissue sensitivity to insulin. All the said genetic variants have not been studied in the context of their pathogenicity. However, there are papers

reporting alterations in prolidase activity in metabolic diseases. Patients with T2D accompanied by nephropathic complications show increased plasma prolidase activity (PPA) compared to healthy volunteers [18]. Increased PPA has also been detected in patients with advanced T2D and foot ulcers [19] or co-occurrence of microalbuminuria [20], while in patients with T2D, neuropathy [6] and osteoporosis PPA was reduced [99]. The clinical significance of prolidase activity in these conditions remains unexplained and requires further studies. It is likely that increased activity of prolidase in diabetes and its complications is associated with enhanced collagen breakdown [100]. In contrast, reduced prolidase activity in bone metabolism may result from reduced bone resorption in diabetic patients. In the CNS, the relationship between prolidase and diabetic neuropathy remains unknown. There were attempts to use prolidase activity as an anti-cancer therapy approach. Mittal et al. [21,101,102] synthesized a dipeptide containing C-terminal proline. Since prolidase activity is increased in cancers, the prodrug is released in cancer cells. The results from both in vitro and in vivo experiments are satisfying as they demonstrate inhibited cancer progression. There is available data presenting promising effects of chlorambucil [103] and nitrosoureas [104] linked to L-proline in the MCF-7 breast cancer cell model. However, it is necessary to confirm these results in in vivo models. In summary, numerous literature reports confirm the significant role of prolidase in the clinical aspects associated with both collagen metabolism disorders as well as metabolic and oncological conditions. Further studies need to be conducted to explain the mechanism of prolidase activity in these disorders as T2D or cancers affect more and more people worldwide.

4. Concluding Remarks and Future Perspectives

Prolidase is involved in numerous biological processes at the cellular level. PEPD as an enzyme is regulated by signaling pathways dependent on the β_1-integrin receptor, IGF-1 receptor, and TGF-β_1 receptor. The catalytic function of PEPD enables the provision of proline or hydroxyproline, which modulate intracellular signaling in the PI3K/Akt/mTOR and ERK1/2 pathways as well as energetic processes. As a result, the cell switches to pro-survival mode promoting DNA synthesis and cell proliferation. In addition, prolidase also has biological properties independent of its enzymatic activity. It plays a regulatory role in the function of other biological molecules. Prolidase is an EGFR and HER2 ligand regulating signaling pathways dependent on these receptors, such as PI3K/Akt/mTOR, ERK1/2, and JAK/STAT3. Under physiological conditions, prolidase stimulates these pathways and may serve as an interface in the regeneration processes under inflammation or tissue damage. Under the overexpression of EGFR and HER2 receptors commonly observed in cancers, the ligand contributes to internalization and lysosomal degradation of these receptors. In the PEPD-p53 complex, prolidase prevents p53 from activation. In plasma, prolidase activates coagulation factors by maintaining its concentration at a normal level. PEPD also participates in immune response by stimulating the expression and maturation of the interferon α/β receptor. Taken together, prolidase could act as a "friend" or "foe" – anti-tumor and pro-tumor enzyme. Promoting EGFR and HER2 degradation seems to be a promising factor in cancer cells, however, prolidase supplying proline stabilizes HIF-1α and, thus, promotes cellular survival in the hypoxic conditions. Similarly, prolidase-PRODH/POX axis could be a crucial mechanism for switching cancer cell mode between ATP (survival) or ROS (apoptosis) generation. Generated ROS can cause DNA damage leading to p53 activation, however, prolidase can protect this transcriptional factor. In the context of cancer milieu, prolidase seems to play an emerging role and its biological activity may be the starting point for further research. Prolidase-based new therapeutic approaches in numerous diseases, including prolidase deficiency, cancers, metabolic disorders, or viral infections may be developed.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

Akt	Protein kinase B
Ala-Pro	Alanyl-proline
ATM	Mutated in ataxia telangiectasia
ATR	ATM and RAD3-related
BAX	Bcl-2-associated X
BCL-2	B-cell lymphoma 2
cGMP	Cyclic guanosine monophosphate
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
EGF	Epidermal growth factor
EP	Enoxaparin
ErbB1/EGFR	Epidermal growth factor receptor
ErbB2/HER2	Epidermal growth factor receptor 2
ErbB3	Epidermal growth factor receptor 3
ECM	Extracellular matrix
FAK	Focal adhesion kinase
Glut-1	Focal adhesion kinase
Gly	Glycine
Gly-Pro	Glycyl-proline
Grb2	Growth factor receptor-bound protein 2
HB-EGF	Heparin-binding EGF-like growth factor
HIF-1α	Hypoxia-inducible factor 1 alpha
Hyp	Hydroxyproline
IFNAR1	Interferon alpha/beta receptor 1
IGF-1R	Insulin-like growth factor 1 receptor
iNOS	Inducible nitric oxide synthase
IκBα	Nuclear factor kappa alpha
JAK	Janus kinase
K_d	Dissociation constant
Leu-Pro	Leucyl-proline
MAPK/ERK	MAP kinase/Extracellular signal-regulated kinase
MDM2	Murine double minute 2
MDM4	Murine double minute 4
Met-Pro	Methionyl-proline
mTOR	Mammalian target of rapamycin
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NADP+	Nicotinamide adenine dinucleotide phosphate
NADH	Reduced nicotinamide adenine dinucleotide
NAD+	Nicotinamide adenine dinucleotide
NF-κβ	Nuclear factor kappa beta
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NS5	Non-structural protein 5
NSAID	Nonsteroidal anti-inflammatory drug
OATT	Ornithine aminotransferase
P5C	Pyrroline-5-carboxylic acid
P5CS	Pyrroline-5-carboxylic acid synthase
PD	Prolidase deficiency
PEPD	Prolidase

Phe-Pro	Phenylalanyl-proline
PI3K	Phosphoinositide 3-kinase
PPA	Plasma prolidase activity
Pro	Proline
PRODH/POX	Proline dehydrogenase/proline oxidase
PYCR1/2/L	Pyrroline-5-carboxylic acid reductase 1/2/L
ROS	Reactive oxygen species
SNP	Single nucleotide polymorphism
Src	Proto-oncogene tyrosine-protein kinase
STAT3	Signal transducer and activator of transcription 3
T2D	Type 2 diabetes
TGF-β	Transforming growth factor beta
TGF-β_1R	Transforming growth factor beta 1 receptor
Val-Pro	Valyl-proline
VEGF	Vascular endothelial growth factor
VHL	Von Hippel–Lindau tumor suppressor
WIP1	Wild-type p53-induced phosphatase

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Prolidase Stimulates Proliferation and Migration through Activation of the PI3K/Akt/mTOR Signaling Pathway in Human Keratinocytes

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Abstract: Recent reports have indicated prolidase (PEPD) as a ligand of the epidermal growth factor receptor (EGFR). Since this receptor is involved in the promotion of cell proliferation, growth, and migration, we aimed to investigate whether prolidase may participate in wound healing in vitro. All experiments were performed in prolidase-treated human keratinocytes assessing cell vitality, proliferation, and migration. The expression of downstream signaling proteins induced by EGFR, insulin-like growth factor 1 (IGF-1), transforming growth factor β_1 (TGF-β_1), and β_1-integrin receptors were evaluated by Western immunoblotting and immunocytochemical staining. To determine collagen biosynthesis and prolidase activity radiometric and colorimetric methods were used, respectively. Proline content was determined by applying the liquid chromatography coupled with mass spectrometry. We found that prolidase promoted the proliferation and migration of keratinocytes through stimulation of EGFR-downstream signaling pathways in which the PI3K/Akt/mTOR axis was involved. Moreover, PEPD upregulated the expression of β_1-integrin and IGF-1 receptors and their downstream proteins. Proline concentration and collagen biosynthesis were increased in HaCaT cells under prolidase treatment. Since extracellular prolidase as a ligand of EGFR induced cell growth, migration, and collagen biosynthesis in keratinocytes, it may represent a potential therapeutic approach for the treatment of skin wounds.

Keywords: prolidase; PEPD; EGFR; keratinocytes; wound healing

1. Introduction

Wound healing is a physiological process restoring skin functionality. It consists of four phases occurring in proper time and order. This process starts from hemostasis, then inflammation followed by proliferation and eventually tissue remodeling. The sequence of events during wound healing is strictly programmed and any disturbances may impair normal tissue repair. In the microenvironment of the damaged tissue, there are numerous biological factors and cell types involved in this process. One of them is keratinocytes, which proliferate and migrate to the wounded area during the proliferative phase induced by growth factors [1].

Stimulated epidermal growth factor receptor (EGFR) and its downstream signaling proteins are responsible for cell proliferation, differentiation, growth, and migration [2]. Recent reports have demonstrated that prolidase (PEPD) is a ligand of EGFR [3]. PEPD is a protein that evokes intracellular and extracellular functions. Intracellularly it has an enzymatic activity [EC.3.4.13.9]

splitting imidodipeptides with C-terminal proline or hydroxyproline [4,5] supplying proline for protein biosynthesis, mainly collagen. Moreover, this enzyme is involved in the regulation of cell growth at transcriptional (e.g., nuclear factor kappa beta (NF-κβ)) and post-transcriptional (e.g., hypoxia-inducible factor 1 alpha (HIF-1α)) levels [6–9]. Extracellularly, prolidase as a ligand of EGFR induces growth-promoting signaling [3]. Both functions, intracellular and extracellular contribute to the upregulation of anabolic processes. Therefore, prolidase expression and the enzyme activity could be of great importance in tissue regeneration processes. Although the intracellular function of prolidase in collagen biosynthesis is well recognized [10], its role in the activation of EGFR-dependent signaling in an experimental model of wound healing has not been established.

EGFR, known also as ErbB, is a member of four related tyrosine kinase receptors that upon ligand binding undergo dimerization leading to receptor autophosphorylation. This process initiates a cascade of phosphorylation of many signaling proteins inducing different cellular events [11]. The most specific signaling proteins downstream of EGFR are phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR), Ras/Raf/extracellular signal-regulated kinase (ERK), and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways [12] that represent progrowth and proproliferation signaling. However, the signaling pathway could not function without cross-talk with adhesion receptors. In keratinocytes and fibroblasts, the most abundant are integrin receptors. In the context of the anabolic and growth-promoting activity, the signaling induced by the β_1-integrin receptor is well characterized. This signaling pathway is known to induce prolidase activity [13] and collagen biosynthesis [14]. Upon stimulation, the β_1-integrin receptor induces autophosphorylation of non-receptor focal adhesion kinase pp125FAK (FAK) that is specific for adhesion receptors. Then, the kinase can interact with growth factor receptor-bound protein 2 (Grb2), through proto-oncogene Src and Shc proteins. The further cascade of signaling pathway involves son of sevenless 1 (Sos1), Ras and Raf proteins, and subsequently two mitogen-activated protein (MAP) kinases: ERK1 and ERK2 [15,16]. This signaling pathway is amplified by EGFR signaling, leading to the induction of transcription factors that stimulate the expression of genes for several proteins involved in the regulation of cell growth, differentiation, and metabolism [17]. The evidence for the coordinate regulation of prolidase activity and collagen biosynthesis by β_1-integrin signaling was documented [18]. Although the functional significance of EGFR and integrin signaling in anabolic and growth-promoting processes is well established, the mechanism for regulation of the processes in tissue regeneration is not well understood. Since prolidase is a ligand of EGFR and EGFR signaling activates cell proliferation, we hypothesized that extracellular prolidase may accelerate the experimental wound healing.

2. Results

2.1. Prolidase Promotes Proliferation and Migration of HaCaT Keratinocytes in a Model of Wound Healing

During the re-epithelization phase of wound healing keratinocytes proliferate and migrate closing the wound and restoring the epithelial layer [19]. To test whether keratinocytes proliferate under conditions of mechanical damage we scratched the cell monolayer. DNA fluorescence assay was employed to study the effect of prolidase on the proliferation of keratinocytes. The cells were treated with various concentrations of prolidase (1–100 nM) for 24 and 48 h. As a positive control for cell proliferation, the epidermal growth factor (EGF) at a concentration of 10 nM was used. As shown in Figure 1A, prolidase at all studied concentrations similarly stimulated cell proliferation, however, exhibited lower potency than EGF. It was found that the proliferation of keratinocytes was noticeably increased in a time-dependent manner.

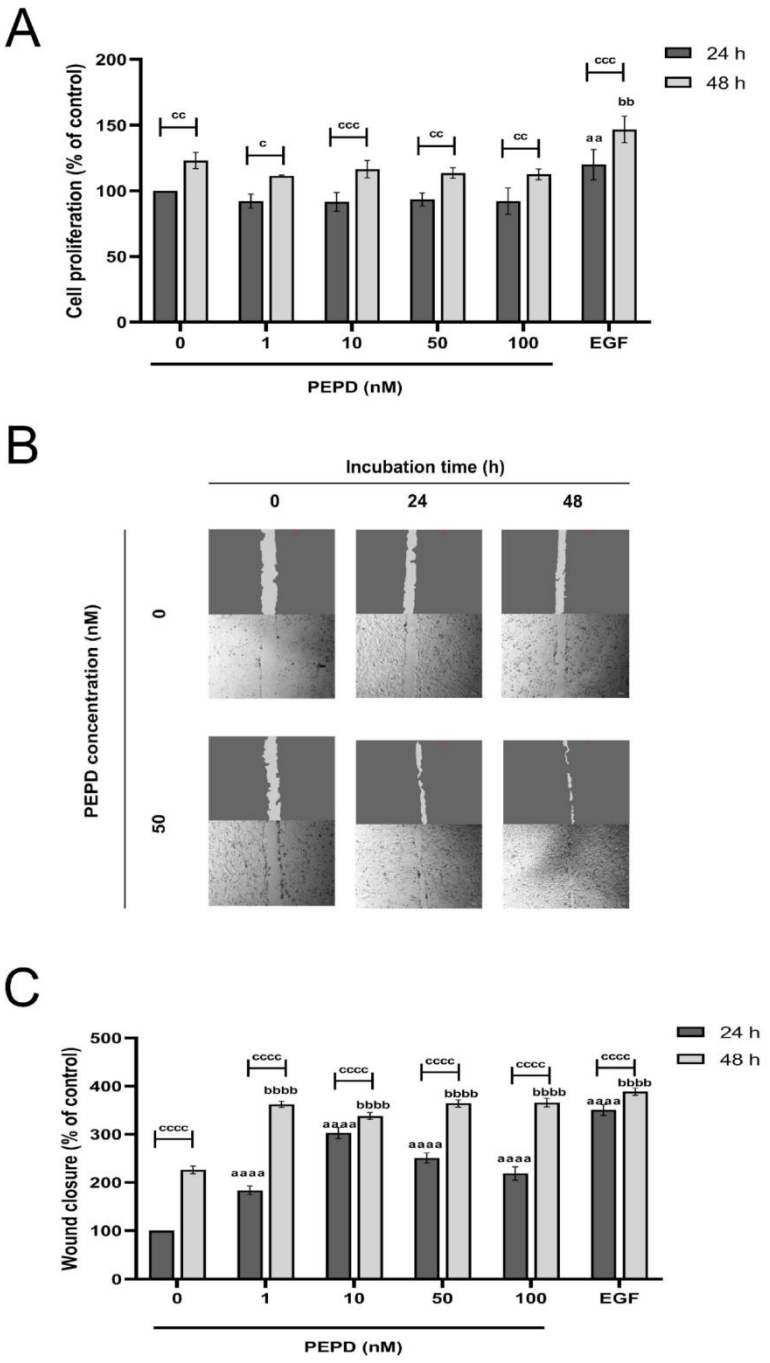

Figure 1. Prolidase promotes proliferation and migration of HaCaT cell lines. (**A**) Cell proliferation was evaluated by CyQuant Proliferation assay after 24 and 48 h upon prolidase supplementation. (**B**) Microscopic images of wound closure in scratched assay monitored using an inverted microscope (40× magnification) after 24 and 48 h incubation with prolidase (50 nM) compared to control cells. Supplementary Materials contain microscopic images in triplicates (Supplementary Figure S1). (**C**) The wound closure rate was evaluated by ImageJ software in HaCaT cells treated for 24 and 48 h with prolidase at concentrations of 1–100 nM. EGF was used as a positive control. A mean ± SEM of three replicates is presented. The results are significant at $c < 0.05$, *aa*, *bb*, or $cc < 0.01$, $ccc < 0.001$, and *aaaa*, *bbbb*, or $cccc < 0.0001$ and are marked as *a* vs. control (0 nM of PEPD) of 24 h incubation, *b* vs. control (0 nM of PEPD) of 48 h incubation, and *c* marked 24 h incubation cells vs. 48 h incubation cells at the same concentration of PEPD or EGF, respectively.

Subsequently, we investigated whether prolidase stimulates the migration of HaCaT cells followed by a scratch of the cell monolayer using an in vitro wound closure/scratch assay. EGF (10 nM), as a factor promoting cell proliferation, was used as a positive control in a model of wound closure. The results demonstrated that prolidase contributed to progressive wound closure in a dose- and time-dependent manner. As presented in Figure 1B prolidase treatment of the cells for 48 h resulted in almost complete closure of the wound, whereas the wounded area of control cells (without prolidase) remained unchanged. Quantification of the wounded area revealed that HaCaT cells subjected to prolidase treatment migrated faster to the wounded area than those in control. After 48 h incubation, the rate of wound closure was similar for both EGF and prolidase (Figure 1C).

