PANCREATIC RIBONUCLEASE FUNCTIONS AS AN EPIDERMAL GROWTH FACTOR RECEPTOR LIGAND INDEPENDENTLY OF ITS ENZYME ACTIVITY AND CONTRIBUTES TO CETUXIMAB RESISTANCE

Heng-Huan Lee

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PANCREATIC RIBONUCLEASE FUNCTIONS AS AN EPIDERMAL GROWTH FACTOR RECEPTOR LIGAND INDEPENDENTLY OF ITS ENZYME ACTIVITY AND CONTRIBUTES TO CETUXIMAB RESISTANCE

By
Heng-Huan Lee, M.S.

APPROVED:

___________________________________
Mien-Chie Hung, Ph.D., SUPERVISOR

___________________________________
Dihua Yu, M.D., Ph.D.

___________________________________
Stephanie S. Watowich, Ph.D.

___________________________________
Zhen Fan, Ph.D.

___________________________________
Min Gyu Lee, Ph.D.

APPROVED:

___________________________________
Dean, The University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences
PANCREATIC RIBONUCLEASE FUNCTIONS AS AN EPIDERMAL GROWTH FACTOR RECEPTOR LIGAND INDEPENDENTLY OF ITS ENZYME ACTIVITY AND CONTRIBUTES TO CETUXIMAB RESISTANCE

A

DISSERTATION

Presented to the Faculty of
The University of Texas Health Science Center at Houston
and
The University of Texas M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

Heng-Huan Lee, M.S.

Houston, Texas
August 2014
Dedication

This thesis is dedicated to

my mother and sisters

for their love and support
Acknowledgments

I would like to express appreciation to my advisor, Dr. Mien-Chie Hung, for providing me a great opportunity to pursue Ph.D. degree is his group. Indeed, it is a really special journey to study and work in our lab. In these years, I have experienced and learned a lot in both science and life. I really appreciate his full support and kind guidance during all the process of my Ph.D. training.

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I would like to thank all our collaborators, Drs. Xifeng Wu, Huamin Wang, Yuanqing Ye, and Jun Yao for their comments and technical supports.

Finally, I would like to express sincere gratitude to my family and friends who love and support me all the time.
PANCREATIC RIBONUCLEASE FUNCTIONS AS AN EPIDERMAL GROWTH FACTOR RECEPTOR LIGAND INDEPENDENTLY OF ITS ENZYME ACTIVITY AND CONTRIBUTES TO CETUXIMAB RESISTANCE

Heng-Huan Lee, M.S.
Supervisory Professor: Mien-Chie Hung, Ph. D.

Ribonuclease (RNase) with its catalytic enzyme activity to degrade RNAs has been shown as a diagnostic serum marker for pancreatic cancer and has also been suspected to have an unidentified cell surface receptor. Epidermal growth factor receptor (EGFR), a well-characterized receptor tyrosine kinase is an effective therapeutic target in multiple cancer types. However, clinical trials targeting EGFR have not demonstrated improved therapeutic efficacy in pancreatic cancer. Here, we show that both bovine pancreatic RNase A (bRNaseA) and human RNase 5 (hRNase5) act as EGFR ligands and directly activate EGFR to promote epithelial-mesenchymal transition. This ligand-like activity is independent of RNases’ enzymatic activity. In addition, Gln93 and Tyr94 of hRNase5, which are highly conserved between hRNase5 and EGF, are critical for efficient binding to EGFR. A statistically positive correlation between hRNase5 and activated EGFR expression in human pancreatic tissue microarrays further supports the pathological relevance of hRNase5. Strikingly, hRNase5 also enhances resistance to cetuximab therapy in pancreatic cancer. Thus, there may be an interplay between serum RNase and overexpressed EGFR in human cancers, especially in pancreatic cancer, that leads
to aggressive cancer behaviors. Our findings uncover a potential opportunity for therapeutic intervention by rational combination therapy of hRNase5 inhibition and cetuximab.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP</td>
<td>Adaptor proteins</td>
</tr>
<tr>
<td>AR</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>bRNaseA</td>
<td>Bovine ribonuclease A</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BTC</td>
<td>Betacellulin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DSP</td>
<td>Dithiobis-succinimidylpropionate</td>
</tr>
<tr>
<td>ECD</td>
<td>Extracellular domain</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-mesenchymal transition</td>
</tr>
<tr>
<td>EPG</td>
<td>Epigen</td>
</tr>
<tr>
<td>EPR</td>
<td>Epiregulin</td>
</tr>
<tr>
<td>ERC</td>
<td>Endocytic recycling compartment</td>
</tr>
<tr>
<td>eRNAs</td>
<td>Extracellular RNAs</td>
</tr>
<tr>
<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
</tr>
<tr>
<td>GBM</td>
<td>Glioblastoma multiforme</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin-binding EGF-like growth factor</td>
</tr>
<tr>
<td>hRNase5</td>
<td>Human ribonuclease 5</td>
</tr>
<tr>
<td>ICD</td>
<td>Intracellular domain</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>K_D</td>
<td>Dissociation constants</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
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<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
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<tr>
<td>NGC</td>
<td>Neuroglycan C</td>
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<td>NRGs</td>
<td>Neuregulins</td>
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<tr>
<td>NSCLC</td>
<td>Non-small cell lung cancer</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PDAC</td>
<td>Pancreatic ductal adenocarcinoma</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
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<td>PLC_\gamma</td>
<td>Phospholipase C gamma</td>
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<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TGF-\alpha</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TKI</td>
<td>Tyrosine-kinase inhibitor</td>
</tr>
<tr>
<td>TR</td>
<td>Tomoregulin</td>
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Chapter 1

Introduction
1.1 Structural perspective of EGFR and its family members

Growth factors and their cognate receptors play essential roles in governing life and death of cells and organisms. Among them, the epidermal growth factor receptor (EGFR/ErbB1/HER1) and its family members including ErbB2 (HER2/Neu), ErbB3 (HER3) and ErbB4 (HER4), have long been recognized as a primordial type of receptor tyrosine kinases which contribute to key steps in both human physiology and diseases (Hynes & Lane, 2005; Lemmon & Schlessinger, 2010). As single chain transmembrane glycoproteins, the epidermal growth factor receptor family preserves structural resemblance within its four members, which are composed of an N-terminal extracellular ligand-binding ectodomain, a lipophilic transmembrane domain, a short juxtamembrane region, a tyrosine kinase domain and a tyrosine-rich regulatory domain at C-terminal. To date, both EGFR and HER4 are reported as complete functional receptor tyrosine kinases with corresponding ligands and functional tyrosine kinase domains. However, HER2 is an orphan receptor without cognate ligands found so far (Brennan et al., 2000), and HER3 is not a functional tyrosine kinase since it loses intrinsic kinase activities (Guy et al., 1994). The N-terminal extracellular domain can be further divided into four subdomains which are L1 (Leucine-rich/domain I), CR1 (Cysteine-rich/domain II), L2 (Leucine-rich/domain III) and CR2 (Cysteine-rich/domain IV) in a linear order from N- to C-terminal. Domain I and III are responsible for direct ligand binding to receptors, whereas domain II and IV constitute dimerization loop and contribute to receptor-receptor interaction. Without ligand stimulation, domain II and IV are bound with each other through intramolecular binding, this interaction forces the receptor in a closed and
inactive monomer conformation which can keep domain I and III away from extracellular ligands. However, in the presence of a soluble ligand, domain I and III can be bound by the ligand in a direct manner, this conformational change can remove the intramolecular interaction between domain II and IV and shift a receptor from a closed and inactive conformation to an open and active orientation. In an open and active orientation, the dimerization region from domain II of one receptor can be exposed and associated with the other receptor at the same region to further facilitate the formation of a receptor dimer. The balance between the open and closed conformation of a receptor is usually kept at steady state and can be modulated by a variety of ligands binding to switch monomeric receptors to become either homo-dimers or hetero-dimers, depending on the type of ligands and corresponding receptors (Eccles, 2011).

1.2 Endocytic trafficking of EGFR

When receptors are bound by ligands to cause their conformational change and dimerization, they will be further internalized as vesicles and undergo endosomal sorting and trafficking to different subcellular location inside the cell (Conner & Schmid, 2003; Doherty & McMahon, 2009; Sorkin & von Zastrow, 2009). Internalization of receptors can occur through clathrin-dependent or clathrin-independent pathways to form vesicles, followed by adaptor proteins (AP2) and growth factor receptor-bound protein 2 (Grb2) binding and fusion with early endosomes (Mayor & Pagano, 2007; Orth et al., 2006; Sigismund et al., 2005; Sorkin, 2004). Concomitant recruitment of E3 ubiquitin ligase Cbl to receptors
through direct or indirect binding (Grb2-dependent) will catalyze receptor ubiquitination in either monomeric or polymeric forms on certain lysine residues of their kinase domain, which can be further recognized by ubiquitin-binding proteins such as epsin1 and Eps15 for subsequent sorting and targeting through endosomal sorting complex required for transport (ESCRT) within multivesicular bodies and subject to lysosomal degradation (Gruenberg & Stenmark, 2004; Saftig & Klumperman, 2009; Sorkin & Goh, 2009; Williams & Urbe, 2007; Woodman, 2009). Under certain circumstances such as pH-dependent decreased stability of ligand-receptor complex or AMSH-mediated deubiquitination process, activated receptors can go through recycling pathway to escape from lysosomal degradation (Baldys & Raymond, 2009). As reported by previous studies, recycling of receptor kinases can occur via Rab4- and Rab35-dependent pathway to plasma membrane or Rab11-regulated route through perinuclear endocytic recycling compartment (ERC) (Ceresa, 2006; Grant & Donaldson, 2009; Maxfield & McGraw, 2004). Increased receptor recycling instead of lysosomal degradation after its activation may prolong its signaling duration and succeeding mitogenic effects.

Notably, a growing body of evidence has shown that other than traditional routes to lysosomal degradation and endocytic recycling, EGFR and other membrane receptors can undergo nuclear translocation through a retrograde pathway (Brand et al., 2013; Carpenter & Liao, 2009; Lin et al., 2001; Lo, 2010; Massie & Mills, 2006; Mosesson et al., 2008; Ni et al., 2001; Offterdinger et al., 2002; Seshacharyulu et al., 2012; Wang & Hung, 2012; Wang et al., 2010a). Inside the nucleus, EGFR can serve as a kinase, transcription factor or protein scaffold to
modulate nuclear signaling and gene expression. This alternative route of EGFR to nucleus can contribute to DNA repair, replication of tumor cells and confer resistance to chemo- and radio-therapies (Dittmann et al., 2011; Huang et al., 2011; Huang & Hung, 2009; Wang et al., 2006; Wang et al., 2010b). In addition, nuclear EGFR has the clinical relevance to be associated with poor patient prognosis in diverse cancers, including breast, ovarian, and oropharyngeal and esophageal squamous cell cancers (Hadzisejdic et al., 2010; Hoshino et al., 2007; Lo et al., 2005; Psyrri et al., 2008; Psyrri et al., 2005; Xia et al., 2009) (Figure 1-1).

1.3 Ligands of ErbB family

A group of ligands are known to activate ErbB family members including EGF, transforming growth factor alpha (TGF-α), amphiregulin (AR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF), epiregulin (EPR), epigen (EPG), neuregulins 1-4 (NRGs), neuroglycan C (NGC) and tomoregulin (TR) (Citri & Yarden, 2006; Harris et al., 2003). These ligands are expressed as plasma membrane-anchored precursor proteins and are processed to mature forms via proteolytic release by certain metalloproteinase such as TACE/ADAM17 and ADAM10, etc (Nakamura et al., 1995). Among them, EGF, TGF-α, AR and EPG are specific ligands to EGFR, whereas BTC, HB-EGF and EPR can bind to both EGFR and HER4. Moreover, NGC is a specific ligand to HER3, and both NRG3 and NRG4 are particular ligands to HER4. NRG1 and NRG2 can bind to either HER3 or HER4. To date, there is no specific ligand found for HER2 and its activation are reported to be caused by either receptor overexpression or heterodimerization with another
Figure 1-1. ERBB trafficking.

