

Supplementary Material

Pepsin Promotes Activation of Epidermal Growth Factor Receptor and Downstream Pathways, at Slightly Acidic and Neutral pH, in Exposed Hypopharyngeal Cells

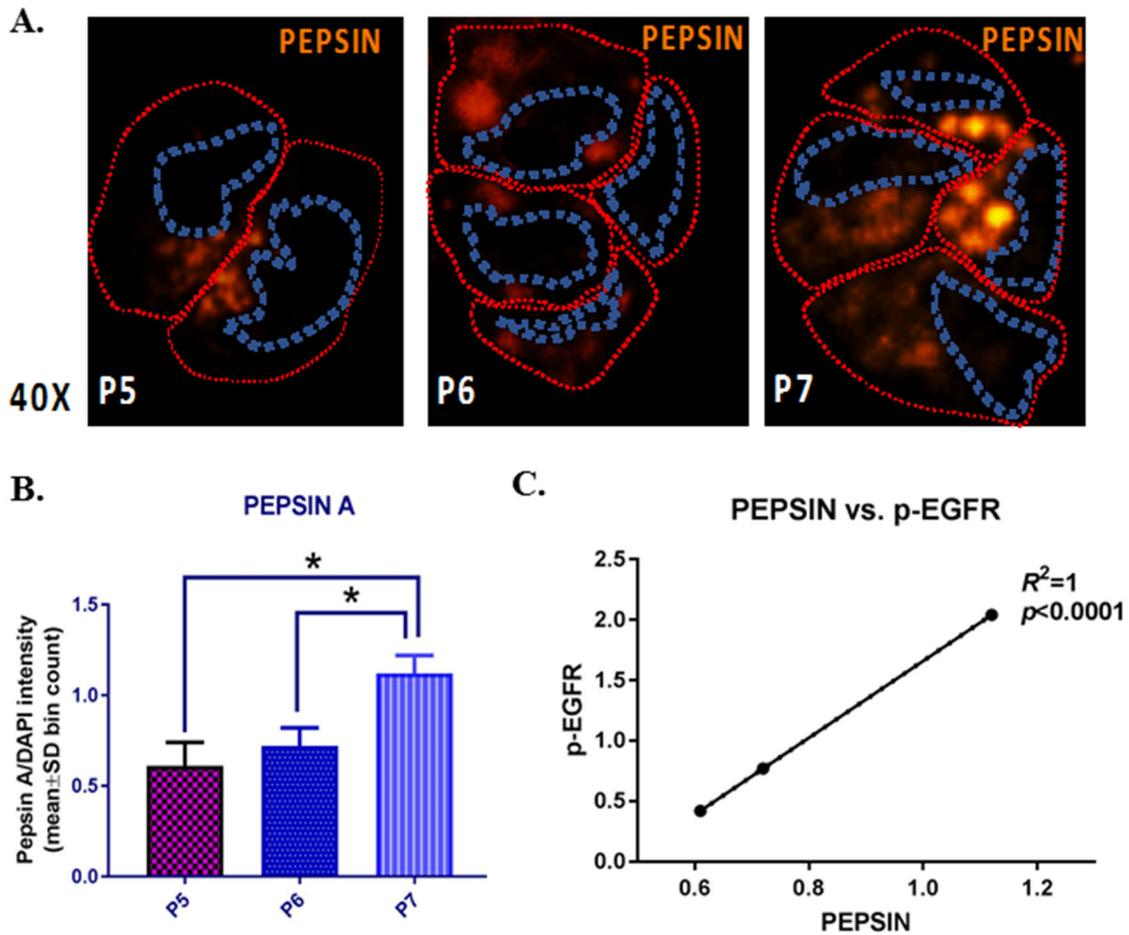
Supplementary methods

Enzyme-Linked Immunosorbent Assay for Total MSH2 Quantification: We performed a direct enzyme-linked immunosorbent assay (ELISA) to quantify cytoplasmic p-EGFR, and nuclear NF- κ B, and p-STAT3 and expression levels. Nunc MaxiSorp™ 96-well plates (Invitrogen by Thermo Fisher Scientific) were coated with 100 μ L of cytoplasmic and nuclear protein extracts from human hypopharyngeal primary cells (HCs), at a concentration of 10 μ g/mL, in 1X coating buffer [protein added to coating buffer and mixed for 15 min; 1X coating buffer diluted from 5X stock (BUF030A; BIORAD) in dH₂O and mixed for 15 min]. The plates were covered and incubated at 4 °C overnight. The next day, the plates were washed 3 times in wash buffer (PBST; 0.05% v/v Tween-20 in PBS), incubated in 150 μ L/well of blocking solution (1% w/v BSA in PBS) for 60 min at 37 °C, and then washed 4 times in wash buffer and incubated for 1 h at 37 °C, in 100 μ L/well of primary p-EGFR (Clone F-3), p-STAT3 (clone B-7), NF- κ B (Clone F-6), and β -actin (Clone C4; which was used as a reference control for cytoplasmic protein normalization) or Histone 1 (AE-4; which was used as a reference control for nuclear protein normalization) mouse monoclonal antibodies HRP (Santa Cruz Biotechnology). (We used 0.3 μ g of each antibody per 1-2 μ g of protein; antibodies were diluted in 1%BSA/PBS). Finally, the plates were washed 3 times in wash buffer