2.2. Prolidase Does not Change the Vitality of HaCaT Keratinocytes

The effect of prolidase on cell vitality was determined by the measurement of intracellular thiols concentration. As demonstrated in Figure 2A, prolidase treatment (1–100 nM, 24 h) did not affect cell vitality in control and scratched cell models.

2.3. Extracellular Prolidase does not Affect Intracellular Prolidase Activity in Keratinocytes

Prolidase exhibits dual biological activity as an enzyme and an EGFR ligand [20]. To exclude the possibility that extracellular prolidase affects intracellular prolidase activity, the enzyme activity was measured in prolidase treated keratinocytes. There was no difference in prolidase activity upon prolidase treatment (1–50 nM, 24 h) of HaCaT cells in both control and scratched cell models (Figure 2B).

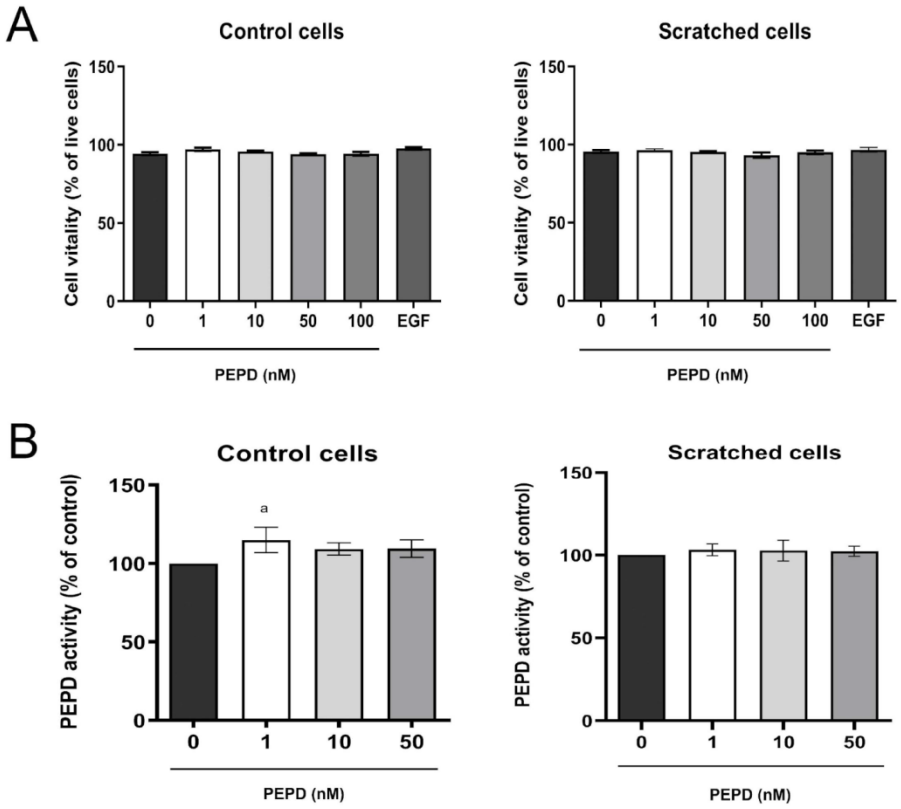

Figure 2. Prolidase does not affect the vitality and prolidase activity of keratinocytes. (**A**) Cell vitality and (**B**) PEPD activity in control and scratched HaCaT cells, treated with different concentrations of prolidase (1–100 nM) and EGF (10 nM) for 24 h. A mean ± SEM of three replicates is presented. The results are significant at $a < 0.05$. Statistically significant differences were calculated vs. control (0 nM of PEPD).

2.4. Prolidase Activates EGFR and Downstream Signaling Proteins in HaCaT Cells

The potency of PEPD to phosphorylate EGFR and downstream signaling proteins was compared to the activity of EGF (10 nM), used as a positive control. Kinetic analysis of the activation of the EGFR signaling pathway in a time course of 0, 5, 15, 30, 60, 120, and 240 min and 24 h was performed using Western immunoblotting. The time course of EGFR activation by these ligands was similar indicating that PEPD is a potent inducer of this receptor. Treatment of HaCaT cells with 10 nM PEPD resulted in significant phosphorylation of EGFR and its key signaling proteins such as mTOR, Akt, and ERK1/2. PEPD present in cell culture medium activated these molecules through phosphorylation of p-EGFR (Tyr1068), p-mTOR (Ser2448), p-Akt (Ser473), and p-ERK1/2 (Thr202/Tyr204; Figure 3A,B). In PEPD-treated HaCaT cells, EGFR was activated within 5 min upon treatment and then gradually decreased up to 4 h, similarly to the activation by EGF. We found that phosphorylation of EGFR instantly entailed phosphorylation of Akt and ERK in response to PEPD treatment. Whereas these signaling proteins were activated rapidly, activation of mTOR was slower, as p-mTOR reached a maximal level at 1 h after treatment with PEPD. These results demonstrated that prolidase could elicit EGFR transactivation leading to sustained phosphorylation of Akt/mTOR and MAPK signaling pathways in keratinocytes.

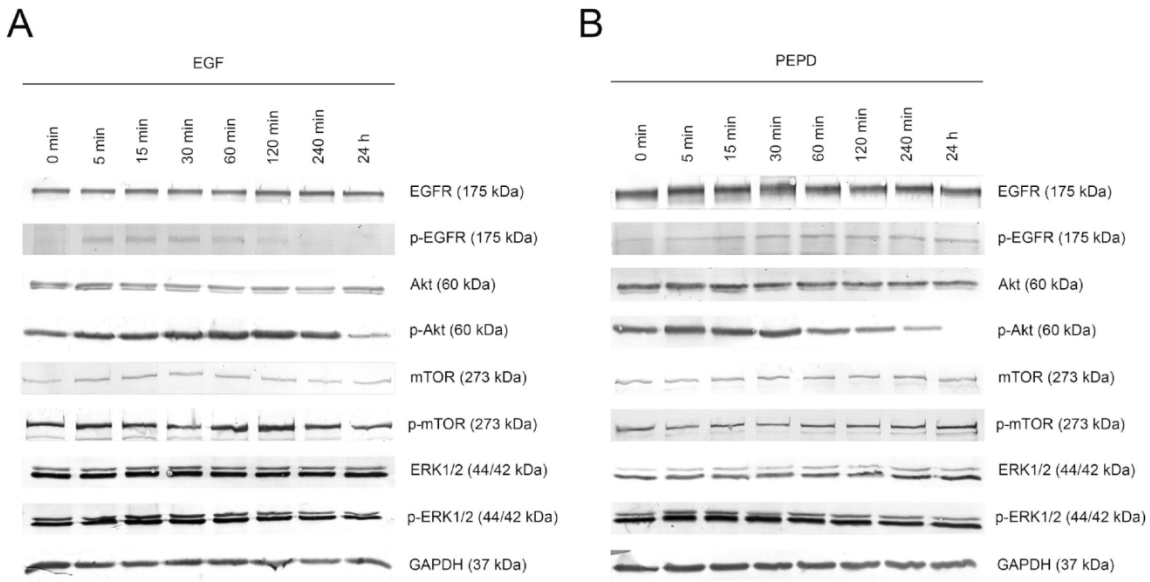

Figure 3. Kinetic analysis of phosphorylation of the epidermal growth factor receptor (EGFR) and its downstream signaling proteins induced by EGF (**A**) and PEPD (**B**) at concentration of 10 nM after 0, 5, 15, 30, 60, 120, and 240 min and 24 h upon supplementation. Representative blot images are shown (densitometric analysis of presented protein is included in Supplementary Materials (Supplementary Figure S2)).

2.5. Prolidase Elicits Biological Effects in HaCaT Cells through EGFR

To establish whether prolidase-dependent functions undergo through EGFR, the expression of downstream signaling proteins was measured in prolidase-treated keratinocytes. As shown in Figure 4A, in prolidase-treated cells (1–50 nM, 24 h) the expressions of the total forms of EGFR, PI3K (p85), Akt, mTOR proteins were increased in a dose-dependent manner. To prove that prolidase activates EGFR in the HaCaT cell line under normal and scratch conditions, Western immunoblotting was applied to evaluate the phosphorylated forms of all aforementioned signaling proteins. As can be seen in Figure 4A, HaCaT cells treated with prolidase (1–50 nM, 30 min) caused phosphorylation of EGFR (Tyr1068) and downstream molecules including PI3K p85 (Tyr458)/p55 (Tyr199), Akt (Ser473),

and mTOR (Ser2448). Figure 4B presents a schematic illustration of the PEPD-dependent stimulation of the EGFR-downstream signaling pathway.

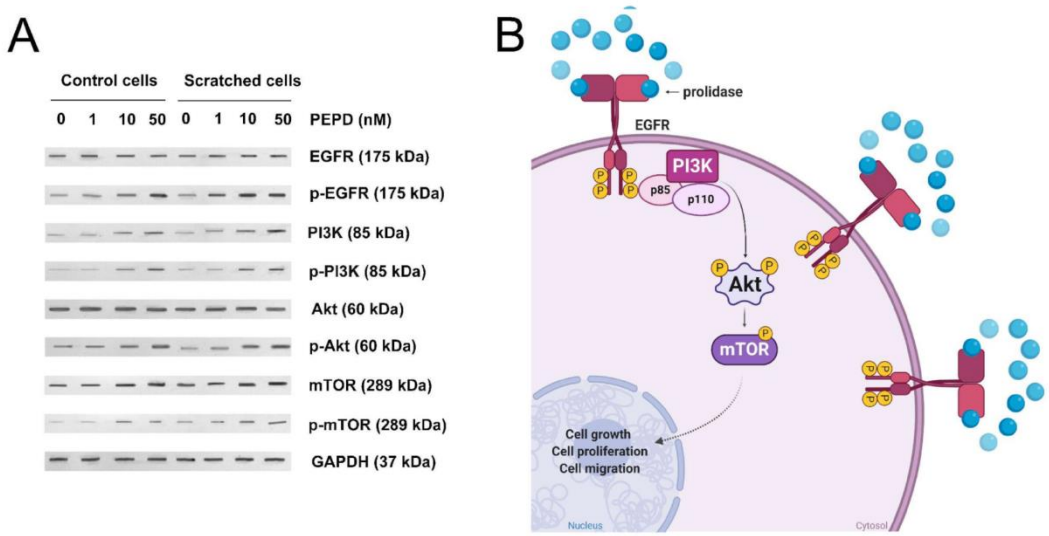

Figure 4. Prolidase induces EGFR-downstream signaling pathway. (**A**) The proteins of the EGFR-downstream signaling pathway analyzed by Western immunoblotting in lysates of control and scratched prolidase-stimulated HaCaT cells. GAPDH was used as a loading control. Representative blot images are shown (densitometric analysis of presented protein is included in Supplementary Materials (Supplementary Figure S3)). (**B**) An illustration of the prolidase-dependent EGFR-downstream signaling pathway activation. Created with BioRender.com.

Prolidase-induced EGFR signaling was confirmed by an experiment showing that blocking EGFR abolished PEPD-dependent effects. The cells pretreated with Gefitinib (2 µM, 2 h), a specific EGFR inhibitor, suppressed PEPD-induced EGFR-downstream signaling. The inhibitor strongly diminished PEPD-related EGFR and Akt phosphorylation and the number of total proteins of EGFR and Akt (Figure 5A). Subsequently, we tested cell migration in the presence of prolidase and Gefitinib to investigate the effect of prolidase on migratory ability upon blockade of EGFR. We observed that HaCaT cells pretreated with the EGFR inhibitor and then supplemented with prolidase (10 nM) lost their capacity to migrate (Figure 5B). Quantification of the scratch area demonstrated that prolidase-treated HaCaT cells remained unchanged compared to control after 24 h (Figure 5C).

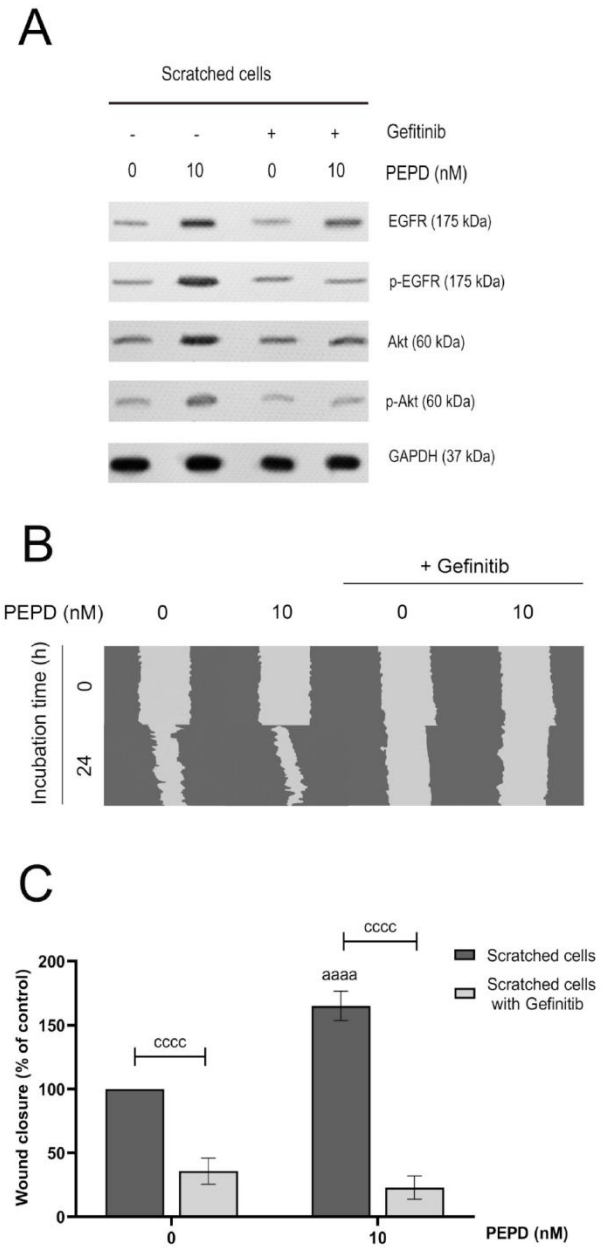

Figure 5. Prolidase elicits migration in HaCaT cells through EGFR. (**A**) The level of expression of EGFR and Akt proteins analyzed by Western immunoblotting in lysates of scratched HaCaT cells incubated with PEPD (10 nM) and an inhibitor of EGFR (Gefitinib, 2 μM) for 30 min and 24 h. Representative blot images are shown (densitometric analysis of presented protein is included in Supplementary Materials (Supplementary Figure S4)). (**B**) Prolidase- and Gefitinib-treated keratinocytes were scratched and monitored using an inverted microscope at 0 and 24 h of incubation. Supplementary Materials contain microscopic images in triplicates (Supplementary Figure S5). (**C**) The wound closure rate of scratched HaCaT cells was evaluated by ImageJ software. The results represent mean ± SEM of three replicates and are significant *aaaa*, or *cccc* < 0.0001 and are marked as *a* vs. control (0 nM of PEPD) of scratched cells, *b* vs. control (0 nM of PEPD) of scratched cells after treated with Gefitinib, and *c* marked scratched cells vs. scratched cells after treated with Gefitinib in the same concentration of PEPD, respectively.

2.6. PI3K/Akt/mTOR Signaling Pathway is Involved in Prolidase-related Proliferation and Migration of HaCaT Keratinocytes

The PI3K/Akt/mTOR signaling pathway is involved in the proliferation and migration of keratinocytes [21]. To demonstrate the role of prolidase-induced PI3K/Akt/mTOR signaling pathway in these processes during wound healing the HaCaT cells were pretreated with the specific PI3K inhibitor, LY294002 (50 μM, 2 h). The expression of both total and phosphorylated forms of EGFR, PI3K, Akt, and mTOR was evaluated. Figure 6A shows that the inhibition of this pathway by LY294002 noticeably abolished prolidase-induced intracellular signaling through PI3K, Akt, and mTOR. To test the functional impact of the blockade of this pathway, proliferation and migration of keratinocytes were measured. Although in prolidase-treated keratinocytes proliferation of the cells was not much stimulated (Figure 6B), the migration of the cells was increased as it is demonstrated in Figure 6C,D.