Exposing the cell to epidermal growth factor (EGF) results in the autophosphorylation of surface EGF receptor (EGFR), which might result in EGFR moving out of lipid rafts. This activation also results in the ubiquitylation of the receptor and its removal (endocytosis) from the cell surface into an early endosomal compartment. The ligand-bound receptor activates several signaling pathways at the cell surface by activating Ras and phospholipase Cγ (PLCγ). EGFR interacts with and activates signalling molecules while its cytoplasmic tail projects into the cytoplasm, as occurs in the early endosome. From the early endosome the receptor
can be recycled back to the cell surface (a) or sorted into the late endosome and the vesicles within this compartment that are called multivesicular bodies (b). If sorted into multivesicular bodies, the cytoplasmic tail is occluded from the cytoplasm and this prevents further activation of signalling cascades. Following a fusion event between the late-endosomal and lysosomal compartments the internalized receptor is incorporated into the lysosome and degraded. A third possibility is that, having been internalized and incorporated into the early endosome, the receptor is delivered to the nucleus (c). The route that is taken to the nucleus by ERBBs is unclear, but might include proteolytic cleavage, membrane fusion or chaperone-mediated dissociation of the receptor from the membrane. This is potentially a direct route to mediate changes in gene expression and DNA repair in response to stimuli. Nuclear translocation is also a property of certain adaptor proteins that are associated with early endosomes, including APPL (adaptor protein containing a pleckstrin homology domain, a PTB domain and a leucine zipper motif) and HIP1 (huntingtin-interacting protein 1). Additional, and less-common, trafficking routes for cargo arriving in the early endosome include retrograde trafficking steps to compartments such as the endoplasmic reticulum (ER) and Golgi apparatus that are associated with the biosynthesis and post-translational modification of proteins. Viruses and toxins commonly use these trafficking routes to ultimately gain access to the cytoplasm by expulsion from the ER. P, phosphate; Ub, ubiquitin. (This figure was adapted from MASSIE, C. & MILLS, I. G. (2006). The developing role of receptors and adaptors. Nat Rev Cancer, 6(5), 403-409; the permission was received from the journal.)
receptors including EGFR, HER3 or HER4 through their corresponding ligand binding and stimulation (Harris et al., 2003) (Figure 1-2).

1.4 Activation of EGFR signaling

Dimerization of receptors leads to their auto-phosphorylation on particular tyrosine residues through a reciprocal manner, further recruiting protein adaptors, effector proteins and kinase substrates to trigger downstream signaling network. To initiate the phosphotyrosine-based signaling cascade, phosphotyrosine residues from a receptor kinase are specifically recognized by a Src homology 2 (SH2) and phosphotyrosine-binding domain from an adaptor protein. This phosphotyrosine-based molecular recognition can lead to adaptor and effector proteins phosphorylation and elevate their activities, further provoking subsequent occurrence of signaling events including the RAS-RAF-MEK-ERK pathway, phosphoinositide 3-kinase (PI3K)-AKT (Protein kinase B/PKB) signaling, phospholipase C gamma (PLC\textsubscript{γ})-protein kinase C (PKC) pathway, and signal transducer and activator of transcription (STAT) activation (Yarden, 2001). These signaling pathways act as mediators amplify and transmit the original stimuli from the receptor at plasma membrane to intracellular space including cytosol and subcellular organelles, eventually to the nucleus to regulate DNA replication, gene transcription and translation, etc. Through these signaling cascades and molecular interaction events, an ErbB receptor-triggered signaling can modulate and contribute to normal cellular events including proliferation, migration, differentiation, development, etc (Citri & Yarden, 2006; Huang et al., 2009). On the other hand, its
Figure 1-2. Heterodimerization, ligand binding and signaling in the EGFR and ERBB family.

(a) EGFR is a 170 kDa transmembrane protein of the HER family of receptors. Each receptor contains an extracellular ligand-binding domain, a transmembrane lipophilic domain and an intracellular tyrosine kinase domain and, except for HER3, relies upon heterodimerization for transphosphorylation. No ligand for HER2 has
been identified. Ligand binding induces autophosphorylation and causes activation of downstream signaling pathways. Ligands are indicated by circles, PI3K binding sites are indicated by red squares. (b) Diagram of EGFR protein. The extracellular domain of EGFR comprises 621 amino acids and consists of four different subunits, RI–RIV. RI is important in ligand-induced dimerization; RII and RIV are rich in cysteine residues. RIII includes the EGF-binding domain. The intracellular region contains the tyrosine kinase domain and the C-terminus, which has six autophosphorylation sites. (c) The most widely known and best-characterized RAS signaling pathways include MAPK–ERK, the MAPK–JNK and p38 pathways. MAPK cascades are activated through a large variety of extracellular signals, including growth factors, differentiation factors and stress-induced factors. Abbreviations: HB-EGF, heparin-binding EGF-like growth factor; TM, transmembrane region. (This figure was adapted from LINARDOU, H., DAHABREH, I. J., BAFALOUKOS, D., KOSMIDIS, P. & MURRAY, S. (2009). Somatic EGFR mutations and efficacy of tyrosine kinase inhibitors in NSCLC. Nat Rev Clin Oncol, 6(6), 352-366; the permission was received from the journal.)
dysregulation can lead to multiple types of human malignancies and diseases, including cancers.

1.5 EGFR genetic alterations in cancers

Enormous studies and clinical evidence have demonstrated that EGFR plays a key role in various types of cancer development, including lung, head and neck, breast, ovarian, colon and pancreatic cancers, etc. Ectopic expression of EGFR can lead to transformation of mouse NIH3T3 cells in vitro through ligand-dependent manner. Blockage of EGFR signaling impedes growth of multiple types of cancer cells both in vitro and in vivo. Genetic alternations of EGFR such as gene amplification or somatic mutation are frequently observed in both clinical samples and tumor cell lines derived from patients with different types of cancers. In average, EGFR gene amplification can be detected in about 40% of human primary glioblastoma multiforme (GBM) (Hatanpaa et al., 2010; Lopez-Gines et al., 2010) and 5-10% Non-small cell lung cancer (NSCLC) (Gazdar, 2009; Linardou et al., 2009). Activating deletions of EGFR have also been found in a significant population of GBM patients and associated with poor prognosis. N-terminal truncations and deletions of EGFR are reported including EGFRvI (N-terminal truncation), EGFRvII (exon 14-15 deletion), EGFRvIII (exon 2-7 deletion), EGFRvIV (exon 25-27 deletion) and EGFRvV (C-terminal truncation). Among them, EGFRvIII is a major mutant which is known to play a key role in GBM pathogenesis (Shinojima et al., 2003). In addition, it can be also detected in breast, ovarian, prostate and lung cancers and participated in cancer progression. Activating
mutations of EGFR kinase domain are also identified in 10-20% NSCLCs including missense mutations (L858R, G719X and L861Q), in frame deletion of exon 19 or insertion of exon 20, etc (Wieduwilt & Moasser, 2008).

1.6 EGFR-targeted therapies in cancers

Due to its integral role in the pathogenesis of different types of tumors, inhibition of EGFR signaling as a target therapy is developed for years and proved powerful in certain clinical settings (Tomas et al., 2014). Both EGFR-specific monoclonal antibodies (mAbs) and chemical compounds as EGFR tyrosine kinase inhibitors (TKIs) are approved and prevalently used in clinics for different types of cancers (Chong & Janne, 2013; Eccles, 2011; Wheeler et al., 2010). EGFR-specific mAbs such as cetuximab (Erbitux/C225) and panitumumab (Vectibix) are generated to bind to EGFR extracellular domain for preventing from their cognate ligand binding. C225 is commonly used in colon cancers with wild type KRAS status, and it is also applied in head and neck cancers which possess EGFR overexpression with higher frequency (Mendelsohn & Baselga, 2000). On the other hand, the TKIs gefitinib (Iressa) and erlotinib (Tarceva) are both actively applied in the clinical treatment of NSCLC patients with EGFR activating mutations. Higher response rate and extended survival time are reported for NSCLC patients with EGFR activating mutations compared to those who do not have mutations treated with TKIs (Seshacharyulu et al., 2012). In summary, several lines of evidence and studies have suggested that application of anti-EGFR agents to certain types of tumors
including colon, head and neck, NSCLC and pancreatic cancer confers benefits and advantages in terms of overall survival or progression-free survival.

1.7 The role of EGFR in pancreatic cancer

Human pancreatic cancer, attributed to its high mortality rate and poor clinical outcome, currently ranks fourth as cancer death cause in the United States. Due to the early metastasis, late diagnosis and refractory to canonical therapies including chemotherapy and radiotherapy, pancreatic cancer especially pancreatic ductal adenocarcinoma (PDAC) is recognized as one of the most lethal malignancies among human cancers in the last decades (Hidalgo, 2010). Massive efforts have been made to characterize critical genetic alternation and signaling pathways occurring with PDAC development. Based on genetic studies and transgenic mouse models, it is believed that KRAS and INK4A mutation occur in the early stage of PDAC development; follow by alternation of SMAD4 and p53 tumor suppressors (Lunardi et al., 2014). Notably, two decent papers have recently reported that EGFR is required for tumor initiation and development based on PDAC transgenic mouse models (Ardito et al., 2012; Navas et al., 2012). EGFR depletion can impede PDAC tumorigenesis in KRAS\textsuperscript{G12D} and CDKN2A\textsuperscript{flox} mice, whereas it doesn’t affect KRAS\textsuperscript{G12D}-driven lung cancer progression. Interestingly, EGFR overexpression in advanced pancreatic cancer is present from 30% to 95% depending on cases examined. Moreover, several lines of evidence suggest that EGFR or EGF expression has been found to be positively associated with PDAC metastasis in a significant level. These previous research work point out EGFR may play a critical
role in PDAC tumor initiation and contribute to its development and metastasis. However, how EGFR signaling regulates PDAC progression remains unclear and its clinical impact awaits more follow-up studies and clinical trials to delineate. To date, four Phase III trials in advanced PDAC using anti-EGFR target therapies combined with conventional chemotherapeutic agents including gemcitabine or capecitabine were completed (Heinemann et al., 2013; Moore et al., 2007; Philip et al., 2010; Van Cutsem et al., 2009). The benefits from EGFR-targeted therapy are limited, and it remains to be further pursued if it can be improved in the aid of certain predictive biomarkers.

1.8 Bovine pancreatic RNase A

As a most well studied enzyme in 20th century and so far, bovine pancreatic ribonuclease A (RNase A) has been established as a model system for research of protein chemistry, enzymology and structure biology, etc. Ribonuclease A was first identified by René Dubos in 1938, crystallized by Moses Kunitz’s group in 1940, sequenced by Stanford Moore and William Stein in 1963 (through cooperation with Anfinsen’s group), and synthesized through solid-phase by Merrifield’s group in 1969. Four Nobel Prizes were awarded (Anfinsen, Moore, Stein and Merrifield) for their comprehensive and excellent work at RNase studies (Marshall et al., 2008; Raines, 1998). RNase A is a pancreatic endonuclease which is secreted from β cells at the islets of Langerhans. It can cleave phosphodiester bond at single strand RNA between the 5’-ribose of a nucleotide and the phosphate group on a 3’-ribose of the neighboring pyrimidine nucleotide, and further hydrolyze the formed 2’-3’-
cyclic phosphate intermediate to generate 3'-nucleoside phosphate. It exerts substrate preference for different bases on both 5’- and 3’-end of the broken phosphodiester bond. At the 5’-position, RNase A displays higher enzymatic efficiency in an order as A>G>C>U, whereas at the 3’-position, a pyrimidine is required to be present and C is favored than U. Being a secretory enzyme produced from pancreas, RNase A possess signal peptide on the very N-terminal (1-26 of precursor protein) which is responsible for its secretion to extracellular space. In native RNase A protein, there are four pairs of disulfide bonds (Cys26-Cys84, Cys40-Cys110, Cys58-Cys110 and Cys65-Cys72) which contribute to its protein folding and stability. The mature form of RNase A protein (124 amino acids) contains a catalytic triad constituted by amino acid residue His12, Lys41, and His119, where His12 and His119 acts as a respective proton acceptor and donor, and Lys41 can provide a hydrogen bond to stabilize the transition state during catalysis (Cuchillo et al., 2011).