and incubated in 100 μ L/well of TMB Core+ substrate solution (3,3', 5, 5'-tetramethylbenzidine plus hydrogen peroxide) BUF062C; Bio-Rad) for 30 min at room temperature in the dark. We read the absorbance values immediately at 600 nm using a microplate reader (Sunergy1, BIOTEK; Gen5™ software, BioTek Instruments Inc., Winooski, VT, USA). Protein standards for β -actin were used by 1:10 serial dilutions of a highly concentrated protein sample that was positive for p-EGFR. Assays were carried out according to the manufacturer's instructions and performed in triplicate. All experiments were repeated three times, independently.

Western blot analysis: Twenty to 30 μ g of nuclear and cytoplasmic protein extracts from pepsin and control-treated HCs were heated at 70 °C for 10 min in sodium dodecyl sulfate-polyacrylamide gel electrophoresis Laemmli sample buffer (Bio-Rad, Hercules, CA, USA), and separated using 420- μ m Mini-PROTEAN TGX Tris/Glycine precast gels, at 150V for 1 h. We used precision plus pre-stained protein standards (Dual Color or Kaleidoscope, *BIO-RAD*) as molecular-weight size markers. Proteins were transferred onto a 0.45 mm nitrocellulose membrane, using a Trans-Blot Turbo transfer system (Bio-Rad). After blocking in 5% BSA, for 1 h, membranes were incubated with 1:1000 primary antibodies, p-EGFR (Clone F-3) and bcl2 (Clone N-19), p-NF- κ B p65 Antibody (27.Ser 536), and 1:500 of p-STAT3 (Tyr 705) (clone B-7)(Santa Cruz Biotechnology Inc., Dallas, TX, USA), which were diluted in 5% BSA, overnight at 4 °C. Membranes were incubated for 1:30 h with goat anti-mouse horseradish peroxidase-conjugated secondary antibodies