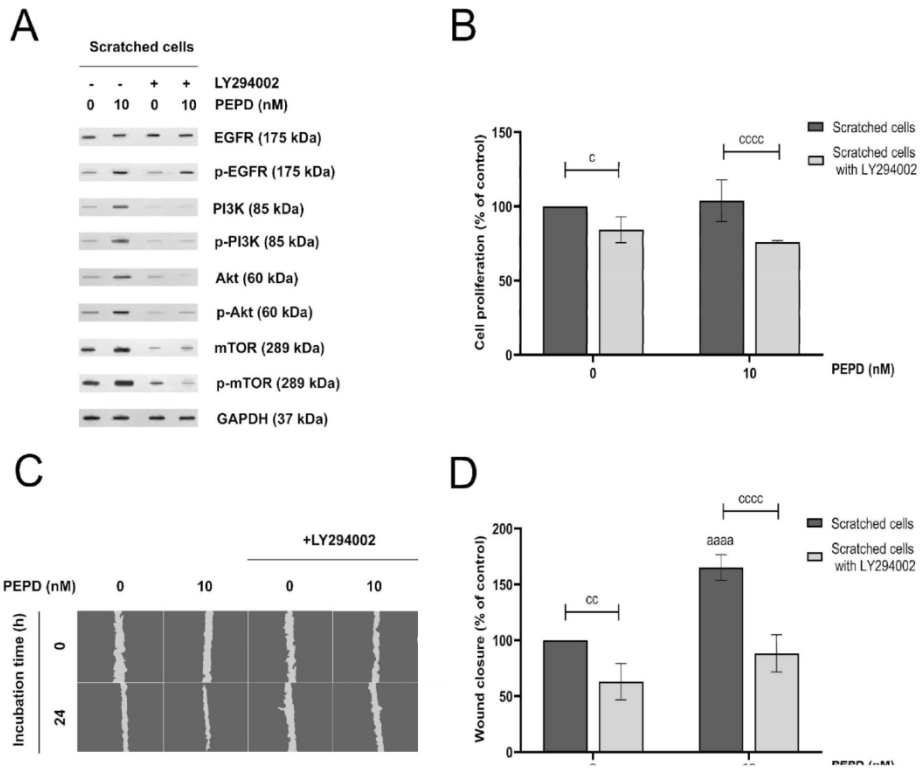

Figure 6. PI3K/Akt/mTOR signaling pathway is involved in prolidase-mediated proliferation and migration of HaCaT cells. (**A**) EGFR-downstream signaling pathway proteins analyzed by Western immunoblotting in lysates of scratched HaCaT cells incubated with prolidase (10 nM) and an inhibitor of PI3K (LY294002) for 30 min and 24 h. Representative blot images are shown (densitometric analysis of presented protein is included in Supplementary Materials (Supplementary Figure S6)). (**B**) Cell proliferation of scratched HaCaT cells incubated with prolidase and LY294002 for 24 h. (**C**) Prolidase- and LY294002-treated keratinocytes were scratched and monitored using an inverted microscope at 0 and 24 h of incubation. Supplementary Materials contain microscopic images in triplicates (Supplementary Figure S7). (**D**) Prolidase and LY294002-stimulated HaCaT cell migration was calculated using ImageJ software and presented as a percent of scratched control cells. A mean ± SEM of three replicates is presented. The results are significant at *cc* < 0.01, and *aaaa*, or *cccc* < 0.0001 and are marked as *a* vs. control (0 nM of PEPD) of scratched cells, *b* vs. control (0 nM of PEPD) of scratched cells pretreated with LY294002, and *c* marked scratched cells vs. scratched cells pretreated with LY294002 at the same concentration of PEPD, respectively.

2.7. Prolidase Induces Expression of the β_1-Integrin Receptor, IGF-1R, and EGFR in Keratinocytes

Prolidase stimulated HaCaT cells to express the β_1-integrin receptor and insulin-like growth factor 1 receptor (IGF-1R). Activation of the β_1-integrin receptor and IGF-1R was accompanied by an increase in the expression of downstream proteins such as FAK, Grb2, and Sos1 (Figure 7A). Phosphorylation of FAK (Tyr397) was detected under prolidase treatment. The potential signaling pathway, which mediates Ras/Raf/ERK signaling is illustrated in Figure 7B. Immunofluorescence analysis confirmed an increase in the expression of EGFR upon prolidase stimulation in a model of scratched cells (Figure 7C). Moreover, it proved that FAK was stimulated in mechanically damaged keratinocytes subjected to prolidase treatment (Figure 7D).

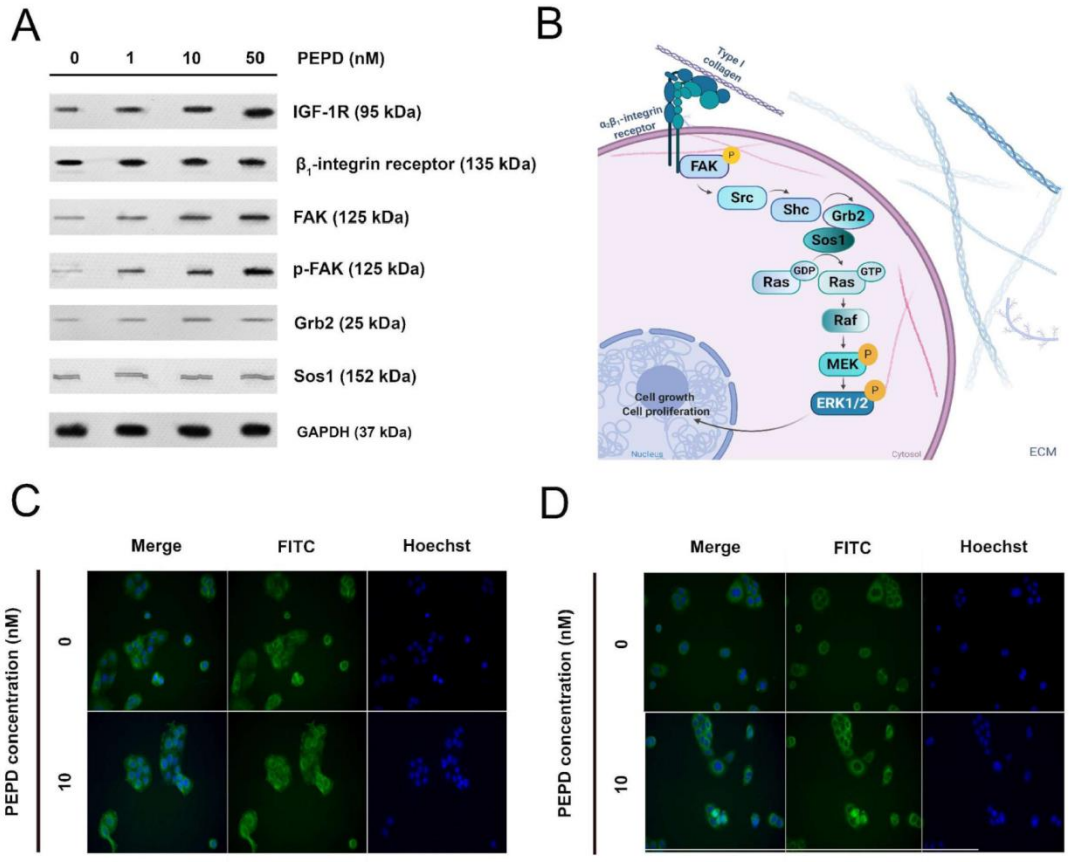

Figure 7. Prolidase stimulates expression of the β_1-integrin receptor, IGF-1R, and EGFR signaling proteins. (**A**) The proteins of the β_1-integrin receptor, IGF-1R, and EGFR-downstream signaling pathways were analyzed by Western immunoblotting in lysates of PEPD-stimulated HaCaT cells for 30 min and 24 h. Representative blot images are shown (densitometric analysis of presented protein is included in Supplementary Materials (Supplementary Figure S8)). (**B**) The scheme for activation of the β_1-integrin receptor-downstream signaling pathway. Created with BioRender.com. (**C**) Immunostaining of EGFR in PEPD-stimulated HaCaT cells for 24 h. (**D**) Immunostaining of FAK in PEPD-stimulated HaCaT cells for 24 h (magnification 200×).

2.8. Extracellular Prolidase Affects Epithelial-to-Mesenchymal Transition in HaCaT Cells

In a scratching experiment, we observed that prolidase enhanced the ability of keratinocytes to migrate. Thus, we tested whether PEPD is involved in the promotion of the epithelial-to-mesenchymal transition (EMT) process. We evaluated the effect of prolidase on this process at the protein level under conditions of mechanical damage in cultured HaCaT cells. Using Western immunoblotting, we found that the levels of expression of key EMT markers such as E-cadherin and N-cadherin were slightly

downregulated and upregulated, respectively (Figure 8A). It is known that non-canonical activation of the TGF-β_1 receptor also mediates EMT through MAPK signaling including ERK1/2 and p38 [22]. To examine whether this pathway was affected by prolidase, we performed Western immunoblotting for the TGF-β_1 receptor (TGF-β_1R) and ERK1/2. We found that the levels of expression of TGF-β_1R and ERK1/2 were upregulated, compared to control cells indicating that EMT occurred. As can be seen in Figure 8B, loss of E-cadherin and the gain of N-cadherin facilitates the migratory phenotype of cells promoting their migration. E-cadherin is involved in the formation of tight cell–cell junctions, thus their lost may result in increased keratinocyte migration.

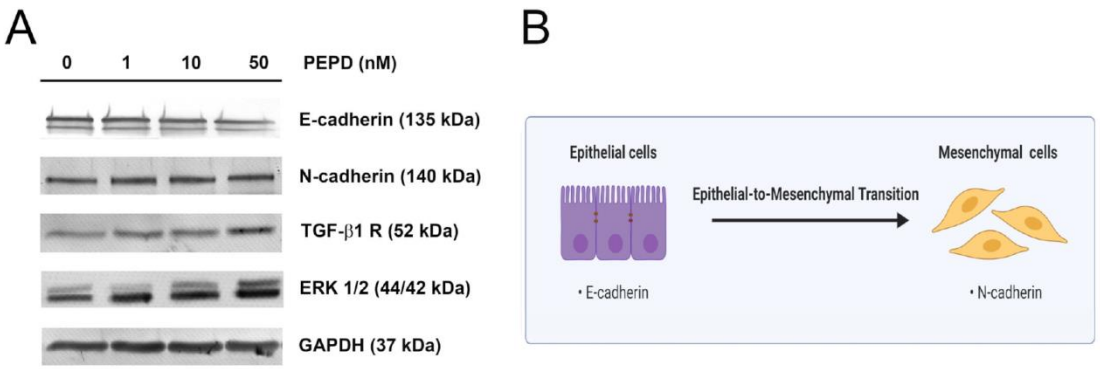

Figure 8. Extracellular prolidase affects epithelial-to-mesenchymal transition in HaCaT cells. (**A**) The proteins of the EMT process were analyzed by Western immunoblotting in lysates of PEPD-stimulated HaCaT cells for 24 h. Representative blot images are shown (densitometric analysis of presented protein is included in Supplementary Materials (Supplementary Figure S9)). (**B**) An illustration of the idea for the epithelial-to-mesenchymal transition process. Created with BioRender.com.

2.9. Extracellular Prolidase Stimulates Collagen Biosynthesis in HaCaT Cells

The last step of the wound healing process comprises extracellular matrix (ECM) remodeling in which collagen is required to restore connective tissue [19]. 5-[3H]-proline incorporation assay was employed to study the effect of prolidase on collagen biosynthesis in HaCaT keratinocytes. The cells were treated with different concentrations of prolidase (1–50 nM) for 24 and 48 h. As shown in Figure 9A,B, prolidase stimulated collagen biosynthesis in control and wounded keratinocytes in a dose-dependent manner. When prolidase was added to cultured cells for 24 h, the rate of collagen biosynthesis was 2-fold higher in a model of wounded cells than in normal HaCaT cells. Complementary Western immunoblotting shows that prolidase inhibited expression of NF-kβ suggesting the mechanism for prolidase-dependent collagen generation (Figure 9C). In the collagen molecule, proline constitutes about 10% of total amino acids [23]. The LC–MS-based analysis revealed that prolidase treatment of HaCaT in a dose-dependent manner increased proline concentration both in control and scratched cells (Figure 9D).

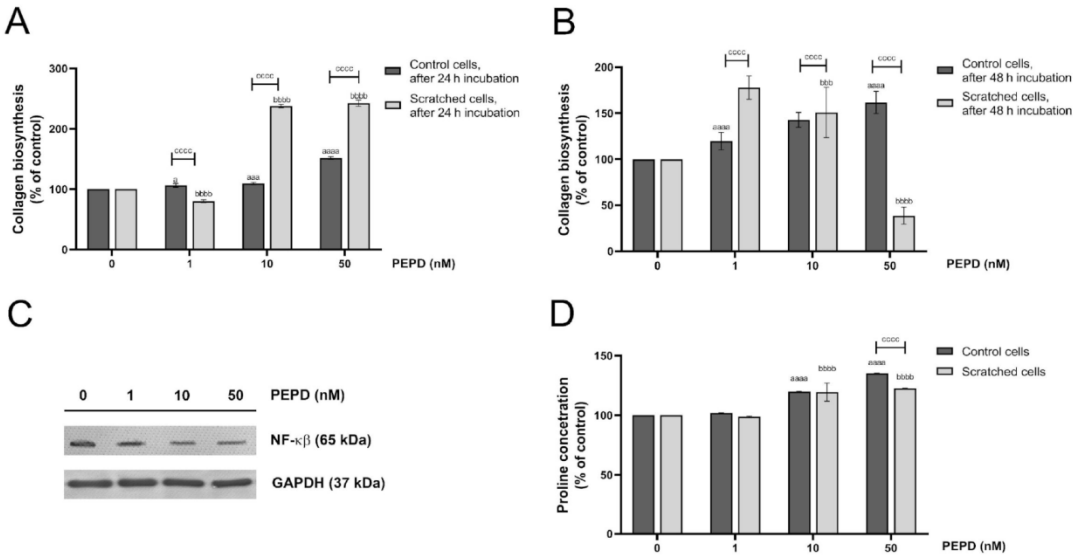

Figure 9. Extracellular prolidase stimulates collagen biosynthesis in HaCaT cells via an increase in proline synthesis and a downregulation of the level of NF-kβ. (**A**,**B**) Collagen biosynthesis was measured in prolidase-treated HaCaT cells (1–50 nM) after 24 and 48 h. (**C**) Analysis of NF-kβ in the cell lysate of keratinocytes upon PEPD supplementation after 24 h using Western immunoblotting. Representative blot images are shown (densitometric analysis of presented protein is included in Supplementary Materials (Supplementary Figure S10). (**D**) LC–MS-based analysis of proline concentration in prolidase-treated HaCaT cells after 24 h incubation. The results represent a mean ± SEM of three replicates are significant at $a = < 0.05$, *aaa*, or *bbb*, < 0.001, and *aaaa*, *bbbb*, or *cccc* < 0.0001 and are marked as *a* vs. control (0 nM of PEPD) of unscratched (control) cells, *b* vs. control (0 nM of PEPD) of scratched cells, and *c* marked unscratched (control) cells vs. scratched cells in the same concentration of PEPD, respectively.

3. Discussion

To the best of our knowledge, this study represents the first investigation of the functional significance of prolidase-dependent stimulation of EGFR signaling in a model of wounded keratinocytes. Based on the original observation by Yang et al. [3] showing that prolidase directly binds to and activates EGFR, we hypothesized that extracellular prolidase may promote cell migration and proliferation facilitating wound repair. Impaired wound healing is observed in numerous conditions such as acute and chronic diseases, aging, or after surgery [24] thus searching for new boosters of tissue regeneration is crucial.

In our study, we found that prolidase in a dose-and time-dependent manner induced keratinocyte proliferation and migration both in control and "wounded" cells. However, these processes were more pronounced in "wounded" cells. The difference cannot be attributed to the vitality of the cells since no changes in this parameter were found. We observed that prolidase, added to the culture medium of human keratinocytes, activated EGFR downstream signaling through PI3K/Akt/mTOR proteins. Activated EGFR induces PI3K/Akt/mTOR and Ras/Raf/ERK pathways [12]. As PEPD does not share an EGF motif with other EGFR ligands [3], we investigated the potency of this protein to phosphorylate EGFR. EGF is known as the most potent inducer of EGFR. Kinetic analysis showed that the prolidase in a time-dependent manner phosphorylated EGFR and its downstream molecules, similarly to that of EGF suggesting that prolidase can elicit sustained phosphorylation of EGFR-downstream signaling proteins.

We found that PEPD significantly improved cell migration to close the scratch area in a model of wound healing in vitro. Under normal conditions, during re-epithelialization, keratinocytes are attracted to the injury site to restore tissue continuity [19]. Given the fact that they evoke epithelial characteristics such as low migratory ability, the epithelial-to-mesenchymal transition has to occur.

Here, we found that PEPD promoted the migration of HaCaT cells suggesting that EMT happened. We observed the upregulation of key EMT markers such as N-cadherin and downregulation of E-cadherin. As induction of TGF-β_1 receptor through canonical (Smad signaling) and non-canonical (MAPK signaling) pathways also mediate EMT [22], we found that the amount of TGF-β_1R and ERK1/2 were increased which indicates EMT process in HaCaT cells. Activation of non-canonical TGF- β_1R signaling during EMT in keratinocytes was also presented in previous studies [22,25]. Our study demonstrated that PEPD could activate keratinocytes and stimulate cell migration through the EMT process facilitating wound healing.