1.9 Human RNase A family

In last decades, a family of vertebrate secretory ribonucleases with sequence and structure homology to bovine RNase A has been found from the human genome. In general, they are recognized as part of host defense system and contribute to immunity especially innate immunity. To date, there are 13 ribonucleases are identified for the RNase A family which are produced and secreted from various types of cell types including epithelial, endothelial and immune cells, etc (Cho et al., 2005). Among them, human RNase 1 is the direct
homologue of bovine RNase A according to their sequence and structure similarity (Peracaula et al., 2000). The RNase A family can be divided into two groups; the canonical ribonucleases (RNase 1-8) and non-canonical ribonucleases (RNase 9-13) (Sorrentino, 2010). In the group of canonical ribonucleases, they maintain classical signal sequences for secretion, particular disulfide-bonded secondary and tertiary structure, conserved active sites and His-Lys-His catalytic triad to exhibit certain degree of catalytic activity against single strand RNA substrate (Cuchillo et al., 2011; Yang et al., 2004). On the other hand, the non-canonical ribonucleases do not act as functional enzymes because they encode either insertions, deletions or mutation at certain essential residues although they still exert microbicidal activities and contribute to host defense system (Cho et al., 2005; Sorrentino, 2010).

1.10 RNases in cancers

In addition to being participants in host defense system, expression of ribonucleases have been demonstrated to be positively associated with cancer occurrence and progression. Elevated ribonuclease activities were detected in several types of cancers including lung, thyroid, breast, ovarian, kidney, colon and pancreatic cancer (Matte et al., 2012; Reddi & Holland, 1976; Tello-Montoliu et al., 2006). Compared to other types of cancers, pancreatic cancer is one of the most studied malignancies with this observation since numerous studies have indicated higher ribonuclease activities can be measured in patient serum compared to control samples. Accordingly, ribonuclease activity was proposed to serve as early diagnostic marker for pancreatic cancer in several studies (Kottel et al., 1978;
Warshaw et al., 1980; Zhao et al., 1998) although some researchers argued it lacks specificity to be useful. In addition to ribonuclease activities, specific members of RNase A family including human RNase 1 and RNase 5 were reported to be involved in the development of pancreatic cancer (Peracaula et al., 2000; Poon et al., 2001; Reddi & Holland, 1976). Both human RNase 1 and RNase 5 protein can be detected in the culture medium of pancreatic cancer cells, and specific isoforms of human RNase 1 with distinct glycosylation pattern were merely detected in tumor cell lines (Peracaula et al., 2003). Moreover, increased human RNase 5 was correlated with pancreatic cancer occurrence and aggressiveness (Shimoyama et al., 1996). In contrast to other members in the RNase A family, accumulated studies have concluded human RNase 5 as an oncogenic protein which can induce blood vessel formation in vitro and in vivo (Hu et al., 1997; Li et al., 2011; Yoshioka et al., 2006). Because its potency to promote angiogenesis, it is also named angiogenin.

1.11 Human RNase 5 (angiogenin)

Human RNase 5 (angiogenin) was first isolated from culture medium of a human colon cancer cell line HT-29. Like other RNase A family members, human RNase 5 exhibits microbicidal function to protect from pathogen infection. However, it is also a potent factor to stimulate angiogenesis. It is a close homologue of human RNase 1 with 33% in sequence identity and 65% homology. Human RNase 5 contains a classical secretory signal sequence (1-24 of a precursor protein) and a conserved catalytic triad (His13, Lys40 and His114 of the mature form with 123 amino acids) (Padhi et al., 2012; Strydom, 1998). In spite of its high similarity to
human RNase 1 in tertiary structure, human RNase 5 exerts much lower enzymatic activity than RNase 1 (10^5 - 10^6 less in average) (Cho et al., 2005; Sorrentino, 2010). However, it is demonstrated that ribonuclease activity is essential but not sufficient for human RNase 5-induced angiogenesis (Hu, 1998). Its expression is increased during inflammation, pregnancy and certain pathological conditions. Moreover, it was showed that its expression is elevated in several malignant diseases and multiple types of cancers including colon, prostate, ovarian and pancreatic cancer (Matte et al., 2012; Tello-Montoliu et al., 2006). Several lines of evidence suggests human RNase 5 can increase endothelial cell migration and proliferation through initiating intracellular signaling pathways, such as PLCγ, AKT and ERK activation (Kim et al., 2007; Liu et al., 2001). Previous studies also showed that after entry into recipient cells, human RNase 5 can translocate to nucleus and increase rRNA transcription (Ibaragi et al., 2009; Moroianu & Riordan, 1994). Correspondingly, nuclear translocation of human RNase 5 is required for its provoked endothelial cell proliferation and angiogenesis. To search for a potential surface receptor bound by human RNase 5, researchers have demonstrated that human RNase 5 can bind to α-actinin-2 on the surface of smooth muscle cells to promote their proliferation (Hu et al., 2005). In addition, α-actin and 170-kDa cell surface receptor from endothelial cells were identified to be a binding partner of human RNase 5 (Hu et al., 1997). Binding to α-actin can lead to proteolytic activation, which may contribute to basement membrane degradation (Hu et al., 1993). It is believed that this 170-kDa surface receptor could be critical for human RNase 5-provoked signaling and
angiogenic function. However, the identity of this receptor remains ambiguous and awaits more studies to resolve the puzzle.
Chapter 2

Material and Methods
2.1 Materials

The following antibodies and chemicals were purchased commercially: anti-EGFR antibodies (Ab-13, Thermo Scientific; 4267, Cell Signaling; ab62, Abcam); AG1478, mouse IgG, bovine RNase A (bRNaseA), human EGF, anti-RNase1, actin, and tubulin antibodies (Sigma); anti-bRNaseA (ab6611) and bRNaseA biotinylated (ab20588) antibodies (Abcam); anti-myc antibody (Roche); anti-flag antibodies (Sigma; Cell Signaling); TMB (3,3',5,5"-tetramethylbenzidine) substrate, stop solution, anti-Akt, phospho-Akt, phospho-ERK1/2, phospho-EGFR (-Tyr992, -Tyr1045, -Tyr1068, -Tyr1086, -Tyr1148, and -Tyr1173), phospho-ErbB2 (-Tyr1221/1222 and -Tyr1248), EMT (Slug, Snail, E-cadherin, N-cadherin, Vimentin, and ZEB-1), and GST antibodies (Cell Signaling); anti-ErbB-2 (Calbiochem); ELISA 96-well plate, BSA blocking solution, and anti-p68 RNA helicase antibody (Santa Cruz Biotechnology); anti-phosphotyrosine (p-Tyr; 4G10) and ERK1/2 antibodies (Millipore); recombinant human RNase 5/angiogenin, recombinant human EGFR/ErbB1 Fc Chimera (N-EGFR-Fc), Proteome Profiler Human Phospho-RTK Array Kit, Streptavidin-HRP, anti-angiogenin (AB-265-NA), angiogenin biotinylated (BAF265), EGF, and EGF biotinylated (BAF236) antibodies (R&D Systems); gefitinib (ChemieTek); erlotinib (LC Labs); biotin-EGF and SYTO® RNASElect™ green-fluorescent cell stain (Invitrogen); and DyLight 594 labeling kit (Thermo Scientific). Cetuximab was obtained from the Clinic Pharmacy at MD Anderson Cancer Center. The secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. For siRNA experiments, siRNA oligonucleotides targeting EGFR, ErbB2, and nonspecific siRNA control were purchased from Sigma.
The siRNA sequences were EGFR-siRNA-1: 5’-GACAUAGUCAGCAGUGACU-3’; EGFR-siRNA-2: 5’-GAUCUUUCCUUCUUAAAGA-3’; ErbB2-siRNA-1: 5’-GCAGUUACCAGUGCCAUA-3’; ErbB2-siRNA-2: 5’-CAGAAUGGCUCAGUGACCUC-3’.

2.2 Constructs and stable transfectants

For myc-His-tagged constructs, hRNase5 complementary DNA was subcloned into a pcDNA6/myc-His-A vector (Invitrogen). For GST-tagged constructs, bRNaseA and hRNase5 complementary DNAs were subcloned into a pGEX-6P-1 vector (GE Healthcare). The myc-GST-tagged hRNase5 was constructed by PCR from myc-His-tagged hRNase5 with a C-terminal myc tag, and subcloned into a pGEX-6P-1 vector. For HeLa stable transfectants, bRNaseA and hRNase5 complementary DNAs were subcloned into a modified pCDH-CMV-MCS-EF1-Puro vector (CD510B-1, System Biosciences) containing a C-terminal Flag tag. Single-point mutants were generated by site-directed mutagenesis. For AsPC-1-hRNase5 stable transfectants, hRNase5 was subcloned into a pCDH-CMV-MCS-EF1-Puro vector. All stable transfectants were established by lentivirus infection and selected with puromycin. DyLight 594-bRNaseA was generated via fluorescent labeling of bRNaseA recombinant proteins with DyLight 594 dye.

2.3 Cell culture

Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM)/F12 or RPMI 1640 medium (for pancreatic cancer cell lines) supplemented with 10% fetal
bovine serum and antibiotics. Pancreatic cancer cell lines were kindly provided by Dr. Craig Logsdon (SW1990, SU.86.86) and Dr. Paul Chiao (CFPAC-1) at MD Anderson Cancer Center. In this series of experiments, cells were treated with bRNaseA and hRNase5 under serum-starved conditions for 24 h.

2.4 Cell lysis, Western blot, and immunoprecipitation

Cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris [pH 7.5], 1% Nonidet P-40, and protease inhibitor mixture) and by sonication. Total lysates were collected after centrifugation at maximum speed and subjected to Western blot analysis with the indicated antibodies. For immunoprecipitation, cells after bRNaseA treatment were fixed with 1 mM dithiobis[succinimidylpropionate] (DSP, Thermo Scientific) at room temperature for 30 min, and the cell lysates were then immunoprecipitated with EGFR using an anti-EGFR and normal IgG antibodies as a negative control, followed by Western blot analysis with the indicated antibodies.

2.5 Preparation of secreted proteins

Cells were maintained in a serum-starved medium for 24 h, and media containing secreted proteins were collected. After filtration using 0.45 µm filters to remove cell debris, media were concentrated at 5,000×g for 1 h by Amicon Ultra-15 Centrifugal Filter Units (UFC900324, Millipore) to a desired volume of secreted proteins.

2.6 RNase activity detection assay
RNase activity was detected using Ambion® RNaseAlert® Lab Test kit (Invitrogen) with a slight modification. In brief, 5 µl of 10× RNaseAlert buffer was added to one tube of fluorescent substrate, and 45 µl of the test sample was then added to the tube. The mixture was sequentially pipetted into a well of a 96-well plate, and monitored at 37°C to collect real-time fluorescence data at 5 min intervals for the indicated time period using a BioTek Synergy™ Neo multi-mode reader.

2.7 Confocal microscopy

Confocal microscopy was performed as standard procedures described previously (Wang et al., 2010c). Cells were fixed in 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, and blocked with 5% bovine serum albumin for 1 h. After the incubation with primary antibodies overnight at 4°C, cells were then further incubated with the appropriate secondary antibodies tagged with fluorescein isothiocyanate, Texas red, or Alexa 647 (Molecular Probes) for 1 h at room temperature. Nuclei were stained with DAPI contained in the mounting reagent (Invitrogen). Confocal fluorescence images were captured using a Zeiss LSM 710 laser microscope. Three-dimensional image was performed by Imaris software (Bitplane). In all cases, optical sections through the middle planes of the nuclei as determined using nuclear counterstaining were obtained.

