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Novel Angiogenic Peptides in Breast Cancer

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Abstract

Angiogenesis of vascular hematogenous and lymphatic vesicles is the process of formation of new sprouting vessels from preexisting vessels in these systems in order to adequately supply nutrients, oxygen and other growth promoting peptides to tumors that have reached a limited size of 1 to 2 cm. Growth factors that are secreted by breast cancer cells induce the proliferation, migration and sprouting of endothelial cells to the tumor. In this review we will focus on some of the more, novel and more potent angiogenic peptides or growth factors in cancer that can directly regulate angiogenesis or vascular mimicry by tumor cells or indirectly by enhancing the expression of more established angiogenic growth factors.

Keywords: Amidation; Angiogenesis; Breast Cancer; Peptides; Signaling

1. Abbreviations

AKT1	:	Protein Kinase B1
ER	:	Estrogen Receptor
ERK	:	Extracellular Signal-Regulated Kinase
MAPK	:	Mitogen Activated Protein Kinase
NICD	:	Notch Intracellular Domain
PI3-K	:	Phosphoinositide 3-Kinase
PR	:	Progesterone Receptor
Ras	:	Rat Sarcoma
Src	:	Sarcoma-Related Cytoplasmic Tyrosine Kinase
VEGF	:	Vascular Endothelial Growth Factor
VEGFR	:	Vascular Endothelial Cell Growth Factor Receptor.

Introduction

Breast cancer is the most common cancer leading to mortality in women worldwide. Nearly, 270,000 new cases in 2017 in the United States have been diagnosed with this set of diseases and the lifelong risk factor for developing breast cancer is 1 in 8 women over their lifespan [1]. Overall survival rates from 35% in the 1960's to 89% in 2016 have improved for a subset of breast cancer patients. Nearly 500,000 women worldwide die from breast cancer each year. Risk factors for developing breast cancer include age over 40, early menarche, late stage menopause, hormone replacement therapy, a family history of invasive Ductal Carcinoma In Situ (DCIS) arising from premalignant Atypical Ductal Hyperplasia (ADH), breast density, obesity, diabetes and a family history of breast cancer development which accounts for 20-25 % of all breast cancer cases of which 10-15 % are due to genetic predispositions in autosomal dominant mutations in tumor suppressor genes such as p53, Rb-1, BRCA1 and BRCA2 [1-4]. Breast cancer arises from a small subpopulation of tumor initiating cells or Cancer Stem Cells (CSCs) that can arise from multipotent basal Mammary Stem Cells (MaSCs) in the basal layer of the mammary gland or progenitor cells such as luminal progenitor cells in the luminal compartment of the mammary gland [4-6]. There are also bipotent progenitor cells that exhibit properties of both basal and luminal cells which are the two main types of differentiated cells in the mammary gland. The luminal progenitor cells can give rise to either ductal epithelial cells or secretory alveolar epithelial cells during pregnancy from unipotent luminal progenitor cells. CSCs exhibit properties of quiescence, self-renewal, recapitulation of the original tumor phenotype and genotype of the more differentiated breast cancer cells, tumor initiation at low numbers following orthotopic implantation of these cells, and chemo- and radio- resistance [5]. Normal stem cells, progenitor cells and in certain cases trans-differentiation of differentiated breast epithelial cells either of the basal or luminal lineages can be converted to CSCs by oncogenic insult such as activation of components in multiple intracellular signaling pathways that maintain cell proliferation, growth factor receptor tyrosine kinase alterations or intracellular signaling pathway mediators such as Ras mutations

that can result in ligand independent activation or excessive growth factor stimulation either in an autocrine or paracrine fashion [5-9]. In addition, loss of tumor suppressor gene function, radiation, environmental carcinogens and changes in the CSC niche or microenvironment which contains different cell types such as activated stromal/fibroblast cells, mesenchymal stem cells, adipocytes, endothelial cells and immune cells such as CD4 and CD8 T cells, type II macrophages, pro-myeloid cells, neutrophils and eosinophils all of which can secrete multiple lymphokines and cytokines that collectively can maintain the existing CSC subpopulation and/or can increase the number of CSCs as occurs during chronic inflammation [9-11].