Further, we applied an EGFR inhibitor, Gefitinib, to prove that prolidase binds to EGFR on the surface of keratinocytes. Inhibition of prolidase-dependent EGFR activation by Gefitinib led to a decrease in the amount of phospho- and total forms of EGFR and Akt confirming that prolidase is a ligand of this receptor. Then, we found that prolidase-mediated wound closure rate was abolished in the presence of Gefitinib supporting that activation of PEPD driven EGFR is a crucial event in HaCaT cell migration. Similar results presented previously showing that EGFR inhibition caused a reduction of keratinocyte migration [26]. Subsequently, to establish whether PI3K/Akt/mTOR is functionally linked to the proliferation and migration of HaCaT cells, we used a pharmacological inhibitor of PI3K, LY294002. Blocking this signaling pathway resulted in noticeable inhibition of the amount of phospho- and total forms of EGFR-downstream proteins. HaCaT cells were pretreated with LY294002 and then subjected to prolidase treatment followed by cell proliferation and migration assays. LY294002-pretreated cells exhibited decreased cell proliferation and migration. The results showed that the prolidase-stimulated PI3K/Akt/mTOR pathway is functionally required for the proliferation and migration of human keratinocytes. Diminished proliferation and migration of keratinocytes after inhibition of the PI3K/Akt/mTOR pathway were also observed previously by Lee et al. [27].

Moreover, prolidase was found to activate the expressions of the β_1-integrin receptor and insulin-like growth factor 1 receptor (IGF-1R). It is of great importance in wound healing since the role of both receptors in anabolic processes is well established [28]. Their role is particularly important in collagen biosynthesis. Both, the β_1-integrin and IGF-1 receptors transmit signals that induce collagen biosynthesis [10,13]. This process is of critical importance in the last step of wound healing and scar formation [28]. Upon stimulation, the β_1-integrin receptor induces autophosphorylation of FAK, which is then capable of interaction with Grb2, through Src and Shc proteins and then further cascade of signaling pathway through Sos, Ras, and Raf proteins and subsequently two MAP kinases: ERK1 and ERK2 [15,16]. The end-point of the signaling cascade is the induction of transcription factors that stimulate the expression of genes for several proteins involved in the regulation of cell growth, differentiation, and metabolism [17]. Performing analysis of the expression of the selected proteins from this pathway we confirmed prolidase-dependent stimulation of the β_1-integrin receptor and its downstream proteins. We demonstrated that prolidase-stimulated HaCaT cells enhanced collagen biosynthesis as a result of increased expression of β_1-integrin and IGF-1R receptors. Our results are supported by the observation made by Pappas et al. [29] who established that the ERK1/2 level reflects the rate of collagen synthesis. Moreover, we demonstrated that NF-kβ expression was decreased under prolidase treatment in keratinocytes. Since NF-kβ is known as an inhibitor of expression of α1 and α2 subunits of type I collagen [6,7,30]. It suggests that a drop in NF-kβ expression facilitated collagen biosynthesis. We found that proline content increased in the cells treated with prolidase, providing a substrate for the synthesis of collagen [31]. Prolidase as an enzyme participates in recycling proline from imidodipeptides (mostly derived from degradation products of collagen) for resynthesis of collagen and other proline-containing proteins. However, we found that the intracellular activity of prolidase did not change in prolidase-treated cells. Similar results were reported by Yang's study [3]. It is likely that in the experimental conditions proline came from the enzymatic reduction of pyrroline-5-carboxylic acid derived from glutamate or ornithine [32].

Given the activation of EGFR and β_1-integrin receptor signaling by prolidase, it seems that both receptors cooperate by cross-talk mechanism in tissue regeneration processes.

Furthermore, overexpression of prolidase resulted in increased nuclear HIF-1α level and elevated expression of HIF-1α-dependent gene products, vascular endothelial growth factor (VEGF), and glucose transporter-1 (Glut-1) [5,21,23]. Since HIF-1α, EGFR, and Glut-1 are involved in angiogenesis (as one of the tissues regeneration steps), regulation of prolidase expression and its activity could also play a major role in wound healing [33,34]. It is well established that platelet-rich plasma (PRP) facilitates the tissue repair process. Due to the high content of prolidase and growth factors contained in PRP, it has been widely used in regenerative medicine, especially in acute and chronic soft tissue injuries [28,35–38]. Poor wound healing is usually accompanied by impaired clot formation that limits access of all constituents of blood to the injured cells.

The functional significance of prolidase was found in prolidase deficiency (PD). Mutations in the prolidase gene are the molecular basis for PD resulting in decreased enzymatic activity of PEPD. It has been found several mutated alleles of the PEPD gene [39–41]. PD is a rare autosomal recessive disorder characterized by massive imidodipeptiduria, skin lesions, and elevated proline-containing dipeptides in plasma [42–48]. The most specific symptom of PD is reflected by defects in connective tissue metabolism. All symptomatic cases had skin lesions as diffuse telangiectasia, purpuric rash, crusting erythematous dermatitis, or progressive ulcerative dermatitis, particularly on the lower legs. To date, it is believed that PD results from low or a lack of PEPD activity, however, it cannot be excluded that the symptoms result mainly from deficiency of extracellular function of prolidase since supplementation of PD patients with proline or proline-convertible amino acids was unsuccessful in the treatment of the disease [49]. The biological activity of prolidase is of emerging research interest. So far, it is known that prolidase serves as a regulator of p53 function, affects interferon-α/β receptor maturation, and is a ligand of EGFR and epidermal growth factor receptor 2 (HER2) [20]. As our study demonstrated promising effects of prolidase in cell proliferation, migration, and collagen biosynthesis, further investigations are necessary to explore its role in PD.

4. Materials and Methods

4.1. HaCaT Cell Cultures

HaCaT cells were purchased from Cell Line Service (Eppelheim, Germany) and cultured in DMEM cell culture medium (PanBiotech, Germany) supplemented with 10% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified atmosphere of 5% CO_2. The medium was replaced every 3 days until 80% of confluency. For different applications, cells were seeded on various culture dishes (Sarstedt, Nümbrecht, Germany). For Western immunoblotting, cells were cultured on 100-mm dishes at a density of 2×10^6 cells/plate. To evaluate cell vitality, collagen biosynthesis, prolidase activity, and proline concentration HaCaT cells were cultured on 6-well plates at 2×10^5 cells/well. For wound healing assay, cells were plated at a density of 1×10^5 cells/well in 12-well dishes. For proliferation assay and immunocytochemistry, cells were seeded at 5×10^3 cells/well in a 96-well plate.

4.2. HaCaT Treatment

The cells (5–8th passages) were treated with prolidase from the porcine kidney (Sigma Aldrich, Saint Louis, MO, USA) at the concentrations of 1–50 nM. The enzyme, delivered as a lyophilized powder, was reconstituted in sterile phosphate buffer (PBS; pH 7.4, PanBiotech, Germany). For wound healing, proliferation, and Western immunoblotting HaCaT cells were pretreated with LY294002, selective PI3K inhibitor (Cell Signaling Technology, Danvers, MA, USA) at the working concentration of 50 μM for 2 h before treatment with prolidase (1–50 nM, 30 min and 24 h). Keratinocytes were subjected to pretreatment with Gefitinib (Sigma Aldrich, Saint Louis, MO, USA), an EGFR inhibitor at the working concentration of 2 μM for 2 h before treatment with prolidase (1–50 nM, 3 h and 24 h). The cells pretreated with Gefitinib were analyzed by Western immunoblotting and wound healing assay.

4.3. Cell Proliferation Assay

The proliferation of HaCaT cells was evaluated using commercially available CyQUANT® Cell Proliferation Assay (Thermo Fisher Scientific, Waltham, MA, USA). HaCaT cells were submitted to prolidase treatment at concentrations of 1–50 nM, and EGF (10 nM, Gibco, Carlsbad, CA, USA) as a positive control for 24–48 h. After incubation, cells were rinsed twice with PBS (pH 7.4) and frozen at −80 °C until analysis. Before analysis, samples were thawed at RT, and 200 µL of the mixture consisted of CyQUANT® GR dye/cell-lysis buffer was added and incubated for 5 min at RT. The plate was protected from light. Fluorescence was read on Victor X4 Multilabel Reader (PerkinElmer, Waltham, MA, USA) at 480 nm and 520 nm as excitation and emission wavelengths, respectively. The results were presented as the percent of the control value.

4.4. Cell Vitality Assay

The cell vitality was assessed by measurement of the level of intracellular thiols. After incubation with prolidase (1–50 nM, 24 h) or EGF (10 nM) as a positive control, cells were washed twice with warm PBS (pH 7.4) and trypsinized (0.25%). Then, cells were centrifuged (5 min, 500× *g*), the pellet was washed with PBS and stained with Solution 5 (ChemoMetec, Lillerød, Denmark) containing VitaBright-48, acridine orange, and propidium iodide and analyzed using Nucleo Counter NC-3000 (ChemoMetec, Lillerød, Denmark). The results were presented as the percent of the control value.

4.5. Preparation of Lysates

The cells were cultured with prolidase (1–50 nM) for 30 min and 24 h. Before harvesting cells were rinsed twice with cold PBS (pH 7.4). Then, cells were scraped in RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) with protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Roche, Basel, Switzerland) and phosphatase inhibitor cocktail (PhosSTOP, Roche, Basel, Switzerland) and incubated on ice for 15 min. Lysates were sonicated three times (15 sec on and 5 sec off) followed by centrifugation at 4 °C (10 min, 12,000× *g*). The supernatant was transferred to a fresh tube and stored at −80 °C until Western immunoblotting. Protein concentration was measured using the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

4.6. Western Immunoblotting

For Western immunoblotting, equal amounts (5–20 µg/lane) of protein were diluted in RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA) and mixed with Laemmli buffer (120 mM Tris-HCl, 20% glycerol, 0.4% SDS, and 0.02% bromophenol blue, pH 6.8) containing fresh 5% β-mercaptoethanol (Sigma Aldrich, Saint Louis, MO, USA). The samples were denatured at 95 °C for 7 min. The proteins were separated on 7.5–10% SDS-PAGE gels and then blotted onto polyvinylidene difluoride (PVDF; BioRad Laboratories, Hercules, CA, USA) membranes. The membranes were blocked with either 5% non-fat dried milk (Santa Cruz Biotechnology, Dallas, TX, USA) or BSA (Sigma Aldrich, Saint Louis, MO, USA) in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 h at room temperature with agitation. The membranes were incubated with primary antibodies overnight at 4 °C, followed by incubation with alkaline phosphatase-linked goat antirabbit or antimouse antibodies for 1 h at RT. The membranes were washed three times in TBS-T for 5 min. The bands were visualized using 1-Step™ NBT/BCIP Substrate Solution (Thermo Fisher Scientific, Waltham, MA, USA) and their intensities were semiquantitatively measured with ImageJ software (https://imagej.nih.gov/ij/). All experiments were run in triplicates.

4.7. Antibodies

The membranes were incubated with the following primary antibodies purchased from Cell Signaling Technology (Danvers, MA, USA): p44/42 MAPK (ERK1/2) Rabbit mAb (1:1000), mTOR Rabbit mAb (1:1000), EGF Receptor Rabbit mAb (1:1000), IGF-1 Receptor β Rabbit mAb (1:1000), phospho-EGF

Receptor (Tyr1068) Rabbit mAb (1:1000), phospho-p44/42 MAPK (Thr202/Tyr204) Rabbit mAb (1:1000), phospho-mTOR (Ser2448) Rabbit mAb (1:1000), FAK Rabbit mAb (1:1000), phospho-FAK (Tyr397) Rabbit mAb (1:1000), Integrin β_1 Receptor Rabbit mAb (1:2000), Akt Rabbit mAb (1:2000), phospho-Akt (Ser473) Rabbit mAb (1:2000), PI3 Kinase p85 Rabbit mAb (1:1000), phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199) Antibody (1:1000), NF-κβ p65 Rabbit Antibody (1:1000), TGF-β Receptor I Rabbit Antibody (1:1000), E-Cadherin Rabbit mAb (1:1000), N-Cadherin Rabbit mAb (1:1000), and GAPDH Rabbit mAb (1:1000). Mouse anti-Grb2 (1:500) and mouse anti-Sos1 (1:1000) were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Secondary alkaline phosphatase-conjugated antimouse or antirabbit antibodies diluted 1:10 000 were from Sigma Aldrich (Saint Louis, MO, USA).

4.8. Evaluation of Collagen Biosynthesis

Collagen biosynthesis was determined by the incorporation of radioactive 5-[3H]-proline (5 μCi/mL; Hartmann Analytic, Germany) into proteins susceptible to bacterial collagenase according to the Peterkofsky's method [50]. After 24 and 48 h incubation, cells were washed with PBS (pH 7.4), harvested in PBS containing 10 mM proline, and frozen at −80 °C until the day of analysis. For collagen digestion, we used purified *Clostridium histolyticum* collagenase (Sigma Aldrich, Saint Louis, MO, USA). The radiometric analysis was performed applying Liquid Scintillation Analyzer Tri-Carb 2810 TR (PerkinElmer, Waltham, MA, USA). The results were normalized to total protein biosynthesis and were presented as a percent of the control value.

4.9. Determination of Prolidase Activity

The activity of prolidase was determined according to the method published by Besio et al. [51]. Protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). An equal amount (50 μg) of proteins was mixed with 50 mM Tris–HCl (pH 7.8) containing 1 mM MnCl2 and 0.75 mM glutathione and incubated for 1 h at 50 °C. Then, we added 100 mM glycyl-proline (substrate for prolidase) and incubated for 30 min at 50 °C. The reaction was stopped by 0.45 M ice-cold TCA. After centrifugation (15 min, 12,000× *g*), the supernatant was transferred to fresh tubes and incubated with Chinard's reagent (12 min, 90 °C) followed by incubation on ice for 15 min. Absorbance was read at 515 nm on TECAN Infinite® M200 PRO (Männedorf, Switzerland). The results were reported as a percent of the control value.

4.10. In Vitro Wound Healing Assay

At confluence, HaCaT cells were scratched using a sterile 200 μL pipette tip, washed twice with PBS, and incubated with different concentrations of prolidase in 0.5% FBS-containing DMEM for 24 and 48 h. Every 24 h cells were photographed using an inverted optical microscope (Nikon; Minato, Tokyo, Japan) with a 40× magnification for monitoring the wound closure area. The wound closure was measured by ImageJ software (https://imagej.nih.gov/ij/) and its rate was calculated according to the following formula.

$$\text{wound healing rate} = \frac{\text{original wound area} - \text{unhealed wound area}}{\text{original wound area}}$$

All the experiments were performed in triplicates.

4.11. Immunofluorescence Staining and Confocal Microscopy

After 24 incubation with prolidase (10 nM), cells were rinsed with prewarmed PBS twice. For fixation, 3.7% paraformaldehyde was used (10 min). Permeabilization with 0.1% Triton for 5 min was performed before FAK and NF-κβ visualization in contrast to that of EGFR where no permeabilization step was conducted. The next step included blocking with 3% fetal horse serum for 60 min at room temperature (RT). Then, cells were incubated with target primary antibodies (anti-EGFR,

anti-FAK, and anti-NF-κβ) overnight at 4 °C at dilutions 1:500 and 1:100, 1:400, respectively. As a secondary antibody were used antirabbit FITC-linked antibody (Becton Dickinson, Franklin Lakes, NJ, USA) at a concentration of 5 µg/mL for 1 h in the dark at RT. The cell nuclei were stained with Hoechst (1 ng/mL). Immunofluorescence staining was visualized using a confocal laser scanning microscope (BD Pathway 855 Bioimager, Becton Dickinson, Franklin Lakes, NJ, USA) supported with AttoVision software.

4.12. LC–MS-Based Quantitative Analysis

LC–MS analysis of proline concentration in HaCaT cells was conducted according to the method of Klupczynska et al. [52]. Briefly, cells were harvested by scraping in ice-cold methanol with stable isotopically labeled proline (25 µM, d3-proline; Sigma Aldrich, Saint Louis, MO, USA) as an internal standard. Cell lysates were stored at −80 °C until analysis. Samples were analyzed using Agilent 1260 Infinity HPLC system coupled to Agilent 6530 Q-TOF mass spectrometry detector with electrospray ionization (Agilent Technologies, Santa Clara, CA, USA) as an ion source in positive ionization mode. Samples were injected onto a HILIC column (Luna HILIC, 2 mm × 100 mm, 3 µm, Phenomenex, Torrance, CA, USA) thermostated at 30 °C. All samples were randomized before analysis. The results were normalized to protein concentration and presented as a percent of the control value.

4.13. Statistical Analysis

All experiments were carried out at least three replicates and the experiments were repeated at least three times. Data are shown as a mean ± standard error (SEM). For statistical calculations, a one-way analysis of variance (ANOVA) with Dunnett's correction and *t*-test were used. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, USA). Statistically significant differences were marked as $p < 0.05$, *pp* < 0.01, *ppp* < 0.001, and *pppp* < 0.0001 and presented by using letters showing the differences between appropriate controls described in the legend of figures.