2.8 Duolink® in situ proximity ligation assay (PLA)

Cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 for 15 min, and blocked with 5% bovine serum albumin. Cells were
then incubated with mouse anti-EGFR antibody (1:2,000; Thermo Scientific) and rabbit anti-bRNaseA (1:400; Abcam) or anti-flag antibody (1:200; Cell Signaling) for 30 min. The fluorescence signals were detected by the Duolink® in situ PLA probe (Axxora) according to the manufacturer’s instructions and visualized by using a Zeiss LSM 710 laser microscope.

2.9 Cell migration and invasion assays

Cell migration and invasion were performed using BioCoat™ Control Cell Culture Insertsin and BioCoat™ Growth Factor Reduced Matrigel Invasion Chambers (BD Biosciences), respectively. Culture medium containing 10% fetal bovine serum as a chemoattractant was added to the plate and cells (2.5×10^4 cells/24-well chamber) in serum-free culture medium with the indicated treatments were added to the chamber. The chamber was then incubated at 37°C for 24 h (for HeLa cells) or 72 h (for AsPC-1 stable transfectants) to allow cells to penetrate an 8 µm pore size, uncoated membrane or a Matrigel-coated membrane. Cells remained on the upper surface of the membrane were removed with a cotton swab, and those on the underside of the membrane were fixed with 4% paraformaldehyde, stained with 0.5% crystal violet, and microscopically counted from three random fields of each membrane. The average cell number per field for triplicate membranes was used to calculate the mean with SD. The values of cell migration and invasion are shown as number of migrated cells and invaded cells per field, respectively. The relative fold at empty vector without treatment or the first bar was defined as 1.
2.10 Detection of ligands-EGFR binding affinity by ELISA

ELISA 96-well plates were captured with 3 µg/ml anti-EGFR antibody (Abcam) in 0.2 M sodium phosphate buffer (pH 6.5) at 100 µl/well overnight at room temperature. The plates were then rinsed three times with phosphate-buffered saline with 0.05% Tween-20 (PBST) and blocked with 200 µl/well of 1% BSA solution containing 0.05% Tween-20 at 37°C for 2 h. After rinsing three times with PBST, 100 µl/well of A431-RIPA lysates or RIPA buffer only as a negative control were added and incubated at 37°C for 1.5 h. The plates were then washed with 400 µl/well of 1M sucrose three times, shaking 1 min per time, followed by addition of recombinant hRNase5, bRNaseA, or human biotin-EGF at a series of diluted concentrations in RIPA buffer. After incubation at 37°C for 1.5 h, wells were washed with 400 µl/well of 1M sucrose three times, shaking 1 min per time, and 100 µl/well of biotin-conjugated detection antibodies in blocking buffer was added for incubation at 37°C for 1 h. The plated were washed with 1M sucrose for three more times with shaking, and 100 µl/well of streptavidin-conjugated HRP (1:2,000 in blocking buffer) was added and incubated for 30 min at room temperature. The wells were washed again with 1M sucrose three times with shaking, and 100 µl/well of TMB as a peroxidase substrate were added and incubated for 30 min at room temperature. The reaction was terminated by addition of 50 µl/well of stop solution. The optical density was determined at 450 nm, corrected by subtraction of readings at 570 nm, using a BioTek Synergy™ Neo multi-mode reader. The dissociation constant (K_D) and Bmax were estimated by the above binding data and then transformed to
create a Scatchard plot with GraphPad Prism program (version 6; Prism Software Inc., San Diego, USA).

2.11 Patient plasma samples

Total 40 patient plasma samples, collected as per protocols LAB01-526, LAB10-0434 (TexGen biorepository), and LAB05-0131, were obtained from 30 patients with pancreatic adenocarcinoma, who were untreated previously (cancer/case group: 10 males and 20 females; mean age, 58.8 years; range, 37-77 years) and 10 plasma samples from screening patients who do not have cancer (normal/control group: 3 males and 7 females; mean age, 51.0 years; range, 32-54 years). Each patient provided informed consent at the time of plasma withdrawal.

2.12 Tissue microarray

Tissue microarray of 119 cases in pancreatic cancer were analyzed by immunohistochemistry staining using anti-phospho-EGFR-Tyr1068 (1:50, 3777, Cell Signaling) and anti-hRNase5 (1:50, AF265, R&D Systems) antibodies.
Chapter 3

Bovine RNase A exhibits

an EGFR ligand-like function
**Background and Rationale**

Elevated levels of ribonuclease (RNase) have been reported years ago in the serum of pancreatic cancer patients (Reddi & Holland, 1976; Shimoyama et al., 1996; Warshaw et al., 1980) and proposed to serve as a diagnostic marker for pancreatic cancer (Misek et al., 2007; Zhao et al., 1998). Human RNase 5 (also named angiogenin), which promotes malignancy through angiogenesis, was speculated to have an unidentified cell surface receptor (Hu et al., 1997). Bovine pancreatic RNase A (hereinafter referred to as bRNaseA), the first enzyme sequenced as a classic model system for protein structure and enzymatic function (Raines, 1998), is a secreted endoribonuclease commonly used in research to remove RNAs (Benito et al., 2008; Cuchillo et al., 2011; Findlay et al., 1961; Marshall et al., 2008). One of the most well-characterized cell surface receptors, the epidermal growth factor receptor tyrosine kinase (EGFR-RTK), is a target of many cancer therapies. RNases and RTKs from two unrelated superfamilies are associated with distinct functions but they may share a common role in signal transduction as extracellular RNAs (eRNAs) have recently been discovered to act as signaling molecules and even serve as therapeutic biomarkers (Dinger et al., 2008; Fischer et al., 2013).

In an attempt to study how eRNAs might affect cell signaling, which is not fully understood, we unexpectedly discovered that bovine RNase A and its human equivalent, RNase 5/angiogenin, function as unique EGFR ligands and that serum RNase 5 may contribute to resistance to EGFR targeted therapy, cetuximab.
Results

3.1 Bovine RNase A promotes oncogenic transformed phenotypes

To study how eRNAs affect cellular processes and determine their potential clinical significance, we designed a pilot test by treating cells with a recombinant bRNaseA protein to broadly degrade eRNAs. The catalytic activity of bRNaseA was validated by RNase activity detection assay (Figure 3-1A) and sucrose gradient fractionation using p68 RNA helicase as a positive control (Sytnikova et al., 2011) (Figure 3-1B). Interestingly, we found that bRNaseA triggered oncogenic transformed phenotypes. Specifically, bRNaseA induced epithelial-mesenchymal transition (EMT)-like morphological changes in different cancer types (Figure 3-2) accompanied by changes in EMT markers, including decreased expression of E-cadherin and enhanced expression of mesenchymal markers Vimentin, ZEB-1, Slug, and Snail (Figure 3-3). Cells treated with bRNaseA also showed enhanced cell mobility (Figure 3-4) and cell migration and invasion in a dose-dependent manner (Figure 3-5), except for cell growth (Figure 3-6). These results suggest that bRNaseA induces EMT.

3.2 Extracellular RNA is not involved in bovine RNase A-triggered EMT

To further determine whether eRNAs are involved in bRNaseA-triggered EMT, we tested if the catalytic enzyme-deficient mutant of bRNaseA can still induce EMT. To this end, we generated a GST-tagged wild-type bRNaseA (GST-bRNaseA-WT) and a catalytic-deficient mutant (GST-bRNaseA-H119A) bearing a single mutation at His119 (Zegers et al., 1994) (Figure 3-7A). Surprisingly, a comparison between
Figure 3-1. bRNaseA harbors endoribonuclease enzyme activity.

(A) Detection of bRNaseA enzyme activity, at 50 pg/ml, using an RNaseAlert® Test kit monitored by a real-time fluorometer. (B) Sedimentation pattern of p68 RNA helicase, a RNA binding protein as a positive control, through linear 10–40% (top-bottom) sucrose gradients.
Figure 3-2. Treatment of bRNaseA triggers EMT-like phenotypes.

(A and C) Cell morphological change of four pancreatic cell lines, and a lung (H226) and ovarian (SkOV3) cancer cell line treated with bRNaseA for 2 days. (B) Cell morphological change of HeLa cells treated with bRNaseA in a dose-dependent manner for 1 day.
Figure 3-3. Treatment of bRNaseA modulates molecular EMT makers.

AsPC-1 (A), Panc-1 (B), and HeLa cells (C) were treated with bRNaseA at different time points and lysates were subjected to Western blot analysis with the indicated antibodies (EMT markers).
Figure 3-4. bRNaseA enhances cell mobility in a wound-healing assay.

The distance of the wound gaps at the indicated time points determined using the ImageJ software program (version 1.38x; National Institutes of Health) and normalized against the width at time 0 was plotted diagrammatically as shown in the lower panel. Error bars represent SD from three independent experiments. Statistical analysis was performed by Student’s t test. *, P < 0.05; **, P < 0.01.
Figure 3-5. bRNaseA triggers cell migration and invasion in a dose-dependent manner.

Migration assay in transwells and invasion assay in matrigel-coated invasion chambers of HeLa cells. Top, representative images shown. Bottom, quantification of cell migration and invasion. Error bars represent SD. $n = 3$. Statistical analysis was performed by Student’s $t$ test. *, $P < 0.05$; **, $P < 0.01$. 

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Figure 3-6. The effect of bRNaseA on cell proliferation.

Cell numbers were measured by a Coulter® Particle Counter (Beckman Coulter) in different cell lines. Error bars represent SD. \( n = 3 \).
Figure 3-7. Extracellular RNA is not involved in bRNaseA-triggered EMT.

(A) Coomassie blue staining of bacterial-purified GST-tagged plasmids, including GST-bRNaseA-WT and GST-bRNaseA-H119A, and increasing amounts of recombinant bRNaseA.
cells treated with GST-bRNaseA-WT and those treated with GST-bRNaseA-H119A revealed similar morphological changes (Figure 3-7B) and expression levels of ZEB-1 (Figure 3-7C), suggesting that the catalytic activity of bRNaseA is not required. The enzymatic activity of GST-bRNaseA-H119A was abolished as indicated by RNase activity detection assay (Figure 3-7D). Together, these data indicate that bRNaseA itself but not the degradation of eRNAs induces EMT (see more details in Chapter 5).

3.3 Bovine RNase A triggers signaling cascades of tyrosine-phosphorylation

Since bRNaseA can enter cells (Chao & Raines, 2011), we suspected that the internalized bRNaseA may play a role in EMT induction. Indeed, increased uptake of bRNaseA (signified by red color) was readily detectable without significant changes in the intracellular RNA levels as indicated by SYTO green fluorescence staining (Figure 3-8).

Tyrosine kinase cascades have been shown to associate with EMT (Hardy et al., 2010; Huber et al., 2005), and thus we asked whether bRNaseA might induce EMT by activating the tyrosine kinase cascades. To this end, we examined the phospho-tyrosine (p-Tyr) profile in the presence of bRNaseA treatment in cellular lysates by blotting with a p-Tyr antibody. Interestingly, we found that bRNaseA triggered signaling cascades of tyrosine phosphorylation in multiple types of cancer cells (Figure 3-9). Notably, the p-Tyr content corresponding to the RTK-equivalent molecular weight was substantially increased by bRNaseA treatment (170 to 200-kDa).
Figure 3-7. Extracellular RNA is not involved in bRNaseA-triggered EMT.

(B) Cell morphological change of HeLa cells treated with GST-vector, GST-bRNaseA-WT, and GST-bRNaseA-H119A for 3 days. (C) Cell lysates from (B) were
subjected to Western blot analysis with the antibody against the EMT marker, ZEB-1. Tubulin served as loading control. (D) Detection of RNase enzyme activity in GST-vector, GST-bRNaseA-WT, and GST-bRNaseA-H119A using an RNaseAlert® Lab Test kit monitored by a real-time fluorimeter.
Figure 3-8. bRNaseA internalizes into cells with no interference of intracellular RNA level.
Confocal microscopy of RNA level staining with SYTO® RNASElect™ green-fluorescent cell stain and bRNaseA using an anti-bRNaseA antibody (red signals) for a 30-min treatment of bRNaseA in human cervical HeLa (A), pancreatic cancer AsPC-1 (B), and bovine kidney MDBK (C) cells. Nuclei were stained with DAPI. Bar, 5 µm.
Figure 3-9. bRNaseA triggers signaling cascades of tyrosine-phosphorylation.