Breast cancer cells are normally hormone dependent for their growth relying upon either systemic or breast tumor-derived estrogens, progesterone and prolactin that function through receptors such as the Estrogen Receptor (ER), Progesterone Receptor (PR) and Prolactin Receptor (PrlR), respectively [7,12-14].These hormones can in turn tightly regulate the expression of various endogenous growth factors such as Epidermal Growth Factor (EGF), Transforming Growth Factor Alpha (TGFα), amphiregulin, Wnt4, RANKL and insulin-like growth-1 in the normal breast and in breast cancer cells or in the surrounding stroma (paracrine) [7,11,12,15]. Eventually, most hormone-dependent breast tumors have a tendency to lose their requirements for these local or systemic steroid or polypeptide hormones and become hormone-independent which increases their aggressive behavior as they become resistant to anti-estrogens (i.e. Tamoxifen), antiprogestins (i.e. APR19) or aromatase inhibitors [16]. Under these circumstances multiple growth factors can be produced in an uncontrolled fashion at excessive levels as autocrine peptides by the breast tumor cells (Table 1) which have either proliferative and/or angiogenic activity (refer to next section).

Established Peptides
Epidermal Growth Factor (EGF)
Transforming Growth Factor α (TGFα)
Amphiregulin (AR)
Heparin Binding-Epidermal Growth Factor (HB-EGF)
Betacellulin (BTC)
Insulin-like Growth Factor-1 (IGF-1)
Platelet-derived Growth Factor AA/ BB (PDGF)
Transforming Growth Factor ß 1/2 (TGFß)
Fibroblast Growth Factor 1/2/3(int2)/4 (hst)/5/6/7 (Fgf)
Vascular Endothelial Growth Factor A/B (VEGF)
Angiopoetin 1/2 (Ang)
Placental-Derived Growth Factor (PLGF)
Midkine (MK)
Pleiotrophin (PTN)
Novel Peptides
Cripto-1
Nodal
Apelin/Salcut-NH2
Adrenomedullin
Gastrin Releasing Peptide

Table 1: Established and Novel Angiogenic Peptides in Breast Cancer.

Furthermore, paracrine-derived growth factors from the adjacent stroma such as TGFβ1 and Hepatocyte Growth Factor (HGF) can also drive breast cancer growth and progression by initiating Epithelial-Mesenchymal Transition (EMT) which is an embryonic process that is reactivated in multiple types of cancer whereby epithelial cells lose their phenotypic properties such as a loss in cell adhesion by down regulation of adherens junctional proteins such as E-cadherin and tight junctional proteins such as claudins and occludins [17,18]. These cells become more mesenchymal in their phenotype as they upregulate vimentin, smooth muscle actin, fibronectin and N-cadherin. Mesenchymal cells are more invasive and migratory and can move through the breast stroma and can give rise to distant end organ metastases in the lung, liver, brain and bone after extravasation into the hematogenous or lymphatic vessels near the outer rim of breast tumors. In addition, EMT can also initiate the formation of new CSCs which can contribute to the pool of Circulating Tumor Cells (CTCs) that results in end organ colonization in the lung, brain, liver and bone marrow following intravasation from the lymphatic or hematogenous systems which as the first step in gaining access to metastatic tissue sites where these cells undergo a Mesenchymal-Epithelial Transition (MET) to engage in colonization at these end organ metastatic sites [19,20]. In addition, novel embryonic signaling pathways such as the TGFβ/Smad, HGF, canonical Wnt/β-catenin/Lef, Notch and Hedgehog signaling pathways can be activated in breast CSCs or in the surrounding stroma and can also contribute to CSC maintenance and EMT formation or MET formation by Bone Morphogenic Proteins (BMPs), Periostin and Tenascin C in the lungs and bone [10,21-23].