5. Conclusions

The data presented in this report suggest that extracellular prolidase acting through EGFR induced growth, migration, and collagen biosynthesis in cultured keratinocytes (Figure 10). Therefore, prolidase may represent a therapeutic approach to treat skin wounds.

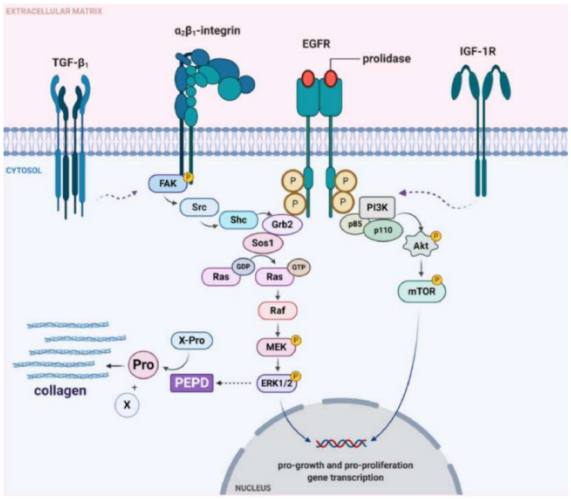

Figure 10. Graphical illustration of prolidase-dependent EGFR, β_1-integrin receptor, TGF-β_1, and IGF-1R-downstream signaling. Under experimental conditions of mechanically damaged HaCaT cells, prolidase causes activation of the ERK1/2 and PI3K/Akt/mTOR pathways resulting in increased collagen biosynthesis and cell proliferation and migration. Created with BioRender.com.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/21/23/9243/s1.

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Abbreviations

Akt	protein kinase B
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK1/2	extracellular signal-regulated kinase 1/2
FAK	focal adhesion kinase $pp125^{FAK}$
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Glut-1	glucose transporter-1
Grb2	growth factor receptor-bound protein 2
HER 2	epidermal growth factor receptor 2
HIF-1α	hypoxia-inducible factor 1 alpha
IGF-1R	insulin-like growth factor 1 receptor
JAK	Janus kinase
LY294002	phosphoinositide 3 kinase inhibitor
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
mTOR	mammalian target of rapamycin
NF-κβ	nuclear factor kappa beta
PD	prolidase deficiency
PEPD	prolidase
PI3K	phosphoinositide 3 kinase
Pro	proline
PRP	platelet-rich plasma
Shc	SHC adaptor protein 1
Sos1	son of sevenless 1
STAT	signal transducer and activator of transcription
Src	proto-oncogene Src
TGF-β_1R	transforming growth factor-beta 1 receptor
VEGF	vascular endothelial growth factor

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Recombinant Prolidase Activates EGFR-Dependent Cell Growth in an Experimental Model of Inflammation in HaCaT Keratinocytes. Implication for Wound Healing

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This study was conducted to investigate the proliferative capacity of recombinant human prolidase (rhPEPD) in a human model of inflammation induced by IL-1β in HaCaT keratinocytes. In this report, we provide evidence that IL-1β stimulates keratinocyte proliferation, and rhPEPD significantly augmented this process through activation of epidermal growth factor receptor (EGFR) and downstream signaling proteins as phosphorylated Akt, ERK1/2, and STAT3, which are implicated in keratinocyte migration, proliferation, and epithelialization during the wound healing process. Inhibition of PEPD-dependent EGFR signaling by gefitinib supported the finding. Moreover, during activation of EGFR in the presence of IL-1β the epithelial-to-mesenchymal transition (EMT) occurred *via* downregulation of E-cadherin and upregulation of N-cadherin. The phenomenon was accompanied by an increase in the activity of matrix metalloproteinase-9 (MMP-9), suggesting extracellular matrix (ECM) remodeling during the inflammatory process. MMP-9 activation may result from nuclear translocation of NF-κB through IKK-mediated IκBα degradation. Interestingly, some mutated variants of PEPD (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) evoked the ability to induce EGFR-dependent HaCaT cell proliferation. To the best of our knowledge, this is the first report on the cross-talk between PEPD and IL-1β in the process of keratinocyte proliferation. The data suggest that both enzymatically active and inactive rhPEPD may activate EGFR-dependent cell growth in an experimental model of inflammation in HaCaT keratinocytes and the knowledge may be useful for further approaches for therapy of wound healing disorders.

Keywords: recombinant human prolidase, PEPD, EGFR, keratinocytes, inflammation

INTRODUCTION

Proper wound healing is a physiological process precisely regulated by numerous factors consisting of four overlapping phases: Hemostasis, inflammation, repairment, and finally, tissue remodeling. Any disturbances occurring during these steps may contribute to a delay in wound healing and form chronic ulcers and/or excessive scarring (Ellis et al., 2018). Among other organs, skin injuries are predominant and involve various cell types specializing in distinct functions e.g., keratinocytes, fibroblasts, macrophages, and endothelial cells. In the complex regulatory mechanisms of the healing process metalloproteinases (MMPs), cytokines, and enzymes are key players. Close cooperation between cells and biomolecules functionally contributes to wound contraction, re-epithelialization, and maturation processes (Yang et al., 2017).

Keratinocytes comprise about 95% of cells in the epidermal layers. As the first line cells, they encounter environmental difficulties such as pathogenic bacteria, viruses, UV radiation, and allergens leading to the production of pro-inflammatory mediators (tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-8, etc), and progression of chronic skin inflammation. In a response to inflammation in keratinocytes, mitogen-activated protein kinases (MAPKs) and the nuclear factor kappa beta (NF-κB) are mainly stimulated. The inflammatory signal causes translocation of transcription factors into the nucleus, such as activator protein-1 (AP-1) and NF-κB, ultimately leading to the production of a variety of proinflammatory cytokines including TNF-α, IL-1β, IL-8, and IL-6 (Nguyen and Kim, 2020). Under non-activated conditions, NF-κB occurs in the cytoplasm and is coupled to IκBα, its inhibitor protein. Upon activation by a variety of external stimuli, including bacterial lipopolysaccharide, IκBα is phosphorylated and degraded *via* the proteasomal degradation pathway. This event further leads to the release of NF-κB, which then is translocated to the nucleus and binds to the promoter region (κB binding site) of several genes, including iNOS and Cox-2 (Kumar et al., 2013). Similarly, MAPKs, such as ERK1/2, p38, and c-jun N-terminal kinase1/2 (JNK1/2), are components of the inflammatory signal transduction pathways that also regulate iNOS and Cox-2 expression in a variety of cells through the activation of NF-κB (Kundu and Surh, 2005).

During skin inflammation extracellular matrix (ECM) remodeling and epithelial-to-mesenchymal transition (EMT) occur as a result of various signaling pathways, e.g., Cox-2, NF-κB, MAPKs (Eberhardt et al., 2000; Lee et al., 2008; Neil et al., 2008; Räsänen and Vaheri, 2010; Hahn et al., 2016). For ECM degradation, MMPs are required, particularly MMP-2, and -9, which are activated upon external stimuli such as various cytokines and growth factors (Ranzato et al., 2017). Downregulation of E-cadherin and upregulation of N-cadherin are the typical biochemical event in EMT enabling cells to relax membrane integrity and increase cell mobility. Both activated MMPs and mobile cells functionally coordinate the wound healing process. Type-2 EMT is a part of the regeneration and restoration of physiological conditions following mechanical and inflammatory damage (Marconi et al., 2021).

Among growth factor receptors inducing cell proliferation, differentiation, growth, and migration, epidermal growth factor receptor (EGFR) exert the most potent anabolic processes (Wee and Wang, 2017). Once ligand-EGFR binding occurs, the receptor dimerizes and leads to its autophosphorylation. As a result, a cascade of downstream protein phosphorylation is induced (Yarden and Sliwkowski, 2001). The most specific are protein kinase B (Akt), Ras/Raf/extracellular signal-regulated kinase (ERK), and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) (Lurje and Lenz, 2009). Finally, the signal is transduced to the nucleus involving transcription factors that regulate the expression of genes coding proteins responsible for cell growth, differentiation, and metabolism (Labat-Robert and Robert, 2000). Recently, a new potent EGFR ligand, prolidase (PEPD) has been identified (Yang et al., 2013). It is an enzyme biologically active both intra- and extracellularly. In the cytoplasm, it acts as an enzyme [EC.3.4.13.9] by cleaving C-terminal proline or hydroxyproline-containing imidodipeptides (Jackson et al., 1975; Yaron and Naider, 1993) and thus supplying proline for protein biosynthesis, particularly collagen. Additionally, this enzyme regulates the cellular growth-promoting signaling at transcription (e.g. NF-κB) as well as post-transcriptional [e.g., hypoxia-inducible factor 1 alpha (HIF-1α)] level (Kouba et al., 1999; Rippe et al., 1999; Jaakkola et al., 2001; Surazynski et al., 2008). In the extracellular space, PEPD binds to EGFR and contributes to cell proliferation (Yang et al., 2013). Since PEPD can upregulate anabolic processes PEPD expression and enzyme activity may play a key role in tissue regeneration processes. Recently, it has been established that prolidase stimulates proliferation and migration of keratinocytes *via* EGFR (PI3K/Akt/mTOR axis) in an experimental model of wound healing (Misiura et al., 2020).

Human immortalized keratinocytes (HaCaT cells) maintain full epidermal differentiation capacity (Boukamp et al., 1988). Since neutrophils show increased expression of IL-1β that promotes proliferation of keratinocytes (Ellis et al., 2018), the administration of IL-1β, a pro-inflammatory cytokine, was here applied as an experimental model to investigate inflammation-associated behavior in HaCaT cells. In particular, this study was focused on explaining, in an experimental model of inflammation in HaCaT keratinocytes (IL-1β treated), the effect of recombinant human PEPD (rhPEPD) on the expression of EGFR-downstream proteins, NF-κB pathway, and MMPs activity which are known to be involved in the EMT process and ECM remodeling typically observed in the inflammatory phase of skin wound healing. Moreover, mutant forms of rhPEPD (Besio et al., 2013) were also tested for the potential to induce EGFR-downstream signaling in the experimental model.

MATERIALS AND METHODS

HaCaT Cell Cultures

HaCaT cells (CLS Cell Lines Service, 300493; Eppelheim, Germany) were cultured in a DMEM cell culture medium (PanBiotech, Aidenbach, Bayern, Germany) containing 10%

fetal bovine serum (Gibco, Carlsbad, CA, United States) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA, United States) in a cell incubator (37°C, 5% CO_2). The medium was replaced every 3 days until cells reached up to 80% of confluency. Cell cultures were checked for *mycoplasma* infection regularly using Hoechst 33258 and confocal microscopy (BD Pathway 855 Bioimager; Becton Dickson, Franklin Lakes, NJ, United States).

Production of Recombinant Human Prolidase in *E. Coli* Expression System

The constructs for wild-type rhPEPD and mutant forms (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) were prepared as previously described (Lupi et al., 2006a; Besio et al., 2013). *E. Coli* BL21 (DE3) competent cells (Thermo Fisher Scientific, Waltham, MA, United States) were transformed with the vector of rhPEPD using the heat shock method and cultured in Luria–Bertani (LB) broth medium (Bioshop, Burlington, Ontario, Canada) with the addition of 100 g/ml ampicillin (Bioshop, Burlington, Ontario, Canada) and grown at 37°C with shaking to 200 RPM for 13 h. Then, cells were stimulated with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Bioshop, Canada) for 18 h at 18°C. Cells were then centrifuged (15 min, 4500 RPM, 4°C) and resuspended in lysis buffer (300 mM NaCl, 20 mM Tris-HCl pH 8.0, 20 mM imidazole, 1 mM EDTA, 10% glycerol). After centrifugation, The supernatant containing each recombinant protein expressed as a soluble form was purified twice. Firstly, it was loaded onto a HisTrap column (BioRad Laboratories, Hercules, CA, United States) with Ni-NTA affinity resin (IMAC) equilibrated with 0.1 M NiSO4 for purification of polyhistidine-tagged proteins. The column was eluted with elution buffer (300 mM imidazole, 300 mM NaCl, 20 mM Tris-HCl, pH 8.0, 20 mM imidazole, 1 mM EDTA, 10% glycerol, 1 mM TCEP). The following step of purification included the concentration of the eluted mixture to 10 ml using ultracentrifugation filters Amicon-Ultra 10 (Merck Millipore, Burlington MA, United States) and loaded onto a Superdex 200 (Pharmacia, New Jersey, NJ, United States) gel filtration column. The recombinant proteins were activated by 1 mM Mn^{2+} at 37°C for 1 h followed by dialysis against PBS for 12 h at 4°C. The concentration of each recombinant protein was determined using a Pierce™ BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, United States) according to the instructions.

HaCaT Treatment

The cells (5–8th passages) were treated with human recombinant IL-1β (10 ng/ml; Sigma Aldrich, Saint Louis, MO, United States) and human recombinant prolidase (rhPEPD^{WT}, rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) at concentrations of 10, 25, 50, 100, 250 nM for the selected time intervals (15 min, 60 min, and 24 h). For specific applications, keratinocytes were subjected to pretreatment with gefitinib (Sigma Aldrich, Saint Louis, MO, United States), an EGFR inhibitor, at the working concentration of 2 µM for 2 h before treatment with rhPEPD (1–50 nM, 15 min and 24 h) and then cell lysates were subjected to Western immunoblotting.

Cell Viability Assay

Cell viability of HaCaT cells was measured using Cell Titer Blue assay as described in the manufacturer's protocol (Promega, Madison, WI, United States). Cells, seeded at 5×10^3 cells/well in a 96-well plate, were submitted to rhPEPD^{WT} treatment at concentrations of 10–250 nM for 24 h. Briefly, cells were incubated with a resazurin-containing solution at 37°C for 2 h. Absorbance was read on TECAN Infinite® M200 PRO (Tecan Group Ltd., Männedorf, Switzerland) at 570 and 600 nm as a reference wavelength. The results were presented as a percent of the control value.

Cell Proliferation Assay

The proliferation of HaCaT cells was evaluated using commercially available CyQUANT® Cell Proliferation Assay (Thermo Fisher Scientific, Waltham, MA, United States). HaCaT cells, seeded at 5×10^3 cells/well in a 96-well plate, were submitted to rhPEPD^{WT}, rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K treatment at concentrations of 10–250 nM for 24 h. After incubation, cells were rinsed twice with PBS (pH 7.4) and frozen at −80°C until analysis. Before analysis, samples were thawed at room temperature (RT), and 200 µL of the CyQUANT® GR dye/cell-lysis buffer–containing mixture was added to each well and incubated for 5 min at RT. The plate was protected from light. Fluorescence was read on Victor X4 Multilabel Reader (PerkinElmer, Waltham, MA, United States) at 480 and 520 nm as excitation and emission wavelengths, respectively. The results were presented as the percent of the control value.

Cell Cycle Analysis

HaCaT cells (seeded on 6-well plates at 2×10^5 cells/well) were treated with 50 nM of rhPEPD^{WT}, rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K for 24 h. After incubation, cells were subjected to the protocol as published previously (Misiura et al., 2021b). Ethanol-fixed cells were analyzed using an image cytometer NC-3000 (ChemoMetec, Allerod, Denmark).

Cell Migration Assay

Confluent HaCaT cells, seeded at the density of 2×10^5 cells/well at 6-well plate, were scratched with a sterile 200 µL pipette tip, rinsed with PBS, and incubated with 25 nM of rhPEPD for 24 h. The gap area was monitored using an inverted optical microscope (40×; Nikon; Minato, Tokyo, Japan).

Preparation of Lysates

The cells were seeded at the density of 2×10^6 cells/plate and cultured with rhPEPD^{WT}, rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K for 30 min and 24 h. Cells were rinsed twice with cold PBS (pH 7.4) and harvested with RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, United States) containing protease inhibitor (cOmplete™ Protease Inhibitor Cocktail, Roche, Basel, Switzerland), phosphatase inhibitor cocktail (PhosSTOP, Roche, Basel, Switzerland) and viscolase (A&A Biotechnology, Gdańsk, Poland). Then, lysates were incubated on ice for 10 min and sonicated 3 times (15 s on and 5 s off) and centrifuged (4°C, 10 min, 12,000 × g). The

supernatant was aliquoted in 200 µL strip tubes and frozen at −80°C until protein analysis. The Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, United States) was employed for the quantification of protein concentration.

Western Immunoblotting

For Western immunoblotting, equal amounts (15 µg/lane) of proteins were diluted in RIPA lysis buffer (Thermo Fisher Scientific, Waltham, MA, United States) and mixed with Laemmli buffer (120 mM Tris-HCl, 20% glycerol, 0.4% SDS, and 0.02% bromophenol blue, pH 6.8) containing fresh 5% β-mercaptoethanol (Sigma Aldrich, Saint Louis, MO, United States). The samples were denatured at 99°C for 7 min. The proteins were separated on 10% SDS-PAGE gels and then blotted onto polyvinylidene difluoride (PVDF; BioRad Laboratories, Hercules, CA, United States) membranes. The membranes were blocked with either 5% non-fat dried milk (Santa Cruz Biotechnology, Dallas, TX, United States) or BSA (Sigma Aldrich, Saint Louis, MO, United States) in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.6) for 1 h at room temperature with agitation. The membranes were incubated with primary antibodies (listed below) overnight at 4°C, followed by incubation with alkaline phosphatase-linked goat antirabbit or antimouse antibodies for 1 h at RT. The membranes were washed three times in TBS-T for 5 min. The bands were visualized using 1-Step™ NBT/BCIP Substrate Solution (Thermo Fisher Scientific, Waltham, MA, United States) and their intensities were semiquantitatively measured with ImageJ software (https://imagej.nih.gov/ij/). All experiments were run in triplicates.