(A) HeLa, (B) pancreatic, (C) breast, ovarian, lung cancer cells as well as (D) bovine kidney cells were treated with or without bRNaseA, and lysates were subjected to Western blot analysis with a phospho-tyrosine (p-Tyr) antibody.
3.4 EGFR-RTK is the primary target of bovine RNase A

We then performed a non-biased antibody array for human phospho-RTKs to determine which RTK(s) might be the target(s) of bRNaseA. Among 49 RTKs in the array, only EGFR and ErbB2 had increased phosphorylation after bRNaseA treatment in both cervical (HeLa) and pancreatic (AsPC-1) cancer cells (Figure 3-10). We further validated the results using the phospho-tyrosine antibodies specific for EGFR and ErbB2 and found that most tyrosine phosphorylation peaked at 5 min after bRNaseA treatment (Figure 3-11), a phenomenon similar to EGFR ligand stimulation (Avraham & Yarden, 2011). To further address which of these two RTKs is required for bRNaseA-dependent tyrosine phosphorylation, we knocked down EGFR and ErbB2 by small interfering RNAs (siRNAs). Silencing EGFR attenuated ErbB2 tyrosine phosphorylation without affecting ErbB2 expression, but not vice versa (Figure 3-12A). We also observed similar results in EGFR-knockdown stable cells expressing small hairpin RNA (shRNA) targeting EGFR (Figure 3-12B). Together, these results suggest that ErbB2 tyrosine phosphorylation induced by bRNaseA is dependent on EGFR. Interestingly, in MDA-MB-453 breast cancer cells that do not express endogenous EGFR, bRNaseA treatment had no effect on pan-tyrosine phosphorylation (Figure 3-12C), which strongly suggests that EGFR, but not ErbB2, is the predominant target of bRNaseA.
Figure 3-10. EGFR and ErbB2 show increased phosphorylation after bRNaseA treatment in human phospho-RTK antibody array.

HeLa and AsPC-1 cells were treated with or without bRNase A, and lysates were subjected to human phospho-RTK antibody array analysis.
Figure 3-11. brNaseA treatment activates EGFR and ErbB2.

HeLa (A) and AsPC-1 (B) cells were treated with brNaseA at different time points, and lysates were subjected to Western blot analysis with the indicated antibodies.
Figure 3-12. ErbB2 tyrosine phosphorylation induced by bRNaseA is EGFR kinase-dependent.

(A) Western blot analysis of lysates from HeLa cells transfected with the individual siRNAs against EGFR and ErbB2 in the presence or absence of bRNaseA.
Figure 3-12. ErbB2 tyrosine phosphorylation induced by bRNaseA is EGFR kinase-dependent.

(B) Western blot analysis of lysates of HeLa EGFR-deficient stable transfectants (EGFR-A and EGFR-B) in the presence or absence of bRNaseA. (C) MDA-MB-453 and SKBR3 cells were treated with or without bRNaseA, and lysates
3.5 Bovine RNase A activates EGFR downstream signaling pathways

To further pursue whether bRNaseA activates the downstream signaling cascades of EGFR, another antibody array for human phospho-kinases was performed. The results showed that six serine and threonine signaling pathways, including p38α, ERK1/2, Gsk-3α/β, Akt, CREB, and RSK1/2/3, were commonly phosphorylated in two cells after bRNaseA treatment (Figure 3-13), in which some candidates, such as ERK1/2 and Akt pathways, were confirmed using the indicated antibodies (Figure 3-14). Pretreating these cells with small-molecular tyrosine-kinase inhibitors (TKIs) targeting EGFR (gefitinib and AG1478) blocked the bRNaseA-triggered EGFR, and subsequently Akt and ERK, activation (Figure 3-15). These results indicate that bRNaseA triggers EGFR-downstream signaling in an EGFR kinase-dependent manner.

3.6 Bovine RNase A associates with EGFR in vivo and in vitro

Since bRNaseA stimulates tyrosine phosphorylation of EGFR and its downstream signaling, we asked whether bRNaseA functions as an EGFR ligand. To this end, we first validated the association between EGFR and bRNaseA by multiple methods, including in vivo co-immunoprecipitation, in vitro binding assay, confocal microscopy, and Duolink in situ proximity ligation assay (PLA). bRNaseA co-immunoprecipitated with EGFR using an anti-EGFR antibody in vivo (Figure 3-16A), and the interaction was primarily through the extracellular domain (ECD) of EGFR (Figure 3-16B). In addition, bRNaseA interacted with the N-terminal EGFR recombinant protein (N-EGFR-Fc) that contained only the extracellular ligand-
Figure 3-13. bRNaseA activates EGFR downstream signaling pathways in a human phospho-kinase antibody array.

HeLa and AsPC-1 cells were treated with or without bRNase A, and lysates were subjected to human phospho-kinase antibody array analysis.
**Figure 3-14. bRNaseA treatment activates ERK and Akt.**

HeLa (A) and AsPC-1 (B) cells were treated with bRNaseA at different time points, and lysates were subjected to Western blot analysis with the indicated antibodies.
Figure 3-15. EGFR-TKIs blocks bRNaseA-triggered EGFR signaling.

HeLa cells were pretreated with EGFR-TKIs (gefitinib and AG1478), followed by bRNaseA treatment, and lysates were subjected to Western blot analysis with the indicated antibodies.
Figure 3-16. bRNaseA associates with EGFR.

(A) HeLa cells underwent immuneprecipitation with antibodies targeting EGFR or normal IgG. Precipitates were analyzed by Western blot the indicated antibodies. The right panel shows the input lysates. (B) In vivo binding assay of bRNaseA in 293T cells transfected with myc-His-tagged EGFR containing full-length (FL), extracellular domain (ECD), and intracellular domain (ICD) plasmids, pulled down using His beads.
binding domain in vitro (Figure 3-16C). Consistent with the biochemical studies, we observed co-localization of EGFR and bRNaseA using confocal immunofluorescence assay as indicated by yellow signals in the merged image (Figure 3-17A), which was further validated by examining sequential photosections of a cell in multiple layers in three-dimensions (Figure 3-17B). This EGFR/bRNaseA association (indicated by yellow merged signals) was also detected by a non-antibody-based approach using bRNaseA recombinant proteins labeled with DyLight 594 dye that generated red fluorescence for confocal analysis (Figure 3-18). In addition, a new and more sensitive Duolink in situ PLA technology, which utilizes detectable DNA molecules amplified by binding of two protein-DNA conjugates in close proximity (Fredriksson et al., 2002), demonstrated a number of interactions between EGFR and bRNaseA (Figure 3-19). These results from these assays support the bRNaseA/EGFR association both in vitro and in vivo.

3.7 Bovine RNase A acts as an EGFR ligand to convey intracellular EGFR signaling

We further found that cetuximab, a monoclonal EGFR antibody that blocks binding of ligands to EGFR, inhibited bRNaseA-induced activation of EGFR and its downstream Akt signaling (Figure 3-20). Disrupting the interaction between the N-terminal ligand-binding domain of EGFR and bRNaseA by competition with an N-EGFR-Fc recombinant protein or EGFR-ECD also decreased EGFR activation triggered by bRNaseA (Figure 3-21). Collectively, the results suggest an unrecognized role of bRNaseA as an EGFR ligand to convey EGFR signaling.
Figure 3-16. bRNaseA associates with EGFR.

(C) *In vitro* binding assay of bRNaseA and N-EGFR-Fc, an N-terminal EGFR recombinant protein, pulled down using protein G beads. Normal IgG and BSA served as negative controls.
Figure 3-17. brNaseA co-localizes with EGFR using confocal immunofluorescence assay.
(A) Confocal microscopy of HeLa cells stained with DAPI (blue), anti-bRNaseA (green), and anti-EGFR (red) antibodies. Boxed areas are shown in detail in the insets. Bar, 5 µm. (B) A single HeLa cell was dissected into 32 focal sections with a thickness of 0.5 µm each. The nucleus spanned between focal plans 04-12. Colocalization of EGFR and bRNaseA, as indicated by the yellow signals in the merged images, is shown from plans 06-10 in the insets (white arrows). Boxed areas are shown in detail in the insets. Bar, 5 µm.
Figure 3-18. DyLight 594-labeled bRNaseA co-localizes with EGFR.

(A) Confocal microscopy of HeLa cells stained with DAPI (blue), DyLight 594-labeled bRNaseA (red), and an anti-EGFR antibody (green). Boxed areas are shown in detail in the insets. Bar, 5 µm. (B) Intensity profile of two yellow signals shown in Inset 1 of (A).
Figure 3-19. bRNaseA interacts with EGFR using Duolink in situ PLA technology.

Detection of EGFR and bRNaseA interaction by Duolink in situ PLA technology. HeLa cells were stained with EGFR and bRNaseA antibodies. The number of interactions per cell is shown on the right. Error bars represent SD. n = 3.
Figure 3-20. Cetuximab attenuates bRNaseA-induced activation of EGFR and its downstream Akt signaling.

HeLa cells were pretreated with cetuximab (10 µg/ml) at 4°C for 1 h, followed by bRNaseA stimulation for 5 min, and lysates were subjected to Western blot analysis with the indicated antibodies.
Figure 3-21. Blockage of the association between EGFR and bRNaseA inhibits bRNaseA-induced EGFR signaling.

(A) HeLa cells were treated with bRNaseA pre-incubated with or without N-EGFR-Fc, as indicated, and lysates were subjected to Western blotting with the indicated antibodies. (B) HeLa cells transfected with the extracellular domain of EGFR (EGFR-ECD) were treated with bRNaseA treatment, and lysates were subjected to Western blotting with the indicated antibodies.
Chapter 4

Human RNase 5 serves

as an EGFR ligand
Background and Rationale

The human RNase A superfamily consists of 13 members and all are secretory proteins (Cho et al., 2005). In addition to their catalytic activities against specific RNAs, they possess a number of physiological functions, e.g., digestion of dietary RNA (RNase 1), angiogenesis (RNase 5/angiogenin; hereinafter referred to as hRNase5), and antiviral host defense (RNases 2, 3, 7) (Li & Hu, 2012; Rosenberg, 2008; Sorrentino, 2010). Among them, human pancreatic RNase 1 and hRNase5 are most evolutionarily closely related to bRNaseA (Peracaula et al., 2000; Rosenberg, 2008). We next investigated whether the newly discovered function of bRNaseA, which acts as an EGFR ligand and promotes transformed phenotypes, also exists in human RNase A members.

Results

4.1 Human RNase 5 is predominantly secreted in pancreatic cancers

We examined the levels of secreted hRNase5 and RNase 1 in a panel of 10 pancreatic cancer cells and found all cell lines secreted hRNase5 whereas only one cell line (Capan-1) secreted RNase 1 (Figure 4-1), suggesting that hRNase5 is the RNase predominantly expressed in pancreatic cancers. Furthermore, we analyzed the correlation of EGFR with ANG (gene encoding hRNase5 protein) and RNASE1 in pancreatic ductal adenocarcinoma using the cancer microarray database ONCOMINE (Rhodes et al., 2004). EGFR correlated positively with ANG1 ($P < 0.0001$) (Figure 4-2) but not RNASE1.
Figure 4-1. hRNase5 is secreted in a panel of 10 pancreatic cancer cells.

Analysis of the expression level of hRNase5 and RNase 1 proteins secreted in pancreatic cancer cells.
Figure 4-2. *hRNase5* correlates positively with *EGFR* in pancreatic ductal adenocarcinoma.