Breast cancer is not a uniform disease but is heterogeneous both within any given tumor and between different types of tumors that consist of multiple molecular subtypes [4,6,24,25]. There are several different molecular subtypes of breast cancers and interconversion between these molecular subtypes can occur due to the cellular plasticity of CSCs or progenitor cells that is dictated by local niche cellular and soluble factors [6,10]. These molecular subtypes include luminal A, luminal B, normal breast-like, HER2 (erbB2)-enriched, basal-like, mesenchymal-like and claudin-low based upon gene expression analysis and clustering. Actually, the discrimination level of subtypes has increased to ten different classifications with an additional 6 in the TNBC group which

accounts for approximately 20% of all the breast cancer subtypes. The basal-like, claudin-low and mesenchymal-like (metaplastic) groups plus the three additional subgroups are classified as Triple Negative Breast Cancers (TNBC). TNBC cancers are Er-, PR- and HER2- but overexpress the EGF receptor (EGFR). TNBC are extremely resistant to chemo- and radio- therapy and there is no effective agent to clinically enhance the regression of TNBC which in patients with this subtype tend to exhibit an extremely low response rate to the effects of chemo- or radiotherapy as far as long-term patient survival and metastasis [26-28]. In this respect TNBC subtypes particularly the basal-like and claudin-low subtypes are enriched for CSCs and an EMT-like phenotype [25-28]. Each of these molecular subtypes of breast cancer can arise from a hierarchical order of multipotent MaSCs (claudin-low subtype) and bipotent progenitor cells (basal/luminal-like). In this respect, mesenchymal-like TNBC and claudin low TNBC resemble the more undifferentiated and primitive Adult Mammary Stem Cells (aMaSCs) from the adult postnatal mammary gland while more epithelial-like TNBC stem cells resemble bi-potent Fetal Mammary Stem Cells (fMaSCs) found in the embryonic day 18 mammary placodes that first start to exhibit branching morphogenesis from the developing nipple area, respectively [6]. Multipotent luminal progenitor cells can give rise to basal-like TNBC due to mutations in BRCA1, p53 or hyper-activation of Notch1 or Notch4 signaling [21]. HER2-enriched breast cancers probably arise from a more lineage restricted unipotent luminal progenitor cells while the more differentiated ER-/PR- or ER+/PR+ luminal B and A subtypes arise from more differentiated and mature ductal or alveolar luminal cells, respectively.

Angiogenesis

Tumor cells require external sources of oxygen, nutrients such as glucose and metabolites such as proline and glutamine to survive when they exceed 1 to 2 cm in size. Blood (hematogenous) and lymphatic vessels need to be located within 100 to 200 µm of the tumor perimeter to initiate and facilitate angiogenesis and to facilitate reoxygenation and nutrient delivery to tumors [29]. In addition, these newly formed vascular vessels need to penetrate the tumor perimeter and to enter into the tumors through sprouting and vessel enlargement. Recruitment of new vessels to the growing tumor is initiated by a process known as angiogenesis [29-31]. Without new vessel innervation, tumors can die and fail to metastasize. Angiogenesis is the process of forming new vessels by sprouting from preexisting vascular hematogenous or lymphatic vessels. These newly formed vessels as they sprout or branch also migrate toward the tumor through the surrounding stroma due to the local release by breast cancers of multiple angiogenic peptides (Table1) such as VEGFs, FGFs and Angiopoietins (Ang) which are the most potent of angiogenic factors especially in breast cancer (Table 1). These peptides increase Micro-Vessel Density (MVD) and endothelial cells exhibit a higher proliferative rate. Expression in breast tumors of angiogenic growth factors such as VEGFA, TGFα, TBFβ2 and FGF2 are also associated with poorer patient prognosis [32-38]. Finally, a small molecule tyrosine kinase inhibitor AZD457, similar to Avastin which is a humanized monoclonal antibody that blocks VEGFA activity in breast cancers, can inhibit FGFR1-3 activity and block CSCs formation in the HER-2 expressing subtype of breast tumors. This may be particularly important since FGFR-3 expression is increased in tamoxifen resistant breast tumors and by Nodal [39,40].