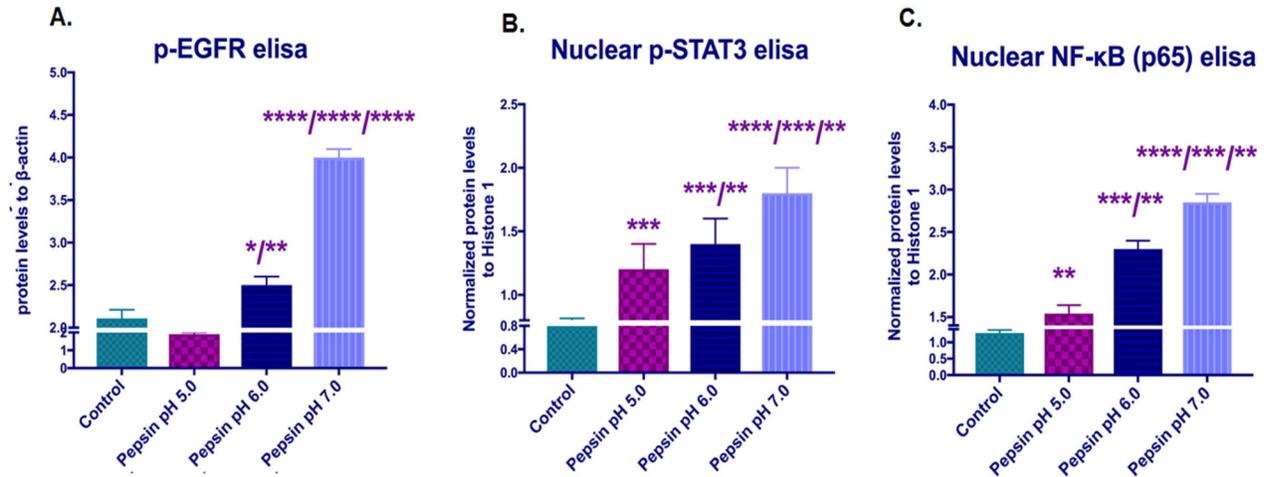
(EMD Millipore, Burlington, MA, USA) at 1:5000, and chemiluminescence was determined using an enhanced chemiluminescence detection system (Clarity Western ECL Substrate, Bio-Rad). Membranes were also stripped using Restore™ Western Blot Stripping buffer (Pierce Biotechnology, Rockford, IL, USA) and reported with β -actin (C4; Santa Cruz Biotechnology Inc., Dallas, TX, USA) for cytoplasmic extracts and Histone 1 (AE-4; Santa Cruz Biotechnology Inc., Dallas, TX, USA) for nuclear extracts normalization. Protein levels were quantified by the Gel imaging system (Bio-Rad, Hercules, CA, USA) in each nuclear or cytoplasmic cellular compartment (Image Lab 5.2 analysis software, Bio-Rad, Hercules, CA, USA).

Supplementary Figure S1.



Supplementary Figure S1: Internalized-pepsin in human HCs under exposure at weakly acidic pH 5.0, slightly acidic pH 6.0 and neutral pH 7.0. **A.** Immunofluorescence staining for Pepsin A reveals that HCs exposed to pepsin at pH 7.0 (P7) presented the highest levels of internalized pepsin compared to slightly acidic pepsin at pH 6.0 (P6) or weakly acidic pepsin at pH 5.0 (P5). **B.** Columns of graph created by Graph Pad Prism 7.0 present the internalized-pepsin levels in HCs at pH 5.0, 6.0 and 7.0 (* $p<0.05$; t-test). **C.** Diagram presents the correlation coefficient between internalized pepsin A and p-EGFR ($r=0.999207$, $p<0.0001$; Graph Pad Prism 7.0; by Pearson)

(orange red: Pepsin A; red distinct line: cell membrane; blue distinct line: nuclear membrane: scale bar $20 \pm \mu\text{m}$ by Zen imaging software).



Supplementary Figure S2: Quantification of cytoplasmic p-EGFR(Tyr1092), and nuclear p-STAT3 (Tyr705) and NF- κ B(p65), by ELISA, in HCs exposed to pepsin at variable pH and controls. Graphs created by GraphPad Prism 7.0 depict (A) cytoplasmic p-EGFR(Tyr1092), (B) nuclear p-STAT3(Tyr705), and (C) nuclear NF- κ B(p65) in HCs exposed to controls at pH 7.0, and pepsin at pH 5.0, 6.0 and 7.0. (Controls: cells treated with media at pH 7.0 without pepsin; data obtained from three independent experiments; * $p < 0.05$; ** $p < 0.005$; *** $p < 0.0005$, **** $p < 0.00005$; by t -test; multiple comparisons by Holm-Sidak; GraphPad Prism 7.0; means \pm SD).