Heat map analysis of *EGFR* and *hRNase5* in pancreatic ductal adenocarcinoma (PDAC) via a cancer microarray database, ONCOMINE, plotted diagrammatically in the lower panel. n = 87.
4.2 Human RNase 5 functions as an EGFR ligand to activate EGFR signaling

We then investigated whether hRNase5 also functions as an EGFR ligand. Indeed, similar to bRNaseA, hRNase5 bound to the N-terminal domain of EGFR directly (Figure 4-3A), which was attenuated by pretreatment of cetuximab (Figure 4-3B, lane 3 vs. lane 4). In addition, hRNase5 also activated EGFR and its downstream targets in a dose-dependent manner (Figure 4-4). These hRNase5-mediated EGFR activations were blocked by pretreatment of cetuximab (Figure 4-5) and EGFR-TKIs (Figure 4-6), further supporting that like bRNaseA, hRNase5 also functions as an EGFR ligand to convey intracellular signaling by binding to the extracellular ligand-binding domain of EGFR.

4.3 Human RNase 5 triggers cell migration and invasion through the binding to and activating EGFR

To determine whether EGFR is required for hRNase5-triggered oncogenic functions in pancreatic cancers, we generated AsPC-1 stable transfectants that express hRNase5 (designated as AsPC-1-hRNase5; Figures 4-7A and 4-7B) at a level comparable to CFPAC-1 cells that express high levels of hRNase5 (Figure 4-7C) and performed cell migration and invasion assays. Compared with vector control, hRNase5-expressing stable cells exhibited enhanced cell migration and invasion (Figures 4-8A and 4-8B), but these effects were significantly repressed by pretreating cells with cetuximab, EGFR-TKIs, or hRNase5 neutralizing antibody (ANG Ab). These results support the notion that hRNase5 triggers pancreatic cancer cell migration and invasion via binding to and activating EGFR.
Figure 4-3. Cetuximab attenuates *in vitro* interaction between hRNase5 and EGFR.

(A) Left: *in vitro* binding assay of a GST-tagged plasmid expressing hRNase5 (GST-hRNase5) and N-EGFR-Fc, pulled down using protein G beads. An empty vector (GST) alone is a negative control. (B) *In vitro* binding assay of GST-hRNase5 and N-EGFR-Fc, which was pre-incubated with cetuximab. An asterisk represents binding and expression of GST-hRNase5.
Figure 4-4. hRNase5 treatment activates EGFR and the downstream signaling pathways.

HeLa cells were treated with increasing concentrations of hRNase5 for 5 min and lysates were subjected to Western blotting with the indicated antibodies.
Cetuximab attenuates downstream Akt signaling. HeLa cells were pretreated with cetuximab (10 µg/ml) at 4°C for 1 h, followed by hRNase5 stimulation for 5 min, and lysates were subjected to Western blot analysis with the indicated antibodies.

Figure 4-5. Cetuximab attenuates hRNase5-induced activation of EGFR and its downstream Akt signaling.

HeLa cells were pretreated with cetuximab (10 µg/ml) at 4°C for 1 h, followed by hRNase5 stimulation for 5 min, and lysates were subjected to Western blot analysis with the indicated antibodies.
Figure 4-6. Pretreatment of EGFR-TKIs inhibits hRNase5-mediated EGFR activation.

AsPC-1 (A), Panc-1 (B), and HeLa (C) cells were pretreated with EGFR-TKIs (erlotinib or gefitinib), followed by hRNase5 treatment for 5 min, and lysates were subjected to Western blotting with the indicated antibodies.
Figure 4-7. The levels of hRNase5 in AsPC-1-hRNase5 stable transfectants are comparable to those in highly-expressed cells.

(A) Western blot analysis of secreted proteins in AsPC-1 stable transfectants expressing hRNase5 (AsPC-1-hRNase5) and empty vector (AsPC-1-vector). A comparison of hRNase5 expression level in AsPC-1-hRNase5 and AsPC-1-vector, to increasing amounts of recombinant hRNase5 proteins (B) and in other pancreatic cancer cell lines (C).
Figure 4-8. hRNase5 triggers cell migration and invasion through the binding to and activating EGFR.

(A) Representative images of cell migration and invasion assays of AsPC-1 stable transfectants expressing hRNase5 treated with an EGFR antibody (cetuximab), an EGFR-TKI (gefitinib), or hRNase5 neutralization antibody (ANG Ab). (B) Quantification of cell migration and invasion from (A). Error bars represent SD. n = 3. Statistical analysis was performed by Student’s t test. **, P < 0.01; ***, P < 0.001.
4.4 The expression level of human RNase 5 is significantly elevated in pancreatic cancer patients

Decades ago, investigators reported that the activities of serum RNases (Reddi & Holland, 1976; Warshaw et al., 1980), and later the expression of hRNase5 (Shimoyama et al., 1996) were elevated in pancreatic cancer patients and suggested that RNases could serve as diagnostic markers in pancreatic cancer progression (Misek et al., 2007; Zhao et al., 1998). We re-evaluated the concentration of RNase 1 and hRNase5 in the plasma samples of pancreatic cancer patients for comparison with non-cancer control group (Figure 4-9). Consistent with the previous report (Shimoyama et al., 1996), a significant increase in plasma hRNase5 was indeed detected in the cancer group ($P = 0.014$; Figure 4-9A) whereas the mean concentrations of plasma RNase 1 between these two groups were not significant ($P = 0.83$; Figure 4-9B).

Interestingly, there were no significant differences in traditional EGFR ligands, EGF and TGF-α, between normal and pancreatic cancer patient samples (Figures 4-9C and 9D); their mean concentrations in patients were approximately 20,000-fold lower than that of plasma hRNase5 (18.1 and 15.6 pg/ml vs. 458.8 ng/ml; Table 4-1).
Figure 4-9. Human RNase 5 is elevated in plasma samples of pancreatic cancer patients.

Analysis of hRNase5 (A), human RNase 1 (B), EGF (C), and TGF-α (D) in plasma samples of pancreatic cancer patients for comparison with non-cancer control group by an enzyme-linked immunosorbent assay (R&D Systems).
Table 4-1. Analysis of hRNase5, human RNase 1, EGF, and TGF-α in plasma samples of pancreatic cancer patients for comparison with non-cancer control group by ELISA.
4.5 A physiological relevance between activated EGFR and human RNase 5 in pancreatic cancers

Importantly, human pancreatic tumor tissue microarrays showed that phospho-EGFR-Y1068, which is indicative of EGFR activation, was positively correlated with hRNase5 expression ($P = 0.03$; Figure 4-10), strongly suggesting a physiological association between activated EGFR and hRNase5 in pancreatic cancers.

<table>
<thead>
<tr>
<th>hRNase5</th>
<th>Low</th>
<th>High</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-EGFR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>77</td>
<td>15</td>
<td>92</td>
</tr>
<tr>
<td>High</td>
<td>17</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>94</td>
<td>25</td>
<td>119</td>
</tr>
</tbody>
</table>

$P=0.03$

Figure 4-10. hRNase5 correlates positively with activated EGFR expression in human pancreatic tissue microarrays.

Pancreatic cancer tissue microarray analysis for the correlation between hRNase5 and phospho-EGFR-Y1068.
Chapter 5

The EGFR ligand-like activity of pancreatic RNase is independent of its enzyme activity.
Background and Rationale

RNase is well known to associate with intrinsic catalytic enzyme activities to degrade RNA (Cuchillo et al., 2011; Marshall et al., 2008); however, as previously mentioned, bRNaseA induced EMT via a catalytic-independent pathway (Figure 3-7). Next, we asked whether hRNase5 and bRNaseA bind to EGFR and activate EGFR signaling pathways, such as Akt and ERK, in a catalytic-independent pathway.

Results

5.1 RNase enzyme activity is not required for tyrosine-phosphorylation cascades triggered by GST-bRNaseA

In addition to an enzymatic activity-independent role of bRNaseA in EMT process, the increased p-Tyr profile and EGFR activation were also observed in the cells treated with enzyme-deficient GST-bRNaseA-H119A mutant, at a similar level compared to that with GST-bRNaseA-WT (Figure 5-1), suggesting that bRNaseA-triggered tyrosine-phosphorylation cascades were independent of its catalytic enzyme activity.

5.2 RNases activate and bind to EGFR independently of their catalytic enzyme activities

Thus, we then asked whether the enzyme activities of bRNaseA and hRNase5 expressed in mammalian cells were also independent of EGFR binding in addition to the activation. To this end, we generated wild-type and catalytic enzyme-deficient
Figure 5-1. bRNaseA-triggered tyrosine-phosphorylation cascades and EGFR activation is independent of its catalytic enzyme activity.

HeLa cells were treated with PBS only, GST-vector, GST-bRNaseA-WT, or GST-bRNaseA-H119A for 5 min, and lysates were subjected to Western blot analysis with the indicated antibodies.
mutants of bRNaseA and hRNase5, either by transient transfection or stably expression in HeLa cells. Media from HeLa cells transiently transfected with empty vector or Flag-bRNaseA plasmids expressing wild-type (Flag-bRNaseA-WT) or catalytic-deficient bRNaseA mutant (Flag-bRNaseA-H119A) were collected and concentrated to evaluate the RNase activities. As shown in Figure 5-2A, RNase activities were abolished in cells expressing the Flag-bRNaseA-H119A mutant but not in those expressing Flag-bRNaseA-WT as indicated by the loss of fluorescence. Western blot analysis demonstrated that their expression levels were not affected (Figure 5-2B). We found that the secreted bRNaseA-H119A mutant lacking RNase activity still activated EGFR and the downstream ERK and Akt pathways at levels similar to bRNaseA-WT (Figure 5-2C). In addition, the lack of RNase activity in the catalytic-deficient mutant did not affect its interaction with EGFR (Figure 5-2D), and these results were validated in stable transfectants (Figure 5-3).

A similar experiment was performed to test the catalytic-deficient mutants of hRNase5 (K40A and H114A; Figure 5-4A) with results indicating that hRNase5 triggered EGFR phosphorylation (Figure 5-4B) and interacted with EGFR (Figure 5-4C). Together, these findings indicate that human RNase 5 and bovine RNase A bind to EGFR and activate EGFR signaling pathways, without requiring their catalytic activities.
Figure 5-2. bRNaseA activates and binds to EGFR independently of their catalytic activities.
(A) Detection of RNase enzyme activity in concentrated culture media collected from cells transfected with Flag-bRNaseA-WT, Flag-bRNaseA-H119A, or empty vector, by using an RNaseAlert® Lab Test kit and monitored by a real-time fluorimeter. (B) Western blot analysis of secreted proteins in the media from HeLa cells expressing Flag-bRNaseA-WT, Flag-bRNaseA-H119A, or empty vector. The relative density at Flag-bRNaseA-WT was defined as 1 using the imageJ software program (version 1.38x; National Institutes of Health) to quantify the signals. (C) Left: HeLa cells were treated with culture media containing secreted proteins, as indicated in (B), for 5 min and lysates were subjected to Western blotting with the indicated antibodies. Right: the quantified signal representing the fold of EGFR phosphorylation against the amounts of the indicated secreted proteins. Error bars represent SD. n = 3. Statistical analysis was performed by Student’s t test. NS, P > 0.05, not significant. (D) Detection of EGFR and bRNaseA interaction by Duolink in situ PLA technology. HeLa cells were treated with the secreted proteins, as indicated in (B), for 15 min, and fixed and stained with anti-EGFR and anti-bRNaseA antibodies. The number of interaction per cell normalized to the amount of the secreted proteins is shown right. Error bars represent SD. n = 3. Statistical analysis was performed by Student’s t test. NS, P > 0.05, not significant.
Figure 5-3. bRNaseA secreted in stable transfectants binds to EGFR independently of its catalytic activity.