This review will focus on other newly identified, novel angiogenic peptides such as Cripto-1, Nodal, Apelin, Salcut-NH2, Adrenomedullin and Gastrin Releasing Peptide in cancer and particularly breast cancer. Different stress factors that can induce angiogenesis include oxygen deprivation (hypoxia) which is common in all tumors especially in the inner tumor regions, low pH, glucose deprivation, inflammation and metabolic stress [29,41-46]. New vessels around tumors can also form by de novo vaculogenesis from bone marrow-derived endothelial cell precursors [29]. Finally, tumor cells including breast cancer cells can transdifferentiate into tubular-like luminal vessels by a process known as vascular mimicry which have similar phenotypic propertiesofhematogenousvesselstocircumventmoreconventional angiogenic processes [45,46]. A sparse population of periocytes also surround the endothelial cells in hematogenous and lymphatic vessels and function as protective agents from environmental stressors and as control cells to maintain vascular tone [29]. Unlike normal vascular vessels, the tumor vasculature can become leaky and highly disorganized due to fenestration and excessive branching within the lumen of the vessels and pore formation due to changes in cellular adhesion between the endothelial cells of the vessel wall, vascular enlargement and excessive branching [29]. In addition, loss of adhesion between endothelial cells can also can contribute to high vascular permeability due to a discontinuous loss of the basement membrane as a functional barrier. This may be due to excessive VEGF and/or Transforming Growth Factor β1 (TGFβ1) expression either in the tumor cells or surrounding tumor stroma can down regulate expression of adhesion molecules such as VE-cadherin in the endothelial cells and enhance pore formation, Inflammation induced by the transcription factor NFκB through an activated Jak2/Stat3 pathway that can be engaged by pro-inflammatory cytokines such as TNFα, IL-6 or IFNγ can also contribute to angiogenesis [41-44]. Likewise, hypoxia (low oxygen) which can induce expression of the hypoxia transcription factor family which includes Hypoxia-Inducible Factors (HIFs) also contributes to angiogenesis [41-44]. In breast cancer, HIF1α is the most prominent in breast cancer [29-38]. HIF1αor NFκB which is induced by chronic inflammation can induce a number of common downstream target genes such as the more established and more potent angiogenic peptides including VEGFs, PDGFs and Ang2 as well as a majority of the noveler angiogenic peptides that were previously mentioned and that will be described in more detail later in this review as well as Nitric Oxide Synthase (NOS) which is an enzyme that produces Nitric Oxide (NO) which is a potent vasodilator [42-48].

Precancerous breast ADH lesions and non-invasive DCIS already exhibit the ability to acquire angiogenic properties at a rate

of 30% compared to normal breast tissue before becoming invasive Ductal Carcinoma in Situ (DCIS) tumor cells which suggests that angiogenesis can occur early in the precancerous state [47-49]. In addition, breast tissue adjacent to tumors is twice as likely to induce angiogenesis as compared to breast tissues from non-neoplastic breasts. Disruption of the tumor vasculature can lead to oxygen deprivation (hypoxia) [50]. Hypoxia can induce the stabilization HIF-1α. HIF-1α can also be induced by chronic inflammation and by the hormones such as estrogen and progesterone [38,41]. HIF-1α plays a significant role in breast tumor progression and metastasis as it can positively regulate in tumors a number of different target genes including the upregulation of VEGFs, Ang2, FGFs, PDGFs, EGF. metalloproteases, glucose transporter genes, wild type p53, the EMT regulated transcription factor Twist, Akt-1, Oct4, Cripto-1, Nodal, adrenomedullin and apelin [27,36,37,51,52]. Of all the molecular subtypes of breast cancer HIFα expression, VEGFA expression and MVD of hematogenous vessels were significantly higher in basal and other TNBC subtypes as compared to the other molecular subtypes [26,28,50-53]. HIf-1α can also induce cripto-1 in developing mouse cardiomyocytes, in ischemic pig and in human cardiomyocytes following hypoxia-induced infarction suggesting that the HIF-1α regulatory pathway may be engaged in other developmental and pathological conditions [54].