Antibodies

The membranes were incubated with the following primary antibodies purchased from Cell Signaling Technology (Danvers, MA, United States): Akt Rabbit mAb (1:2000), Cyclin D Rabbit mAb (1:1000), E-Cadherin Rabbit mAb (1:1000), EGF Receptor Rabbit mAb (1:1000), GAPDH Rabbit mAb (1:1000), HIF-1α Rabbit mAb (1:1000), IKKα Mouse mAb (1:1000), IKKβ Rabbit mAb (1:1000), IκBα Rabbit mAb (1:1000), Lamin A/C Mouse mAB (1:1000), N-Cadherin Rabbit mAb (1:1000), NF-κB p65 Rabbit Antibody (1:1000), p44/42 MAPK (ERK1/2) Rabbit mAb (1:1000), PCNA Rabbit mAb (1:1000), phospho-Akt (Ser473) Rabbit mAb (1:2000), phospho-EGF Receptor (Tyr1068) Rabbit mAb (1:1000), phospho-IKKα/β (Ser176/180) Rabbit mAb (1:1000), phospho-IκBα (Ser32) Rabbit mAb (1:1000), phospho-NF-κB p65 (Ser536) Rabbit mAb (1:1000), phospho-p44/42 MAPK (Thr202/Tyr204) Rabbit mAb (1:1000), phospho-Stat3 (Tyr705) Rabbit Ab (1:1000), Stat3 Rabbit mAb (1:1000), Cox2 Rabbit mAb (1:1000), TGF-β Receptor I Rabbit Antibody (1:1000), Thymidine Kinase 1 Rabbit mAb (1:1000). Secondary alkaline phosphatase-conjugated antimouse or antirabbit antibodies diluted 1:10,000 were from Sigma Aldrich (Saint Louis, MO, United States).

Gelatin Zymography Assay

The activities of MMP-2 and -9 in the medium released from the cells (seeded at the density of 2×10^6 cells/plate) were measured *via* a gelatin zymography protease assay as published by Wechselberger et al. (Wechselberger et al., 2019). After treatment, 5 ml of media were collected and concentrated using Vivaspin® 2 Centrifugal Concentrator (Vivaproducts Inc., Littleton, MA, United States). Protein concentration was measured using the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, United States). 20 µg/lane was loaded on to 1 mg/ml gelatin-10% SDS-PAGE gels. Following electrophoresis, the gels were washed with gelatinase renaturation buffer and subsequently incubated in the gelatinase reaction buffer at 37°C for 18 h. The gels were stained with the Coomassie staining method. The relative changes in the MMP-2 and -9 activities were scanned.

Statistical Analysis

All experiments were carried out at least three replicates and the experiments were repeated at least three times. Data are shown as a mean ± standard deviation (SD). For statistical calculations, a one-way analysis of variance (ANOVA) with Dunnett's correction and t-test were used. Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, United States). Statistically significant differences were marked as *, ^, $^{\#}p < 0.05$, **, ^, $^{\#\#}p < 0.01$, ***, ^, $^{\#\#\#}p < 0.001$ and ****, ^, $^{\#\#\#\#}p < 0.0001$; indicates * vs. control (0 nM of PEPD, without IL-1β) cells, ^ vs. control (0 nM of PEPD, with IL-1β) cells, # significance between groups treated with or without IL-1β.

RESULTS

rhPEPD^{WT} Augments IL-1β-Stimulated Cell Proliferation and Cell Cycle Progression in HaCaT Keratinocytes

The effect of human recombinant wild-type PEPD (rhPEPD^{WT}) on HaCaT cell viability and proliferation was measured by testing the mitochondrial activity and by quantifying DNA content, respectively. It was found that rhPEPD^{WT} at studied concentrations did not affect cell viability or cell proliferation of HaCaT cells (**Figures 1A,B**). However, in the presence of IL-1β, rhPEPD^{WT} slightly improved cell viability (at concentrations of 10–50 nM) and moderately induced cell proliferation (especially at concentrations of 10–25 nM) of HaCaT cells (**Figures 1A,B**).

IL-1β and rhPEPD^{WT} treatment contributed to a significant decrease in the percentage of cells in the G_1 phase (growth) and increased the percentage of cells in the G_2/M phase (mitosis), compared to control (**Figures 1C,D**).

It has been considered whether cell-cycle regulatory proteins may represent the underlying mechanism of rhPEPD^{WT}-dependent action on cell proliferation. Cyclin D controls G_1/S-phase transition and subsequently cell proliferation (Montalto and De Amicis, 2020). Thymidine kinase 1 is responsible for deoxythymidine triphosphate (dTTP) synthesis which is required for DNA biosynthesis

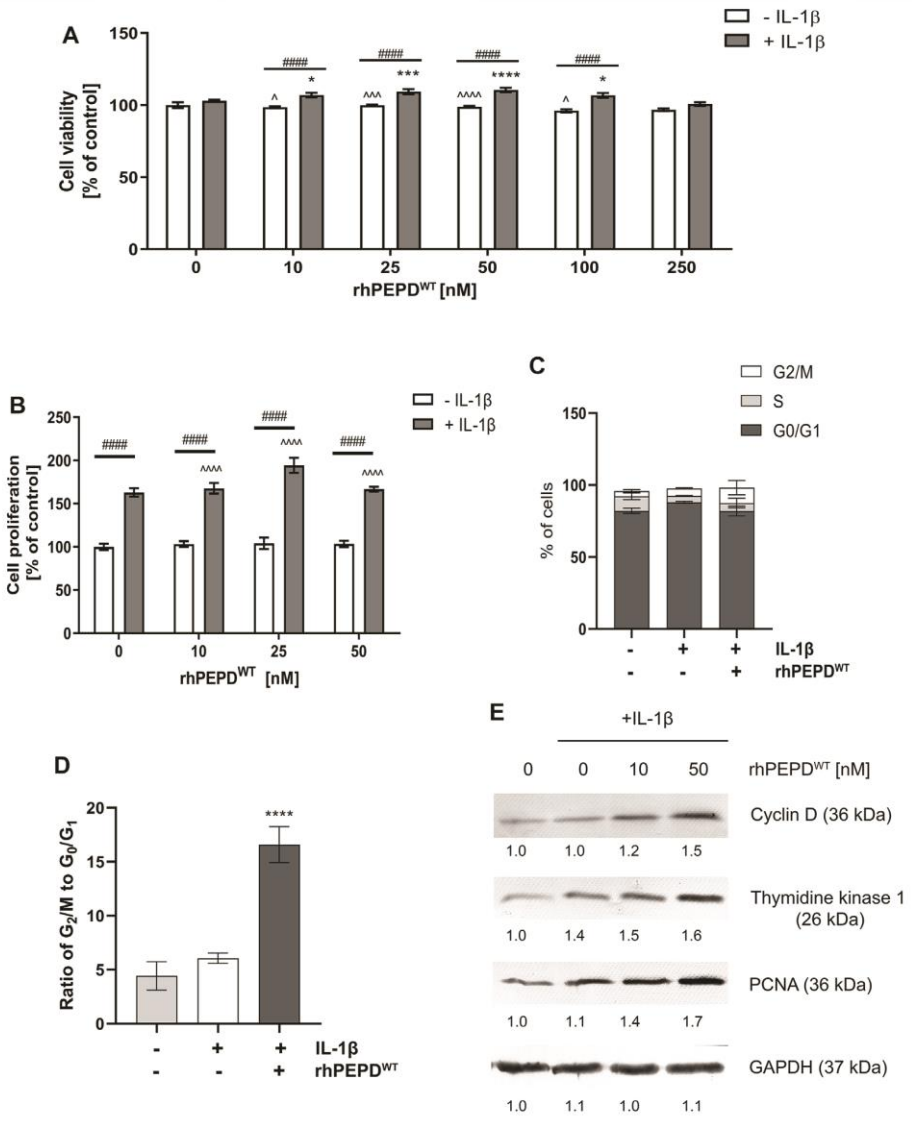

FIGURE 1 | Human recombinant prolidase (rhPEPD^{WT}, 10–250 nM) augments IL-1β (10 ng/ml)-induced cell proliferation in HaCaT cells: **(A)** Cell viability measured by the metabolic capacity of mitochondria; **(B)** cell proliferation measured as the level of incorporated fluorescent dye in DNA; **(C)** the percentage of cells in G_0/G_1, S, and G_2/M phases using DNA content-based cell analysis; **(D)** the ratio of G_2/M to G_0/G_1 phase using DNA content-based cell analysis; **(E)** Expression of the selected cell-cycle regulatory proteins. The data are presented as the mean ± SD, $n = 3$ in each group and compared to the control group. Representative blot images are shown (densitometry of protein stains is presented under protein bands as a ratio versus control; **Supplementary Figure S1**). GAPDH was used as loading control. Statistical significances were expressed as *,^; $^{\#}p < 0.05$, **,^; $^{\#\#}p < 0.01$, ***,^; $^{\#\#\#}p < 0.001$ and ****,^; $^{\#\#\#\#}p < 0.0001$; indicates * vs. control (0 nM of PEPD, without IL-1β) cells, ^vs. control (0 nM of PEPD, with IL-1β) cells, # significance between groups treated with or without IL-1β, respectively.

(Munch-Petersen, 2010). Proliferating Cell Nuclear Antigen (PCNA) participates in the critical step of DNA replication and replication-associated process, namely translation synthesis, error-free damage bypass, break-induced replication, mismatch repair, and chromatin assembly (Boehm et al., 2016). Western immunoblotting analysis showed that the expression of cyclin D, thymidine kinase 1, and PCNA were increased in PEPD- and IL-1β-stimulated HaCaT cells (**Figure 1E**). It suggests that in the presence of IL-1β, PEPD stimulates the proliferation of HaCaT cells *via* upregulation of the expression of cell-cycle regulatory proteins.

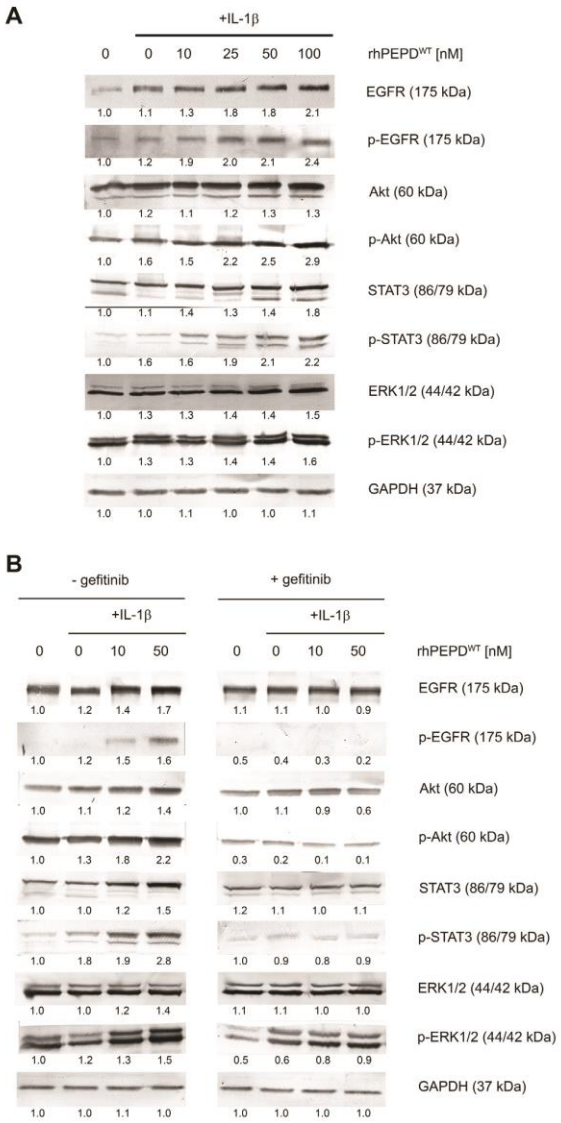

FIGURE 2 | rhPEPD^{WT} augments IL-1β-induced EGFR-downstream signaling pathway in HaCaT cell: **(A)** Western immunoblotting for the proteins of EGFR-downstream signaling pathway in lysates of rhPEPD-treated HaCaT cells (rhPEPD^{WT}, 10, 25, 50, 100 nM) for 15 min and 24 h in presence or absence of IL-1β (10 ng/ml). GAPDH was used as loading control; **(B)** Western immunoblotting for the proteins of EGFR-downstream signaling pathway in lysates of rhPEPD^{WT}-treated HaCaT cells (rhPEPD^{WT}, 10, 50 nM) and pretreated with an inhibitor of EGFR (gefitinib, 2 μM for 2 h) cultured for 15 min and 24 h in presence or absence IL-1β. GAPDH was used as a loading control. The data are presented as the mean ± SD, $n = 3$ in each group and compared to the control group. Representative blot images are shown (densitometry of protein stains is presented under protein bands as a ratio versus control; **Supplementary Figure S1**).

rhPEPD^{WT} Enhances the IL-1β-Induced EGFR-Downstream Signaling Pathway in HaCaT Cells

The effect of rhPEPD^{WT} combined with IL-1β on EGFR-downstream signaling pathways in HaCaT cells was evaluated by Western immunoblot analysis. Signal transduction is mediated by protein phosphorylation leading to activation or deactivation of many enzymes (kinases and phosphatases) and receptors (Ardito et al., 2017). EGFR-downstream signaling pathway involves Akt, ERK1/2, and STAT3. It was found that rhPEPD^{WT} (10–100 nM) in the presence of IL-1β (10 ng/ml) increased the expression of all studied EGFR-downstream signaling proteins (**Figure 2A**). Interestingly, rhPEPD^{WT} induced the expression of both total and phosphorylated forms. Phosphorylation of EGFR at Tyr1068 occurred after treatment with rhPEPD^{WT} in a dose-dependent manner. The expression of p-Akt (Ser473) was significantly increased in prolidase-treated HaCaT cells in comparison to control non-treated cells. Similarly, the phosphorylation of ERK1/2 (Thr202/Tyr204) and STAT3 (Tyr705) was more pronounced in the cells cultured in the presence of prolidase and IL-1β than in non-treated cells.