(A) Detection of RNase enzyme activity in the concentrated media of HeLa stable transfectants expressing Flag-bRNaseA-WT, Flag-bRNaseA-K41A, Flag-bRNaseA-
H119A, and empty vector, using an RNaseAlert® Lab Test kit monitored by a real-time fluorimeter. (B) Western blot analysis of secreted proteins in HeLa stable transfectants expressing Flag-bRNaseA-WT, Flag-bRNaseA-K41A, Flag-bRNaseA-H119A, and empty vector. The relative density at Flag-bRNaseA-WT was defined as 1 using the imageJ software program (version 1.38x; National Institutes of Health) to quantify the signals. (C) Detection of EGFR and bRNaseA interaction by Duolink in situ PLA technology. HeLa cells were treated with secreted WT, K41A, or H119A bRNaseA for 15 min as described in (B) and fixed and stained with anti-EGFR and anti-Flag antibodies. The number of interaction per cell normalized to the amount of the secreted proteins is shown on the right. Error bars represent SD. $n = 3$. Statistical analysis was performed by Student’s $t$ test. NS, $P > 0.05$, not significant.
Figure 5-4. hRNase5 secreted in stable transfectants activates and binds to EGFR independently of its catalytic activity.

(A) Western blot analysis of hRNase5 in the culture media of HeLa stable transfectants expressing Flag-hRNase5-WT, Flag-hRNase5-K40A, Flag-hRNase5-
H114A, or empty vector. The relative density at Flag-hRNase5-WT was defined as 1 using the ImageJ software program (version 1.38x; National Institutes of Health) to quantify the signals. (B) Left: HeLa cells were treated with secreted WT, K40A, or H114A hRNase5 as described in (A) for 5 min and lysates were subjected to Western blot analysis with the indicated antibodies. Right: quantitation of the fold of EGFR phosphorylation against the amount of the indicated secreted proteins. Error bars represent SD. n = 2. Statistical analysis was performed by Student’s t test. NS, P > 0.05, not significant. (E) Detection of EGFR and hRNase5 interaction by Duolink in situ PLA technology. HeLa cells were treated with secreted WT, K40A, or H114A hRNase5 for 15 min as described in (A) and fixed and stained with anti-EGFR and anti-Flag antibodies. The number of interaction per cell normalized to the amount of the secreted proteins is shown on the right. Error bars represent SD. n = 3.
Chapter 6

Characterization of interaction between EGFR and RNases
Background and Rationale

The EGFR ligand-like activity of hRNase5 prompted us to ask whether hRNase5 share sequence homology with traditional EGFR ligands, such as EGF and heparin-binding EGF-like growth factor (HB-EGF).

Results

6.1 The C-terminal hRNase5 possesses highly and evolutionally conserved residuals with those on EGF and HB-EGF

Primary sequence alignment showed that the C-terminal region (known for EGFR binding in EGF and HB-EGF) of hRNase5, EGF, and HB-EGF are highly and evolutionally conserved in different species (shown in red; Figure 6-1).

6.2 The C-terminal domain of hRNase5 (amino acid 74-123) is required for EGFR binding

To further study whether the C-terminal region of hRNase5 plays a role in binding to EGFR, we generated wild-type (WT), N- (ΔN), and C-terminal deletion (ΔC) constructs of hRNase5 and in vitro binding assay was performed. Consistent with the analysis in Figure 6-1, we did not detect any association with EGFR when this C-terminal region (amino acid 74-123) was deleted from hRNase5 (Figures 6-2A and 6-2B), suggesting that this region is required for EGFR binding.
**Figure 6-1.** Sequence alignment of RNase5 to known EGFR ligands (EGF and HB-EGF) in different species.
T-coffee sequence alignment (Notredame et al., 2000; Taly et al., 2011) of EGF, HB-EGF, and RNase 5 between human, mouse, and horse. Regions in red indicate high consistency, orange/yellow as an average consistency, and green/blue as the lowest consistency. Asterisks represent 100% conservation in all sequences; colons and dots represent partial sequence conservation.
Figure 6-2. The C-terminal hRNase5 is required for EGFR binding.

(A) A schematic diagram showing wild-type (WT), N- (ΔN), and C-terminal deletion (ΔC) constructs of hRNase5. The numbers represent amino acid residues. NLS, nuclear localization signal. (B) In vitro binding assay of N-EGFR-Fc with GST-hRNase5 WT and two of its deletion mutants, GST-hRNase5 ΔN and GST-hRNase5 ΔC, pulled down using protein G beads.
6.3 Gln93 and Tyr94 within C-terminal region of human RNase 5 are critical for efficient binding to EGFR

To map the potential EGFR binding site(s) in hRNase5, we compared the C-terminal region between human EGF, hRNase5, and several hRNase5 homologues and found that they are indeed highly conserved (Figures 6-3A and 6-3B).

We identified four conserved residues, including two Cys (C81 and C92), which are generally crucial for protein structure, and Gln (Q93) and Tyr (Y94) that correspond to the side chains in human EGF reported to bind to EGFR (Figure 6-4A) (Li et al., 2005), and when mutated to alanine, their association with EGFR was inhibited (Figure 6-4B), suggesting that they may play a role in binding of hRNase5 to EGFR.

The decrease binding of hRNase5-Q93A to EGFR was further validated in vivo by incubating N-EGFR-Fc with the collected media containing secreted WT and Q93A mutant of hRNase5 following transfection (Figures 6-5A and 6-5B). Interestingly, Y94 hRNase5 mutant was not detected in the media after transfection as the mutation may have affected its secretion (Figure 6-5B). Similarly, no interaction was detectable between EGFR and hRNase5 containing the Cys mutants to alanine, likely due to lack of protein secretion in the media (Figure 6-6). Together, results from the in vitro assays suggest that Q93 and Y94 within C-terminal region of hRNase5 are required for efficient binding to EGFR.
RNase 5 homologues reveals a highly conserved C-terminus.
Figure 6-3. Sequence alignment of human RNase 5 to RNase 5 homologues reveals a highly conserved C-terminus.

(B) T-coffee sequence alignment between different species of RNase 5, including human, mouse, horse, and cow, with human EGF.
Figure 6-4. Gln93 and Tyr94 within the C-terminus of hRNase5 are required for EGFR binding.

(A) Sequence alignment of human RNase 5 and human EGF. Asterisks represent 100% conservation in all sequences; colons and dots represent partial sequence
conservation. (B) Top: in vitro binding assay of N-EGFR-Fc with myc-tagged hRNase5 containing WT, C81A, C92A, Q93A, or Y94A mutant, pulled down using protein G beads. The relative density at myc-GST-hRNase5-WT was defined as 1 using the ImageJ software program (version 1.38x; National Institutes of Health) to quantify the signals. Bottom: the quantified signal representing the fold ratio of hRNase5 bound to EGFR.
Figure 6-5. The *in vivo* association of hRNase5 with EGFR is decreased while Gln93 of hRNase5 is mutated.

(A) Left: binding assay of N-EGFR-Fc with the collected media from 293T cells transfected with WT and Q93A mutant, pulled down using protein G beads. Quantified ratio of hRNase5 bound to EGFR is shown on the right. (B) Western blot analysis of 293T cells expressing myc-His-hRNase5 WT, Q93A, Y94A, or empty vector in the lysates and in culture media (secreted proteins).
Figure 6-6. hRNase5 plasmids containing the Cys mutants to alanine are not secreted in the media.

(A) Binding assay of N-EGFR-Fc with the collected media from 293T cells transfected with myc-GST-hRNase5-WT, the indicated Cys mutants, and empty vector, pulled down using protein G beads. (B) Immunoblotting analysis of 293T cells expressing WT, two of its point mutants, and empty vector within the lysates and secreted proteins.
6.4 The binding affinity of human RNase 5 toward EGFR is within the range of known EGFR ligands with high binding affinity

Next, we measured its binding affinity of hRNase5 for EGFR for comparison with EGF. Notably, the binding of hRNase5 to the N-terminal region of EGFR was relatively low (Figure 6-7A; ~70% binding ratio at 143 nM) compared with that of EGF (Figure 6-7B; ~100% binding ratio at 3.33 nM).

For a more quantitative analysis, we performed enzyme-linked immunosorbent assay (ELISA) captured by an EGFR antibody with or without A431 cell lysates to determine the individual dissociation constants ($K_D$). The estimated $K_D$ of EGF bound to EGFR was 1.87 nM (Figure 6-8A), which was consistent with published values (Bjorkelund et al., 2011; Ozcan et al., 2006). Under the same ELISA condition, the binding affinity ($K_D$) of hRNase5 and bRNaseA for full-length EGFR was 36.6 nM (Figure 6-8B) and 885.3 nM (Figure 6-8C) or about 20-fold and 473-fold less than that of EGF, respectively.

It is worthwhile to mention that there are two classes of human EGFR ligands, including those with high affinity with a $K_D$ ranging between 1-100 nM (e.g., EGF; HB-EGF) and those with low affinity with a $K_D$ greater than 100 nM (e.g., amphiregulin, $K_D = 350$ nM; epigen, $K_D > 500$ nM; murine epiregulin, $K_D = 2.8 \mu$M) (Sanders et al., 2013). Thus, the $K_D$ of hRNase5 falls within the range of high affinity EGFR ligands.
Figure 6-7. The binding of hRNase5 to the N-terminal region of EGFR is determined by using a pull-down assay.

(A and B) Left: *in vitro* binding assay of N-EGFR-Fc across a range of concentrations of hRNase5 (A) or EGF (B), pulled down using protein G beads. BSA served as a negative control. Right: Quantification of hRNase5 (A) or EGF (B) bound to EGFR against the individual input.
Figure 6-8. Binding affinity of EGF, hRNase5, and bRNaseA toward EGFR are detected by ELISA with estimated $K_D$ values.
Left: different concentrations of EGF (A), hRNase5 (B), and bRNaseA (C) were added to an EGFR-captured ELISA plate, with or without A431 cell lysates. Dotted line represents Bmax (maximum number of binding sites). Error bars represent SD. n = 2. Right: data derived using Scatchard plot analysis to determine $K_D$ values for interaction of EGFR with EGF, hRNase5, and bRNaseA.
Chapter 7

Human RNase 5 contributes to cetuximab resistance in pancreatic cancer
Background and Rationale

Cetuximab, a FDA approved anti-cancer drug that prevents EGFR from binding to its activating ligands, was proposed as a treatment modality for pancreatic cancer as EGFR signaling was recently identified to be essential for tumor initiation in pancreatic ductal adenocarcinoma (Ardito et al., 2012; Navas et al., 2012). However, clinical trials using cetuximab as adjuvant therapy with gemcitabine to treat pancreatic cancer did not improve the overall survival (Fensterer et al., 2013; Philip et al., 2010). We therefore asked whether hRNase5 plays a role in resistance to cetuximab therapy.

Results

7.1 hRNase5 enhances resistance to cetuximab therapy in pancreatic cancer

Strikingly, we found that cetuximab-repressed cell migration and invasion of AsPC-1 pancreatic cancer cells was significantly increased in response to hRNase5 treatment in a dose-dependent manner (Figures 7-1A and 7-1B). This suggests that hRNase5 is associated with cetuximab resistance.

7.2 hRNase5-enhanced cell migration and invasion is neutralized by an anti-hRNase5 antibody

In contrast, addition of an anti-hRNase5 antibody neutralized the enhanced cell migration and invasion (Figures 7-2A and 7-2B). Together, the elevated level of serum hRNase5 observed in pancreatic cancer patients is likely sufficient to activate EGFR, which may in turn contribute to malignancy and resistance to cetuximab.
Figure 7-1. Cetuximab-repressed cell migration and invasion is significantly derepressed by hRNase5 treatment in a dose-dependent manner.

(A) Representative images from cell migration and invasion assays of AsPC-1 cells treated with or without cetuximab (10 µg/ml) and various concentrations of hRNase5 (1, 3, 9 µg/ml). (B) Quantification of cell migration and invasion from (A). Error bars represent SD. n = 3. Statistical analysis was performed by Student’s t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 7-2. hRNase5-enhanced cell migration and invasion is neutralized by an anti-hRNase5 antibody.