Cripto-1 and Nodal as Novel Angiogenic factors

Cripto-1 (CR-1) also known as Teratocarcinoma-Derived Growth Factor-1 (TDGF-1) is a glycosylated protein ranging in size from 20kDa to 45kDa depending upon the degree of N- or O-linked glycosylation, fucosylation within the EGF-like domain and potential phosphorylation of various serine and threonine residues within the CR-1 protein (Figure 1) [55,56].

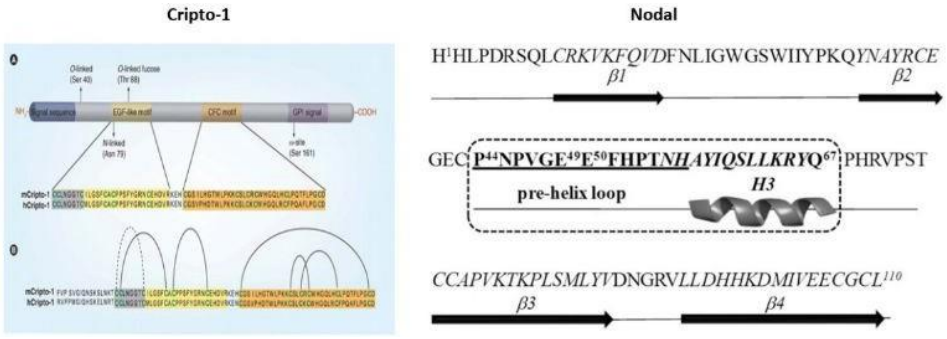

Figure 1: Structure of CR-1 and Nodal Foca A, et al.

In the human CR-1 protein, there are 188 amino acids and the protein has multiple domains and is the founding member of the EGF-CFC family of proteins. CR-1 contains an NH2-terminal signal sequence that is cleaved, an NH2 domain followed by a highly conserved EGF-like domain and a conserved cysteine rich CFC domain which is linked to a hydrophobic COOH-terminus with a motif for a Glycophosphatidylinositol (GPI) region which tethers CR-1 to the cell membrane in cholesterol-rich lipid raft regions within the cell membrane [57,58]. There are 6 cysteine disulfide bridges within CR-1 of which three are in the EGF-like domain and three are in the CFC domain. Unlike EGF which has three loops of cysteine bridges A, B and C, the EGF-like domain in CR-1 lacks the A loop and has a truncated B loop but a complete C loop as compared to EGF The EGF and CFC domains are the functional domains that bind to different proteins such as Nodal, Activins, TGFß1, Alk4, GRP78, Notch, furin-like proteases, canonical Wnts and Frizzeled co-receptors, Lrp5 and Lrp6. Therefore, CR-1 is a multifunctional chaperone protein that can bind to various proteins in a context-dependent manner. CR-1 is the founding member of the EGF-CFC protein family. It is evolutionary conserved along with a related subfamily consisting of cryptic in deutrostomes including sea urchins and amphioxus (lancelet) especially within the EGF-like and CFC domains (Figure 2).