These results were confirmed by an experiment showing that pharmacological blockage of EGFR abolished rhPEPD^{WT}-dependent effects. Gefitinib (2 μM, 2 h), a specific EGFR inhibitor, was used to suppress rhPEPD^{WT}-induced EGFR-downstream signaling (**Figure 2B**). The inhibitor strongly diminished the PEPD-induced of EGFR, Akt, STAT3, and ERK1/2 phosphorylation (**Figure 2B**), indicating that in the presence of IL-1β, rhPEPD^{WT} stimulates anabolic processes through EGFR downstream signaling pathway.

rhPEPD^{WT} Promotes EMT *via* TGF-β1R and Cox-2 Pathway in IL-1β-Treated HaCaT Cells

Since rhPEPD in the presence of IL-1β induces EGFR-downstream signaling and enhances cell proliferation and possibly cell migration, it has been considered whether the mechanism underlying this process may involve EMT. Previous reports demonstrated an EGFR-dependent increase in cell mobility and changes in cellular conjunctions (Wilkins-Port and Higgins, 2007; Stoll et al., 2012). However, the mechanism responsible for EMT is not limited to EGFR activation. TGF-β_1 receptor (TGF-β_1R) mediates EMT through MAPK signaling, including ERK1/2 and p38 (Hahn et al., 2016) and Cox-2 signaling pathway (Neil et al., 2008; Räsänen and Vaheri, 2010). To examine whether EMT occurred, we evaluated the expression of the selected proteins by Western immunoblot. PEPD induced downregulation of E-cadherin and upregulation of HIF-1α, TGF-β_1R, Cox-2, and N-cadherin in the response of IL-1β,

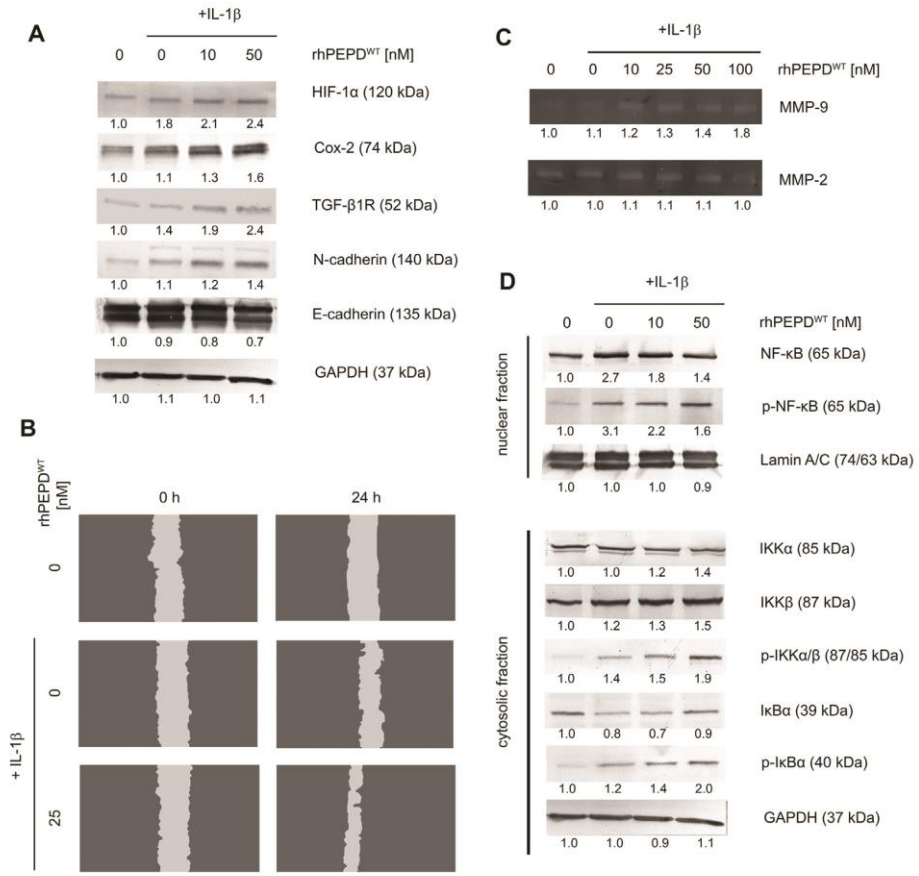

FIGURE 3 | (A) Western immunoblotting for the proteins involved in EMT in lysates of rhPEPD-treated HaCaT cells (rhPEPD^{WT}, 10, 50 nM) and IL-1β (10 ng/ml) for 24 h. GAPDH was used for normalization; **(B)** The effect of rhPEPD on HaCaT cell migration under conditions of IL-1β-induced inflammation; **(C)** The activity of MMP-2 and -9 in the collected culture media from HaCaT cells treated with various rhPEPD^{WT} concentrations (10, 25, 50, 100 nM) and IL-1β; **(D)** Western immunoblotting for the proteins of NF-κB pathway activation in lysates of rhPEPD-treated HaCaT cells (rhPEPD^{WT}, 10, 50 nM) for 30 min in the presence or absence of IL-1β (10 ng/ml). GAPDH was used as a loading control. The data are presented as the mean ± SD, $n = 3$ in each group and compared to the control group. Representative blot images were shown (densitometry of protein stains is presented under protein bands as a ratio versus control; **Supplementary Figure S1**).

suggesting that rhPEPD^{WT}-dependent mechanism for EMT undergoes through TGF-β_1R and Cox-2 pathway leading to increase in cell motility (**Figure 3A**). To support the statement on cell motility, the cell migration assay was performed. As shown in **Figure 3B**, the cell migration was improved upon IL-1β-treatment, however, rhPEPD remarkably augmented this process.

rhPEPD^{WT} Activates MMP-9 Through the NF-κB Pathway in IL-1β-Treated HaCaT Cells

Since EMT occurred as described above, another biochemical event strictly related to this phenomenon was investigated such as the activation of matrix metalloproteinases. Using zymography, the activity of MMP-2 and -9 was evaluated in culture media. rhPEPD^{WT} in the presence of IL-1β induced MMP-9 activity in a dose-dependent manner, while MMP-2 remained slightly activated in comparison to non-stimulated cells (**Figure 3C**). Several reports indicate that the NF-κB pathway is mainly involved in growth factor or cytokines-induced MMP-9 activation (Eberhardt et al., 2000; Lee et al., 2008). Therefore, the expression of the NF-κB family proteins was evaluated by Western immunoblot. Upon rhPEPD^{WT} stimulation IκB kinases, IKKα and β, phosphorylation occurred at Ser176/180. Then, NF-κB was activated by degradation of IκBα releasing p65 subunit from the IκBα/NF-κB complex. Cytosolic IκBα was degraded and accompanied by an increase in the level of p-IκBα and p-IKKα/β (**Figure 3D**). These observations elucidate an increase in nuclear localization of NF-κB in HaCaT keratinocytes resulting in the rise of MMP-9 activity.

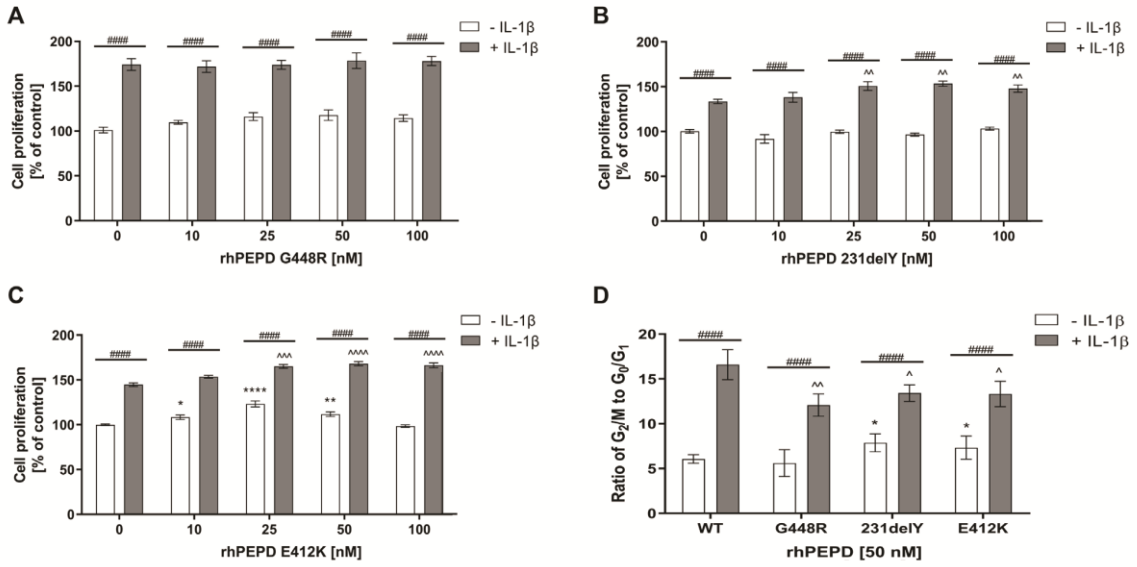

FIGURE 4 | The effect of mutated variants of PEPD **(A)** rhPEPD-G448R; **(B)** rhPEPD-231delY; **(C)** rhPEPD-E412K on HaCaT cell proliferation; **(D)** Cell cycle analysis presented as a ratio of the cell percentage in G_2/M to G_0/G_1 phase in HaCaT cells cultured in the presence and absence of IL-1β (10 ng/ml). The cells were treated with studied compounds for 24 h. Statistical significances were expressed as $^{*,\hat{}, \#}p < 0.05$, $^{**,\hat{}, \#\#}p < 0.01$, $^{***,\hat{}, \#\#\#}p < 0.001$ and $^{****,\hat{}, \#\#\#\#}p < 0.0001$; indicates * vs. control (0 nM of PEPD, without IL-1β) cells, ^ vs. control (0 nM of PEPD, with IL-1β) cells, # significance between groups treated with or without IL-1β, respectively.

Mutated Variants of PEPD Differentially Affect IL-1β-Induced Cell Proliferation and EGFR Signaling in HaCaT Cells

As wild-type PEPD was shown to accelerate proliferation in IL-1β-induced HaCaT cells, it has been considered whether some mutated PEPD (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) would evoke an opposite effect on the process. All these variants occur naturally in patients with prolidase deficiency (PD) and manifested skin ulcers (Besio et al., 2013). Firstly, the effect of mutated variants of PEPD on IL-1β-induced HaCaT cell proliferation and cell cycle was studied. The physicochemical analysis of mutated variants of PEPD was described by Besio et al. (Besio et al., 2013).

Variant rhPEPD-G448R did not induce the proliferation of HaCaT cells both in the presence and absence of IL-1β (**Figure 4A**). However, rhPEPD-E412K and rhPEPD-231delY mutants in the presence of IL-1β increased the cell proliferation significantly, especially at 25, 50, and 100 nM concentrations, while in the absence of IL-1β only rhPEPD-E412K affected the process (**Figures 4B,C**). The analysis of the cell cycle confirmed the findings. HaCaT cultured with the PEPD mutants in the absence of IL-1β did not change significantly the ratio of G_2/M to G_1/G_0. The cells cultured with wild-type PEPD in the presence of IL-1β showed a drastic increase in the ratio, namely significantly decreased the percentage of cells in the G_0/G_1 phase and increased the percentage of cells in the G2/M phase, compared to control cells cultured in the absence of IL-1β (**Figure 4D**). However, treatment of the cells with PEPD mutants in the presence of IL-1β decreased significantly the ratio of G_2/M to G_1/G_0 with the more pronounced effect in the case of rhPEPD-G448R, suggesting lower potency of this mutant protein to induce EGFR-dependent stimulation of cell proliferation.

As shown in **Figure 5**, all studied PEPD mutants in the presence of IL-1β induced phosphorylation of EGFR and some downstream signaling proteins (Akt, ERK1/2, STAT3) as detected by Western immunoblot. rhPEPD-G448R as a ligand of EGFR was able to stimulate downstream signaling proteins, however, cell cycle analysis showed that the response was weaker compared to other PEPD mutants (rhPEPD-231delY and rhPEPD-E412K) that induced expression of p-Akt, p-STAT3, and p-ERK1/2. The data show that some mutated variants of PEPD in the presence of IL-1β evoke the ability to induce EGFR-dependent HaCaT cell proliferation.

DISCUSSION

To the best of our knowledge, this is the first report on the cross-talk between PEPD and IL-1β in keratinocyte proliferation, a process that could be of great importance in wound healing. Impaired wound healing is observed in numerous conditions such as acute and chronic diseases, aging, or post-surgery (Eming et al., 2014), thus understanding the complex regulatory mechanism of tissue regeneration is our interest. Based on the finding of Yang et al. (Yang et al., 2013) that PEPD is an EGFR

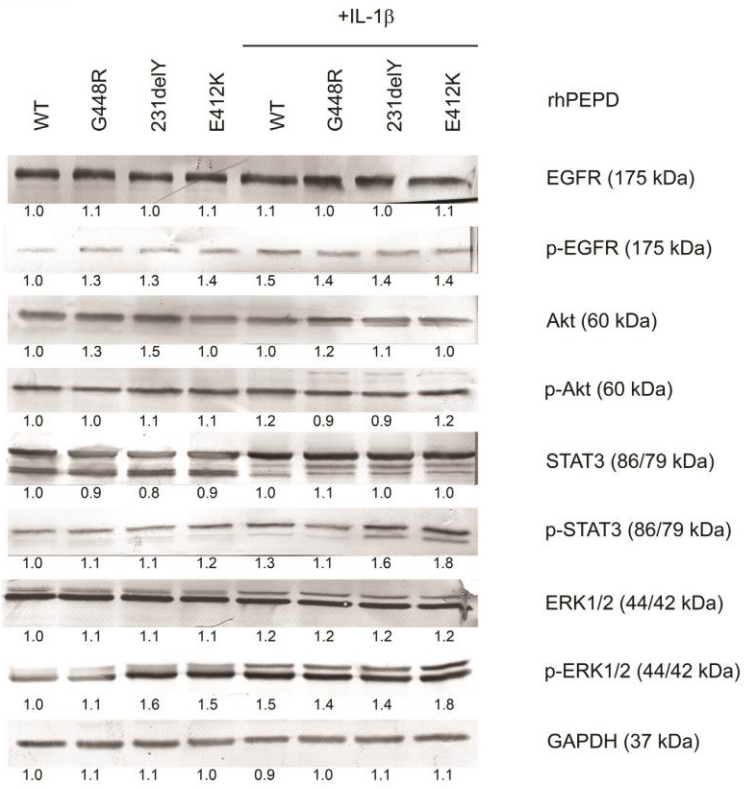

FIGURE 5 | The effect of rhPEPD^{WT} and PEPD mutants (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) on EGFR-downstream signaling proteins in IL-1β treated and non-treated HaCaT cells. GAPDH was used as a loading control. The data are presented as the mean ± SD, $n = 3$ in each group and compared to the control group. Representative blot images were shown (densitometry of protein stains is presented under protein bands as a ratio versus control; **Supplementary Figure S1**).

ligand and our recent study (Misiura et al., 2020), we hypothesized that extracellular PEPD under inflammatory conditions may remarkably contribute to cell proliferation facilitating wound repair. Therefore, this study was conducted to investigate the biological effects of PEPD on human keratinocytes in an *in vitro* model of IL-1β-induced inflammation using HaCaT immortalized human keratinocytes.

We found that PEPD in the presence of IL-1β significantly augmented keratinocyte proliferation through EGFR signaling. Treatment of keratinocytes with IL-1β contributes to the hyperproliferative and migratory phenotype of the cells (Franchi et al., 2009). Indeed, it is well established that IL-1β and EGFR are over-expressed in wounded skin, particularly during the inflammatory phase (Barrientos et al., 2008; Yoshida et al., 2008). The key finding of our study is that PEPD activates EGFR-downstream signaling proteins including Akt, ERK1/2, and STAT3, which are implicated in keratinocyte migration, proliferation, and epithelialization during the inflammatory phase of the wound healing process. PEPD-induced Akt and MAPK signaling in HaCaT cells was reported previously (Misiura et al., 2020).

The finding that PEPD is the ligand of EGFR (Yang et al., 2013) was thoroughly validated. The researchers compared the affinity of EGF and PEPD to the EGFR extracellular domain and found that EGF is a more potent ligand than prolidase. However, EGF can displace PEPD from its complex with EGFR. Interestingly, PEPD and EGFR colocalize the cell membrane indicating a ligand-receptor relationship. In our study, we also addressed the question of whether EGFR-mediated observations are dependent on PEPD. Thus, we employed a pharmacological EGFR inhibitor, gefitinib. Inhibition of PEPD-dependent EGFR activation by gefitinib led to a decrease in the amount of phosphorylated and total forms of EGFR and Akt, ERK1/2, and STAT3 confirming that PEPD is a ligand of this receptor. We found that PEPD exerts cell cycle progression in keratinocytes *via* regulating G_1, S, and G_2/M phases. The entry of eukaryotic cells into mitosis is strictly regulated at several steps including cyclin D, thymidine kinase 1, PCNA, and others. Indeed they all were upregulated in response to PEPD. The data correspond to the study conducted by Kim et al. (Kim et al., 2013) who presented upregulation of cyclin D in growth factor-stimulated HaCaT cells. Thus, the activation of cyclins may influence the epidermal cells to promote the wound healing process.

However, PEPD-dependent stimulation of keratinocyte proliferation requires the participation of IL-1β. Although the

mechanism for the process is not known it has been previously suggested that EGFR and IL-1β signaling synergistically promote keratinocyte proliferation and differentiation (Johnston et al., 2011). We found that during stimulation of EGFR in the presence of IL-1β the EMT occurred, as detected by changes in the expression of EMT markers such as E-cadherin and N-cadherin. Besides the downregulation of E-cadherin and upregulation of N-cadherin, Cox-2 and TGF-β_1R were significantly pronounced upon rhPEPD^{WT} treatment. Several papers suggest that EMT is mediated through the aforementioned pathways which are consistent with our study (Neil et al., 2008; Räsänen and Vaheri, 2010; Stoll et al., 2012). The phenomenon was accompanied by an increase in the activity of MMP-9. MMPs are secreted by keratinocytes to digest ECM constituents in response to external stimuli. The activation of MMPs is essential during the inflammatory and reepithelialization phases of wound healing and regulates the EMT process (Yang et al., 2017).

Moreover, we demonstrated that total NF-κB expression was drastically increased due to IL-1β treatment and gradually decreased under PEPD treatment of keratinocytes, however, NF-κB phosphorylation was remarkably high, similarly to phosphorylated forms of IκB kinases (IKKα/β) and IκBα. It is well established that inflammation activates the NF-κB and MAPK signaling processes (Solt and May, 2008). Activated NF-κB then enters the nucleus, inducing gene transcription involved in the inflammatory response. In quiescent cells, NF-κβ in the cytosol is bound to its inhibitory molecule, IκBα protein. Upon stimulation, IκBα is phosphorylated by the upstream kinases, IKKα and β, which induces the ubiquitination and degradation of IκBα in proteasomes, subsequently leading to the phosphorylation and translocation of NF-κB into the nucleus (Chen et al., 1999). Activated NF-κB binds to specific DNA sequences and regulates the expression of its target genes. Interestingly, in the present study, we found that MMP-9 was activated suggesting the increased ability of HaCaT cells to digest surrounding ECM and migrate. The link between increased MMP-9 activity and activation of NF-κB was indicated by Eberhardt et al. (Eberhardt et al., 2000) who identified the promoter region in the *MMP-9* gene containing a binding site for nuclear factor κB. Thus, in rhPEPD^{WT}-mediated MMP-9 activation, the NF-κB pathway is involved.