Supplementary Table S1: Human genes analyzed by real-time qPCR, in human hypopharyngeal cancer cells (HCs).

Gene	Detected transcripts	Amplicon length (bp)
<i>hGAPDH</i>	NM_001256799, NM_002046	95
<i>BCL2</i>	NM_000633	116
<i>EGFR</i>	NM_005228	80
<i>RELA</i>	NM_201282-4, NM_001145138, NM_001243984-5, NM_021975	107
<i>WNT5A</i>	NM_001256105, NM_003392	105
<i>PKI3CA</i>	NM_006218, XM_006713658	108
<i>TNFα</i>	NM_000594	98
<i>STAT3</i>	NM_003150, NM_139276	95
<i>IL1β</i>	NM_000576, XM_006712496	117
<i>IL6</i>	NM_000600 XM_005249745	107
<i>mTOR</i>	NM_004958, XM_005263438, XM_005263439	66
<i>AKT1</i>	NM_001014431, NM_001014432, NM_005163, XM_005267401	138
<i>PTGS2</i>	NM_000963	68

Supplementary Table S2. Pepsin-induced upregulation of EGFR and its related pathways.

A. Transcriptional levels of EGFR-related genes in pepsin-treated human HCs.

Target gene/ <i>hGapdh</i>* ($\Delta\Delta^{CT}$)	Control pH 7.0	Pepsin pH 5.0	Pepsin pH 6.0	Pepsin pH 7.0
<i>IL6</i>	1.000E-05	2.000E-05	1.580E-03	2.850E-03
<i>EGFR</i>	1.891E-06	1.237E-06	4.182E-05	2.800E-04
<i>IL1β</i>	4.805E-05	4.000E-05	1.900E-04	2.210E-03
<i>RELA</i>	5.100E-04	2.400E-04	3.570E-03	4.430E-03
<i>TNF-α</i>	3.500E-04	1.000E-03	2.930E-03	2.330E-03
<i>AKT1</i>	1.280E-03	1.800E-03	3.150E-03	4.620E-03
<i>mTOR</i>	6.318E-02	6.211E-02	1.795E-01	2.235E-01
<i>BCL-2</i>	4.320E-02	5.662E-02	1.352E-01	1.179E-01
<i>STAT3</i>	2.569E-01	4.264E-01	4.894E-01	4.954E-01
<i>Wnt5a</i>	2.000E-05	4.000E-05	3.000E-05	2.000E-05
<i>PTGS2</i>	6.076E-03	5.970E-03	6.350E-03	6.020E-03
<i>PKI3CA</i>	2.970E-01	3.093E-01	2.998E-01	3.409E-01

* normalization of mRNA levels using *hGapdh*

B. Relative expression levels of EGFR-related genes in pepsin-treated human HCs.

Target gene/ <i>hGapdh</i>* ($\Delta\Delta^{CT}$)	Pepsin 5.0/ Cntl	Pepsin 6.0/ Cntl	Pepsin 7.0/ Cntl
<i>IL6</i>	2.000E+00	1.580E+02	2.850E+02
<i>EGFR</i>	6.545E-01	2.212E+01	1.481E+02
<i>IL1β</i>	8.325E-01	3.954E+00	4.599E+01
<i>RELA</i>	4.706E-01	7.000E+00	8.686E+00
<i>TNF-α</i>	2.857E+00	8.371E+00	6.657E+00
<i>AKT1</i>	1.406E+00	2.461E+00	3.609E+00
<i>mTOR</i>	9.831E-01	2.841E+00	3.537E+00
<i>BCL-2</i>	1.311E+00	3.131E+00	2.730E+00
<i>STAT3</i>	1.660E+00	1.905E+00	1.928E+00
<i>Wnt5a</i>	2.000E+00	1.500E+00	1.000E+00
<i>PTGS2</i>	9.825E-01	1.045E+00	9.907E-01
<i>PKI3CA</i>	1.042E+00	1.009E+00	1.148E+00

* normalization of mRNA levels using *hGapdh*; Cntl: Control pH 7.0