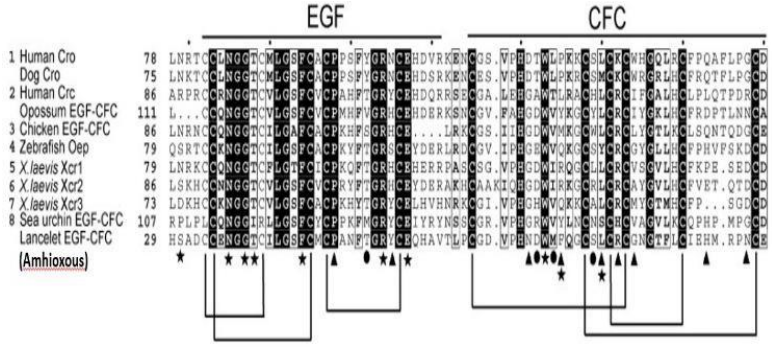

Figure 2: Phylogenetic Conservation of EGF-like and CFC domains in CR-1 [59].

The EGF-CFC family also includes mouse and human Cryptic and Cripto-3 (CR-3) which differs from CR-1 by only five amino acids [59]. Physiologically, CR-1 can be cleaved from the cell membrane by either Phospholipase C (PLC) or by GPI-phospholipase-D which releases a soluble form of CR-1 that can act in a cell-non-autonomous, trans-acting, paracrine manner as a growth factor while the cell tethered formed can function in a cell autonomous, cis-acting, autocrine manner where it functions as a co-receptor for Nodal (see below), the canonical Wnt/β-catenin Frizzeled co-receptors Lrp5 and Lrp6 that bind to various canonical Wnt proteins to amplify this signaling pathway and to proNodal and proNotch receptors to facilitate the processing of these proteins in early endosomes through a series of furin-like proteases [60-62]. Interestingly, mouse CR-1 and human CR-1 are major target genes that are upregulated by the canonical Wnt/β-catenin/Lef pathway during early embryogenesis, in the fetal and adult mouse mammary gland, in normal colon crypt development and in human hepatoma and colon carcinoma cells [55-57,63-66]. Finally, CR-1 has been detected within secreted exosome-like vesicles that are derived from multi-vesicular bodies formed within the lipid raft regions [66]. In fact, secreted CR-1 has been detected in plasma and serum as well as in human milk and may be a potential diagnostic and prognostic serum marker in breast, colon and lung cancers as well as in glioblastomas and germ cell tumors [67-73].

Cell-tethered CR-1 functions as a co-receptor for the TGFβ related proteins such as Nodal and the growth and differentiation factors 1 and 3 (GDF-1/3). CR-1 binds to this subfamily of TGFβ related proteins through the EGF-like domain and then complexes with the type-I receptors Alk4 or Alk7 type-I serine-threonine kinase receptors through the CFC domain which then lead to a trans-phosphorylation of these type-I receptors by the type-II ActRIIB receptor and subsequent activation by phosphorylation of Samd2 and complex formation with Smad3 and Smad4 in early endosomes [55].This then leads to nuclear translocation of this trimeric complex that can then bind to Smad Binding Elements (SBEs) within the promoter region of various target genes in conjunction with other transcriptional co-activators such as FoxH1 to regulate their transcription such as stem cell related pluripotency transcriptional genes such Oct4 and Nanog and Oct4 through a feed-forward loop that can also upregulate including CR-1 and Nodal expression [74-76]. CR-1 can also activate non-canonical, Smad-independent signaling pathways such as the src/ras/MAPK and PI3-K /AKT1 pathways to regulate cellular migration, invasion, EMT and cellular survival (Figure 4). In addition, Glucose Regulated Protein 78 (GRP78) is expressed on the surface of a number of different types of cancer cells and not in the Endoplasmic Reticulum (ER) where it would normally be in tissues and involved in the ER stress response of removing unfolded and dysfunctional proteins through degradation [77]. GRP78 can function as a chaperone protein and binds to CR-1 through the CFC domain and the NH2-terminal domain of GRP78 to amplify Smad-dependent and Smad-independent signaling pathways that are engaged by CR-1. Finally, CR-1 can bind to TGFβ1 and Activins to inhibit their binding to different receptors suggesting that CR-1 is a multifunctional chaperone protein in different signaling pathways in a context-dependent fashion.