Keratinocytes are the source and target for cytokines. A vast range of inflammatory mediators is expressed and secreted by keratinocytes that have multiple consequences not only for inflammatory cells through the promotion of leukocytes migration, amplification of inflammatory responses but also on keratinocytes to promote their proliferation and differentiation processes (Jiang et al., 2020). Thus, under experimental scratch conditions, while the cell membrane is disrupted, a variety of inflammatory mediators may be released from keratinocytes. The interplay between prolidase and secreted mediators of inflammation contributes to the induction of cell proliferation, growth, and migration as presented previously (Misiura et al., 2020). The current research on the effect of prolidase in IL-1β-induced inflammation supports the hypothesis that prolidase in the presence of IL-1β strengthens the proliferative and migratory capacity of keratinocytes. The molecular mechanism underlying PEPD-induced cell proliferation and growth undergoes through EGFR signaling, cell cycle progression, EMT, as well as matrix remodeling. It cannot be excluded that IL-1β stimulates the release of PEPD from keratinocytes, however, it needs further experiments supporting this hypothesis. Based on the experience, it was found that prolidase is expressed by keratinocytes, although PEPD activity is low. To date, the system for prolidase transport outside the cell remains unknown unless the cell membrane is discontinued. Under chemically-induced cell disruption PEPD concentration significantly increases (Yang et al., 2013). Another possible source of prolidase can be platelets in the bloodstream (Guszczyn et al., 2017; Misiura et al., 2021a). Platelets are essential players in the initial stage of inflammation as they carry various inflammatory mediators. Upon activation and degranulation of platelets, growth factors and prolidase-containing load is released close to the wounded area. It is known that platelet-rich plasma is used in regenerative medicine facilitating the recovery from tissue injuries (Marx, 2001; Amable et al., 2013; Etulain, 2018; Emer, 2019).

So far, the functional significance of PEPD was found in PEPD deficiency (PD, OMIM 170100). This is a rare autosomal recessive disorder characterized by massive imidodipeptiduria, skin lesions, and elevated proline-containing dipeptides in plasma (Scriver, 1964; Goodman et al., 1968; Powell and Maniscalco, 1976; Umemura, 1978; Isemura et al., 1979; Freij et al., 1984; Pierard et al., 1984). Currently, it is believed that mutations in the *PEPD* gene explain the molecular basis for PD, and several mutated alleles were found (Endo and Matsuda, 1991; Ledoux et al., 1996; Lupi et al., 2006b). However, it is difficult to indicate the exact cause for PD since clinical phenotype is not always related to genotype (Lupi et al., 2008). To date, PD is diagnosed by low or a lack of PEPD activity, however, the clinical outcome may be due to deprivation of extracellular function of PEPD. This hypothesis would be confirmed since PD therapy was unsuccessful with the application of proline or proline-convertible amino acids (Kitchener and Grunden, 2012). As PD remains incurable, we sought to explore the effect of the selected mutated variants of PEPD (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) on EGFR-downstream proteins under IL-1β-induced inflammation. Interestingly, EGFR-downstream protein analysis showed that some mutated variants of PEPD (rhPEPD-231delY and rhPEPD-E412K) were able to activate EGFR-dependent intracellular signal and induce HaCaT cell proliferation stronger than another mutated variant (rhPEPD-G448R). The possible explanation for weaker ligand properties of rhPEPD-G448R could be its secondary structure abnormality (Besio et al., 2013). Due to the low purification yield of rhPEPD-G448R, Besio et al. were not able to analyze the mutant structure by spectroscopy analysis, however, they found using *in silico* analysis that substitution glycine into arginine at position 448 resulted in the improper protein architecture, suggesting that G448 is necessary for maintaining the enzymatic activity of prolidase. On contrary, the secondary structure compositions of rhPEPD-231delY and rhPEPD-E412K were slightly different from wild-type protein and

characterized by an increase in random coil while the contribution of α-helices and β-sheets were decreased. Given together, it is probable that the EGFR ligand properties of the studied rhPEPD correspond to their architecture. Accordingly, Yang et al. (Yang et al., 2015; 2016) performed research with a mutated form of prolidase (rhPEPD-G278D) and observed that even inactive enzyme acts as a functional ligand. Based on these results a question arise of whether symptoms in PD patients result from low or lack of intracellular activity or lack of extracellular PEPD function. Data presented in this report suggest that the extracellular function of PEPD is of great importance in EGFR-dependent stimulation of keratinocyte proliferation in conditions of experimental inflammation. Our study suggests that cell proliferation and the intracellular responses upon activation of EGFR by PEPD mutants are weaker than in the case of PEPD WT. It seems to match the clinical outcomes as PD patients manifest a wide range of symptoms (Besio et al., 2013). It suggests that not only PEPD activity but an extracellular function of PEPD may be involved in the mechanism underlying prolidase deficiency. Similar effects were reported for a keratinocyte growth factor (KGF), another ligand of EGFR inducing cell proliferation as a mechanism for alveolar epithelial repair (Atabai et al., 2002). Interestingly, IL-1 that was found in elevated concentrations in the pulmonary edema fluid of patients with acute lung injury, promoted *in vitro* alveolar epithelial repair through an EGFR pathway (Geiser et al., 2000). However, the repair effect was independent of its mitogenic effect. It has been suggested that the augmented rate of epithelial repair in these conditions is the result of enhanced cell spreading and migration, but not cell proliferation (Atabai et al., 2002). Whether wild-type PEPD, as well as mutated variants of PEPD, could stimulate similar mitogenic independent effects on the wound healing process requires to be explored.

Thus, the biological activity of PEPD and its genetic variants is of emerging research interest. So far, it is known that PEPD act as a regulator of p53 function, affects interferon-α/β receptor maturation, and is a ligand of EGFR and epidermal growth factor receptor 2 (HER2) (Misiura and Miltyk, 2020). Our results demonstrate for the first time that PEPD activates EGFR-dependent cell growth in an experimental model of inflammation in HaCaT keratinocytes and the knowledge may be useful for further approaches for therapy of wound healing disorders.

CONCLUSION

The data presented in this report suggest that human recombinant wild-type rhPEPD, as well as some mutated variants of PEPD, activate, through EGFR-dependent signaling, cell proliferation, and ECM remodeling in an experimental model of inflammation in HaCaT keratinocytes. The data suggest that both enzymatically active and inactive rhPEPD may modulate, even if with different intensity, EGFR signaling and the knowledge may be useful for further approaches for therapy of wound healing disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization, MN, IO, JP and WM; methodology, MN, RB and IO; investigation, MN, RB and WB; data curation, MN, WB, and IO; writing-original draft preparation, MN; writing-review and editing, JP, AF and WM; visualization, MN and IO; supervision, WM; funding acquisition, WM. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.876348/full#supplementary-material

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11. *Streszczenie*

Gojenie ran to ściśle regulowany proces, w którym występują cztery fazy. Prawidłowe gojenie rozpoczyna się od tworzenia skrzepów, po którym następuje naciekanie komórek zapalnych, proliferacja komórek nabłonka i wreszcie zachodzi przebudowa macierzy. W wyniku zaburzenia gojenia się ran mogą wystąpić przewlekłe stany zapalne i owrzodzenia. Jednym z najczęściej uszkadzanych narządów jest skóra. Zbudowana jest z komórek takich jak keratynocyty, fibroblasty, makrofagi i komórki śródbłonka. W warstwach naskórka około 95% masy komórek stanowią keratynocyty. Jako komórki pierwszej linii stykają się z bakteriami chorobotwórczymi, wirusami, promieniowaniem UV i alergenami co prowadzi do wytwarzania cytokin prozapalnych i progresji stanu zapalnego skóry. Podczas gojenia zaangażowane są również receptory czynników wzrostu, metaloproteinazy (MMP), mediatory stanu zapalnego oraz enzymy ściśle współpracujące z komórkami w celu przywrócenia funkcjonalności uszkodzonej tkanki. Niedawno odkryto, że PEPD jest ligandem EGFR. Ponieważ aktywacja EGFR sprzyja proliferacji, wzrostowi, różnicowaniu i migracji komórek, powstało pytanie, czy prolidaza może być czynnikiem stymulującym gojenie się ran *in vitro*.

Niniejsze badania miały na celu zbadanie zdolności proliferacyjnej prolidazy w modelach doświadczalnego gojenia się ran w warunkach zapalenia indukowanego przez interleukinę (IL)-1β i mechanicznego uszkodzenia keratynocytów linii HaCaT.

Ludzkie keratynocyty linii HaCaT poddano działaniu prolidazy (świńska lub ludzka rekombinowana) i oceniano przeżywalność, proliferację i migrację komórek. Do oceny ekspresji białek zastosowano Western-immunoblot i technikę immunocytochemiczną z wykorzystaniem mikroskopu konfokalnego. Biosyntezę kolagenu oznaczano metodą radiometryczną, a aktywność prolidazy metodą kolorymetryczną. Do pomiaru stężenia proliny zastosowano celowaną analizę metabolomiczną z wykorzystaniem chromatografii cieczowej sprzężonej ze spektrometrią mas. Aktywność MMP oceniano za pomocą zymografii żelatynowej, podczas gdy analizę cyklu komórkowego analizowano za pomocą cytometrii obrazowej.

Przeprowadzone badania ujawniły, że PEPD w warunkach uszkodzenia mechanicznego indukował proliferację i migrację keratynocytów poprzez aktywację sygnalizacji EGFR, w której kluczową rolę odgrywał szlak Akt/PI3K/mTOR. Markery białkowe przejścia nabłonkowo-mezenchymalnego uległy zwiększeniu, co potwierdziło obserwację zwiększonej migracji komórek. Ekspresja receptorów β1-integryny i IGF-1 oraz zależnych od nich kinaz uległa zwiększeniu, czemu towarzyszyło wyższe stężenie proliny i biosynteza kolagenu. W stanach zapalnych PEPD wymagała obecności IL-1β w celu zwiększenia proliferacji

keratynocytów poprzez aktywację EGFR i zależnych od EGFR białek sygnałowych (Akt, ERK1/2 i STAT3). Migrujące komórki wykazywały ekspresję markerów białkowych EMT, takich jak obniżona ekspresja E-kadheryny i zwiększona ekspresja N-kadheryny. Przebudowa macierzy zewnątrzkomórkowej zachodząca w fazie zapalnej została odzwierciedlona poprzez aktywację MMP-9 związaną z aktywacją NF-κB poprzez degradację IκBα z udziałem IKK. Zmutowane PEPD (rhPEPD-G448R, rhPEPD-231delY i rhPEPD-E412K) były również zdolne do pobudzenia proliferacji keratynocytów poprzez szlak EGFR.

Prolidaza zewnątrzkomórkowa działająca poprzez EGFR indukuje wzrost, migrację i biosyntezę kolagenu oraz przebudowę ECM w keratynocytach HaCaT w warunkach eksperymentalnego gojenia ran. Szlaki Akt/PI3K/mTOR, ERK1/2 i STAT3 biorą udział w proliferacji i migracji keratynocytów. Aktywność prolidazy nie jest wymagana do aktywacji EGFR. Enzymatycznie aktywna i nieaktywna prolidaza może modulować sygnalizację EGFR z różną intensywnością. Niniejsze badania sugerują, że nie tylko aktywność PEPD, ale także zewnątrzkomórkowa funkcja PEPD może być zaangażowana w mechanizm leżący u podstaw niedoboru prolidazy. Endogenna prolidaza może służyć jako czynnik stymulujący procesy naprawcze uszkodzonych komórek, którym towarzyszy stan zapalny. Proces ten może stanowić potencjalny punkt uchwytu w terapii ran skóry.

12. Abstract

Recovery from injury, known also as wound healing, is a precisely regulated process in which four phases occur. The proper healing starts from clot formation, inflammatory cell infiltration, re-epithelization, and finally, matrix remodeling. As a result of a delay in wound healing chronic inflammation and ulcers may happen. Skin is one the most frequently injured organ involving a variety of cells playing distinct roles such as keratinocytes, fibroblasts, macrophages, and endothelial cells, however, in the epidermal layers, keratinocytes comprise about 95% of the cell mass. They serve as the first line cells which encounter pathogenic bacteria, viruses, UV radiation, and allergens leading to the generation of pro-inflammatory cytokines and skin inflammation progression. The machinery of the healing process involves also growth factor receptors, metalloproteinases (MMPs), inflammatory mediators, and enzymes that closely cooperate with the cells to restore the functionality of the injured tissue. Recently PEPD was found to be a ligand of the EGFR. As activation of EGFR signaling promotes cell proliferation, growth, differentiation, and migration, the question was raised whether prolidase may be a stimulating factor for wound healing *in vitro*.

This study aimed to investigate the proliferative capacity of prolidase in models of experimental wound healing under conditions of interleukin (IL)-1β-induced inflammation and mechanical damage in HaCaT keratinocytes.

Immortalized human HaCaT keratinocytes were treated with prolidase (porcine or recombinant human) and cell viability, vitality, proliferation, and migration were assessed. Western immunoblotting and immunocytochemical staining coupled to a confocal microscope were employed to evaluate the protein expression. Determination of collagen biosynthesis and prolidase activity was assayed with radiometric and colorimetric methods, respectively. The liquid chromatography coupled with mass spectrometry was applied for the measurement of proline concentration. MMP activity was evaluated with gelatin zymography assay while cell cycle analysis was analyzed with image cytometry.

The study revealed that PEPD, under scratched conditions, induced cell proliferation and migration via activation of EGFR-downstream signaling in which the PI3K/Akt/mTOR pathway played an essential function. The protein markers of epithelial-to-mesenchymal transition were upregulated and supported the observation of enhanced cell motility. The expression of β_1-integrin and IGF-1 receptors and their downstream kinases were upregulated and it was accompanied by higher proline concentration and collagen biosynthesis. While under inflammatory conditions, PEPD required the presence of IL-1β to augment keratinocyte proliferation through activation of EGFR and its downstream signaling proteins (Akt,

ERK1/2, and STAT3). Migrating cells expressed the EMT protein markers such as downregulated E-cadherin and upregulated N-cadherin. Extracellular matrix remodeling occurring in the inflammatory phase was reflected by the activation of MMP-9. It may result from activation of NF-κB via IKK-mediated IκBα degradation. Interestingly, mutated PEPD (rhPEPD-G448R, rhPEPD-231delY, and rhPEPD-E412K) were able to activate EGFR-mediated keratinocyte proliferation.

Extracellular prolidase acting through EGFR induces growth, migration, and collagen biosynthesis and ECM remodeling in HaCaT keratinocytes under the conditions of experimental wound healing. PI3K/Akt/mTOR, ERK1/2, and STAT3 pathways are involved in the proliferation and migration of keratinocytes. Prolidase activity is not required for EGFR activation. Enzymatically active and inactive prolidase may modulate EGFR signaling with different intensities. The study suggests that not only PEPD activity but also an extracellular function of PEPD may be involved in the mechanism underlying prolidase deficiency. Prolidase may serve as a stimulating factor in injured cells accompanied by inflammation and represent a therapeutic approach to treating skin wounds.

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14. Authorship Contribution Statements

Misiura M., Miltyk W.: *Current Understanding of the Emerging Role of Prolidase in Cellular Metabolism*. International Journal of Molecular Sciences. 2020; 21, 5906. doi: 10.3390/ijms21165906.

Author's name and surname	Nature of participation	Contribution in %
PD student – Magdalena Nizioł, M.Sc.	Conception and design of the review, literature review, writing-original draft preparation, visualization, manuscript preparation, writing the responses to the reviewers	85 %
Prof. dr hab. Wojciech Miltyk	Critical revision of the manuscript for important intellectual content and assistance in writing responses to the reviewers	15 %

Misiura M., Baszanowska W., Ościłowska I., Pałka J., Miltyk W.: P*rolidase Stimulates Proliferation and Migration through Activation of the PI3K/Akt/mTOR Signaling Pathway in Human Keratinocytes*. International Journal of Molecular Sciences. 2020; 21, 9243. doi: 10.3390/ijms21239243.

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Weronika Baszanowska, M.Sc.	Co-participation in biochemical investigation	10 %
Dr. Ilona Ościłowska	Co-participation in data curation, assistance in the preparation of figures and manuscript	10 %
Prof. dr hab. Jerzy Pałka	Critical review of the manuscripts and assistance in writing responses to the reviewers	5 %
Prof. dr hab. Wojciech Miltyk	Funding acquisition, review of the manuscripts, and assistance in writing responses to the reviewers	5 %

Nizioł M., Ościłowska I., Baszanowska W., Pałka J., Besio R., Forlino A., Miltyk W.: *Recombinant Prolidase Activates EGFR-Dependent Cell Growth in an Experimental Model of Inflammation in HaCaT Keratinocytes. Implication for Wound Healing.* Frontiers in Molecular Biosciences. 2022; 9:876348. doi: 10.3389/fmolb.2022.876348.

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I hereby declare that all co-authors agreed to use these articles in the dissertation of Magdalena Nizioł.

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which are a part of the doctoral dissertation of Ms. Magdalena Nizioł, my contribution included co-participation in data curation, assistance in the preparation of figures and manuscripts.

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