(A) Representative images from cell migration and invasion assays of AsPC-1 cells treated with cetuximab (10 µg/ml), hRNase5 (2 µg/ml), and ANG neutralization Ab (5 µg/ml), as indicated. (B) Quantification of cell migration and invasion from (A). Error bars represent SD. n = 3. Statistical analysis was performed by Student’s t test. **, P < 0.01; ***, P < 0.001.
Chapter 8

Discussion and Future work
Our study has identified bovine RNase A and its human homologue RNase 5 can serve as specific ligands to activate surface EGFR to initiate its downstream signaling network (Figure 8-1). It brings up one fundamental question that how these secretory ribonucleases can bind to the extracellular domain of EGFR in a specific manner. In our results, ribonuclease binding to EGFR can be impaired by C225 pretreatment, which suggests that ribonuclease binding region on EGFR extracellular domain could be very close to those bound by EGF. It is plausible that ribonuclease may exert certain structural similarity to EGF-like ligands, or ribonuclease could possess distinct and unknown conformation to be associated with EGFR as a complex. Both secondary and tertiary structure of ribonucleases and EGFR canonical ligands are well studied and established. Based on current knowledge about these two protein families, they are very different from each other in terms of disulfide bond linkage and crystal structure. Interestingly, there exist certain homology between human RNase 5 and human EGF in their primary sequence. Among these conserved residues, Q93 and Y94 residues of human RNase 5 are corresponding cognates to Q43 and Y44 of EGF protein, where Q43 and Y44 have been shown to locate on the interface of EGF during its binding to EGFR although both residues are not essential to this interaction. Either Q93A or Y94A mutation can significantly decrease human RNase 5 binding to EGFR protein in vitro. It provides certain evidence to support that the binding region of human RNase 5 with EGFR may be similar to EGF with EGFR in a certain degree. To draw a more delicate and authentic picture of the complex formed by RNase and EGFR, we need to apply X-ray crystallography and NMR spectrometry to analyze their co-
Figure 8-1. A proposed model for mammalian secretory ribonucleases to provoke EGFR signaling pathways and EMT-like phenotypes in human pancreatic cancer.

Bovine RNase A (bRNaseA) and its human homologue RNase 5 (hRNase5) act as EGFR ligands to directly activate cell surface EGFR/EGFR homo-dimer or EGFR/ErbB2 hetero-dimer, initiate its downstream signaling network, leading to cell migration, invasion, and EMT-like phenotypes. This ligand-like activity, which is independent of RNases’ enzymatic activity, contributes to tumor progression and C225 resistance in pancreatic cancer cells.
crystal structure and compared them with well-resolved structure composed of EGFR and conventional ligands like EGF or HB-EGF. More efforts to explore the structural insights of RNase-EGFR complex will provide novel viewpoints of EGFR activation because they may interact with each other through a way quite distinct from EGF-EGFR complex.

Our research has revealed the first example that human RNase 5 can act as EGFR ligand independently of its enzyme activity. It is reasonable to speculate that other members of RNase A family may have similar function to serve as ligands for other receptors, in addition to their conventional roles as ribonucleases and participants in immunity. Notably, among the 13 members of RNase A family, RNase 9-13 are known to lose enzymatic activity as non-canonical ribonucleases. It would be interesting to test if any of them can be a cognate ligand to certain membrane receptor as well. A more systematic primary sequence alignment between RNase A family members and protein growth factors can be conducted to see if there exist any evolutional conservation among them. Moreover, ectopic expression of individual ribonuclease to stimulate recipient cells through conditioned medium combined with analysis through kinase antibody arrays will help to elucidate a potential role of other ribonuclease as a novel ligand to a known receptor. Future studies on this point of view may define certain ribonucleases as specific ligands to receptors to initiate cellular signaling pathways, and it can provide an explanation and biological role for those existing ribonucleases which lose their catalytic activities during evolution. On the other hand, it provides more
opportunities to find out new ligands for membrane receptors especially those without known ligands. Since ErbB2 is an orphan receptor without any known ligand to date, this approach may help to identify a first ligand of ErbB2 in the future.

Previous studies have indicated human RNase 5 can bind to a 170-kDa membrane receptor of endothelial cells to trigger intracellular signaling and promote angiogenesis. Of note, EGFR is a 170-kDa receptor tyrosine kinase and in our study it is identified as the primary receptor of tumor cells to be associated with ribonuclease and initiate downstream signaling including AKT and ERK activation upon ribonuclease stimulation. Our work may provide an adequate answer to a long-term issue for years. According to previous reports, tumor-associated endothelial cells express higher level of EGFR and the EGFR signaling is required for their proliferation and angiogenic function. Since previously the unknown 170-kDa membrane receptor was identified from endothelial cells, it would be worthwhile to investigate if human RNase 5 can bind to EGFR of tumor-associated endothelial cells, like we have observed with tumor cells. The experimental results will help to address if our current model can be applied to endothelial cells through a paracrine manner, since we have demonstrated that secreted ribonuclease can activate EGFR signaling in tumor cells through an autocrine mechanism. The same possibility also exist between tumor and stromal cells, because in pancreatic cancer the stromal cells play a crucial role for cancer aggressiveness and chemoresistance, and through a paracrine pathway that ribonuclease may also stimulate EGFR
signaling in tumor-associated stromal cells to aggravate pancreatic cancer tumorigenesis.

We recently demonstrated that human RNase 5 can activate EGFR signaling in pancreatic cancer cells. In addition, the EGFR activation is essential to human RNase 5-provoked cell migration and invasion. Animal studies including pancreatic cancer orthotopic model or transgenic mice with KRAS$^{G12D}$ and p53 knockout can be conducted in the future to see if human RNase 5 can contribute to pancreatic cancer occurrence and progression. Those mouse models will help us to investigate if RNase 5 level is elevated in the initiation or early phase of pancreatic cancer progression. Accordingly, human RNase 5 level in patient serum is significantly elevated compared to normal donors, and it may confer tumor cells more insensitive to C225 treatment but more sensitive to TKI therapy. A proposed combinational therapy of C225 together with anti-human RNase 5 neutralization antibody or TKI treatment using serum RNase 5 level as a predictive biomarker based on our study can be examined in adequate animal models to see if it can be beneficial to clinical practice in the future.

In our work, secretory ribonuclease including bovine RNase A and human RNase 5 can promote cell migration and invasion, while they cannot induce cell proliferation on 2D culture like conventional EGFR ligands including EGF and HB-EGF. These partial overlapped but distinct biological effects between these two families of ligands implicate that RNases may trigger certain signaling pathways and
gene expression patterns different from typical EGFR ligands. An outstanding question is how those ligands can provoke different biological functions and gene expression by binding to and activating the same receptor. More comprehensive analysis of gene expression such as cDNA microarray and RNA sequencing can be performed in the future to characterize and compare gene expression signatures induced by RNases and conventional EGFR ligands. It will provide a molecular basis for us to study how these ligands can trigger different biological effects through the same receptor activation. Since EGFR activation can promote an array of biological function including anchorage-independent growth, angiogenesis and stem-like properties, it would be worthwhile to examine if RNases can trigger these particular effects through activating EGFR signaling. More efforts to answer these types of questions will provide more solid evidence to depict the biological role of RNase A and RNase 5 as ligands of EGFR in physiology and cancer biology.

Human RNase 5 is characterized to promote angiogenesis both in vitro and in vivo. In our hands, it can stimulate pancreatic cancer cell migration and invasion, leading to EMT process through EGFR activation. During the process, the tumor cell viability is not affected by ribonuclease treatment in a significant level. However, the same stimulation may exert different impact to other type of cells. An interesting finding was made in previous studies that human ribonuclease 5 can suppress immune cells including B and T cell proliferation in vitro, and the same feature was also observed with bovine RNase A treatment although the effect is milder (Matousek et al., 1995; Soucek et al., 1999; Sun et al., 2013). It would be interesting
to ask if increased ribonuclease expression can interfere or impair immune function in vivo, especially during the tumor initiation and progression. Based on previous studies from other groups and our finding, ribonuclease activity and human RNase 5 expression could be elevated in pancreatic cancer patient serum, and it may aggravate cancer progression through two arms: one arm is to activate EGFR signaling of tumor cells to increase their motility and aggressiveness through autocrine or paracrine manner, and the other arm is to block appropriate immune response to eliminate tumor cells. This hypothesis is in need of more experiments both in vitro and in vivo to validate in the future.


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Vita

Heng-Huan Lee was born in Pingtung, Taiwan, on June 12, 1976. He received his B.S. degree in Applied Chemistry at National Chiao Tung University, Hsinchu, Taiwan, in 1998. After serving military service two years in Army, Tainan, Taiwan, he entered Academia Sinica, Taipei, Taiwan, as a research assistant to work on structural analysis of protein glycosylation from 2000 to 2002. He then obtained his M.S. degree in Microbiology from National Taiwan University College of Medicine, Taipei, Taiwan, in 2004. In August, 2007, he entered the Cancer Biology Program in the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences (GSBS). He currently lives in Houston.

Permanent Address:

No.142, Daxue 28th St., Nanzi Dist.,
Kaohsiung City 811, Taiwan (R.O.C.)
Curriculum Vitae

Heng-Huan Lee

Birth: Jun. 12, 1976, male

Citizenship: Taiwan, R.O.C.

Permanent Address: No.142, Daxue 28th St., Nanzi Dist., Kaohsiung City 811, Taiwan (R.O.C.)

E-mail: heng_huan@yahoo.com.tw

Major Fields: Molecular Biology (EGFR signaling and cancer biology)

Virology (reactivation of Epstein-Barr virus)

Glycobiology (structural analysis of protein glycosylation)

Degree-granting Education

Dates: 2002-2004

Degrees and Field: M.S. Microbiology

Institution: National Taiwan University, Taipei, Taiwan


Degrees and Field: B.S. Applied Chemistry

Institution: National Chiao Tung University, Hsinchu, Taiwan

Employment and Training Experience

Dates: 2007-present

Progression experience: Training in Dr. Mien-Chie Hung's Lab
Topics: EGFR signaling and cancer biology

Institution: Department of Molecular and Cellular Oncology, University of Texas at Houston and MD-Anderson Cancer Center

Dates: 2002-2007

Progression experience: Trained in Dr. Ching-Hwa Tsai’s lab

Topics: EBV molecular and cellular biology

Institution: Graduate Institute of Microbiology, National Taiwan University, College of Medicine, Taipei, Taiwan

Dates: 2000-2002

Progression experience: Trained in Dr. Kay-Hooi Khoo’s lab

Topics: Structural analysis of protein glycosylation and glycobiology

Institution: Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan


Progression experience: Military Service

Title: second lieutenant

Institution: Army, Tainan, Taiwan

Dates: 1997-1998

Progression experience: Trained in Dr. Fu-Ming Pan’s lab

Topics: Surface science and semiconductor material analysis technology
Institution: National Nano Device Laboratories, Hsinchu, Taiwan

Dates: 1996-1997

Progression experience: Trained in Chain-Shu Hsu’s lab

Topics: Liquid crystal material science and organic chemistry

Institution: Department of Applied Chemistry, National Chiao Tung University,
Hsinchu, Taiwan

Research Focus

1. Molecular Biology (Kinase signaling and cancer)
2. Molecular biology of EBV reactivation
3. Structural analysis of protein glycosylation and glycobiology

Techniques

GC-MS, HPLC, Cell culture, Transfection, DNA cloning, PCR, Northern blot,
Immunofluorescence assay, Western blot, Luciferase reporter assay, EMSA, DAPA,
Plasmid preparation, Chemical derivatization of glycans, Chemical or enzymatic
analysis of N- or O-linked glycans, Mass-based structural analysis of complex
glycans

Future Research Interests

1. Cancer biology
2. Cell signaling and protein modification
3. Chromatin remodeling and DNA-protein interactions in gene regulation
4. Cell differentiation and development

**Honors and Awards**

2002 Outstanding Publication Award of 16th Federation of Asian and Oceanian Biochemist and Molecular Biologist Symposium

2004 Outstanding Publication Award for Graduate Students of College of Medicine, National Taiwan University

**Publications**


Conference papers


Service

Member, Organization Committee of Cancer Biology Program Retreat, Graduate School of Biomedical Sciences, UT-Health Science Center at Houston and UT MD Anderson Cancer Center (2010).