CR-1 was first identified as a unique gene and isolated from undifferentiated human and mouse embryonal carcinoma cells lines. Subsequently, it was identified in mouse and human embryonic stems cells with Nodal as genes that are essential in maintaining pluripotency and self- renewal by regulating Oct4 and Nanog expression through a Smad-dependent pathway [55,56,64,74]. During early embryonic development, CR-1 and Nodal are important in inducing through a Smad-dependent signaling pathway that regulates anterior-posterior axis formation, formation of the primitive streak during gastrulation by initiating EMT, mesoendoderm formation and later in in left-right organ laterality formation (Cryptic) with Nodal and in early heart development by stimulating cardiomyocyte differentiation. In this regard, cripto-1 can be induced by hypoxia through HIF-1α in developing cardiomyocytes and is elevated in ischemic pig and human hearts following infarction. Germ line deletion of CR-1 is embryonic lethal at day 6.5 in mice [55,56]. More importantly, CR-1 expression is significantly upregulated in numerous types of human cancer including breast, prostate, colon, gastric, liver, pancreatic, ovarian, cervical, endometrial, non-small cell lung adeno and squamous cell, esophageal, head and neck, nasal pharyngeal, bladder and basal cell skin carcinomas as well as in glioblastomas, melanomas and in germ line embryonal carcinoma and non-seminoma testicular carcinomas [56]. In fact, CR-1 expression can be detected in early pre-malignant lesions in gastric metaplasia, colon adenomas and breast hyperplastic alveolar nodules. In breast cancer, overexpression of cripto-1 is highest the both mouse and human TNBC subtypes and can etiologically contribute to TNBC development [53,78]. In breast cancer, CR-1 expression was found to correlate with tumor grade and, an overall decrease in patient survival. This same correlation of an association with high CR-1 tumor expression, poor overall survival and disease-free relapse has been observed in other types of carcinomas such as nasal pharyngeal, head and neck, lung and colon, gastric and cervical tumors. Finally, overexpression of a human CR-1 transgene in the mouse mammary gland at differential post-natal developmental stages can contribute to the formation of premalignant hyperplastic alveolar nodules and to mammary gland tumorigenesis [79,80].

In Non-Small Cell Lung Cancer (NSCLC), there was a significant inverse correlation between expression of the miR 205 family, which promotes an epithelial phenotype by blocking EMT-related transcription factors and CR-1 expression that were expressed in 94% of the NSCLC samples [81]. Those patients with high levels of CR-1 expression had a worse overall prognosis. In NSCLC there was also a significant correlation of CR-1 expression to neovascularization. In fact, resistance of NSCLC patients to EGF Receptor (EGFR) tyrosine kinase inhibitors can lead to an increase in MVD and to the acquisition of an EMT-like phenotype of more mesenchymal-like cells that are more angiogenic where CR-1 was overexpressed [82]. Blockade of CR-1 expression using a c-src tyrosine kinase inhibitor reestablished sensitivity of the NSCLC

cells to EGFR inhibitors and a down regulation in CR-1 expression. In MCF-7 luminal breast cancer cells that ectopically overexpress CR-1 are more angiogenic as evidenced by an increase in CD31 positive endothelial cells and have a higher MVD orthotopically in the cleared mammary fat pad in nude mice than non-CR-1 transduced cells [83]. Soluble CR-1 was able to stimulate both Smad2 phosphorylation and the phosphorylation of c-src, MAPK and AKT1 in Human Umbilical Vein Endothelial Cells (HUVEC) [83]. In HUVEC, VEGFA and FGF2 were able to stimulate *in vitro* proliferation as was CR-1 in a dose-dependent manner. To form new blood vessels in a tumor, endothelial cells must first invade the tumor stroma and extracellular matrix and migrate and invade the basement membrane into the perivascular space. CR-1 and VEGFA were able to significantly stimulate HUVEC migration and invasion *in vitro*. A VEGF receptor 1 tyrosine kinase receptor was able to block the migratory and invasive effects of VEGFA on HUVEC but not these responses that were also stimulated by CR-1. However, a CR-1 neutralizing mouse monoclonal antibody or a c-src inhibitor or PI3-K inhibitor but not MAPK or ALK4 inhibitors were able to significantly inhibit CR-1 induced migration and invasion *in vitro* [83]. HUVEC cells when placed *in vitro* on a Matrigel matrix will form honey-comb-like, tubes in response to different angiogenic factors. VEGF or CR-1 using HUVEC were able to induce tube-like cords in this assay. As with the migration and invasion assays, a CR-1 neutralizing mouse monoclonal antibody or a c-src inhibitor or PI3-K inhibitor but not MAPK or ALK4 inhibitors were able to significantly inhibit CR-1 induced tube formation. *In vivo*, soluble CR-1 treated wild type MCF-7 cells or MCF-7 CR-1 transduced cells induced angiogenesis which could be blocked with the CR-1 neutralizing antibody in both scenarios [83]. In prostate cancer a similar stimulatory effect of CR-1 on migration, invasion, angiogenesis and metastasis was observed [66,84]. Human PC3 or DU145 prostate cancer cells express CR-1 and were then transduced with a CR-1 siRNA. Knockdown of CR-1 mRNA or CR-1 protein expression ranged from 50 to 80 % compared to control siRNA transfected cells [66,84,85]. Knockdown of endogenous CR-1 blocked proliferation of both prostate cancer cell lines by ~50% and were arrested in the G1 phase of the cell cycle which was accompanied by a 50-70 % reduction in Cyclin D1 and Cyclin E1 [84,85]. In addition, *in vitro* migration and invasion of both prostate cancer cells lines was inhibited by two-fold after CR-1 knockdown which correlated with a reduced expression of metalloproteases 2 and 9 in these cells. EMT was also severely impaired in the CR-1 knockdown cells as E-Cadherin was overexpressed while N-cadherin, vimentin and fibronectin expression was severely reduced. The CR-1 knockdown prostate cancer cells were more epithelial-like in their phenotype as compared to the siRNA transfected control cells which were more mesenchymal in appearance. Tube formation of HUVEC was used to assess the ability of secreted CR-1 in the Conditioned Medium (CM) to modify this phenotype. Tube formation of HUVEC using CM from CR-1 knockdown cells reduced by 80% tube formation in HUVEC as compared to CM from siRNA control cells. Examination of the phosphorylation of c-src PI3-K, AKT1 and Glycogen Synthetase Kinase-3β (GSK-3β) which the latter is a regulator of AKT1 activation and the Wnt/β-catenin/Lef pathways were all significantly compromised in the CR-1 knockdown prostate cancer cell [84,85]. Finally, soluble CR-1 that is secreted from either CR-1 transduced kidney HEK293T cells or from NTERA2/D1 human Embryonal Carcinoma (EC) cells that have high levels of endogenous CR-1 expression can function in trans/paracrine manner to influence HUVEC cells [57]. In this context, soluble CR-1 can promote endothelial cell migration as a chemoattractant to facilitate migration and secretion of soluble CR-1 by GPI-PLD that can be enhanced by other growth factors such as HGF, EGF, HB-EGF, TNFα and IL-6 [57]. Nevertheless, tethered CR-1 on tumor cells can induce HUVEC cell sprouting through direct cell-cell interaction [57]. Shedding of CR-1 occurs at the GPI-anchorage site as by the ability of phospholipase C or endogenous GPI- phospholipase D to facilitate CR-1 cleavage [57,58]. Multiple hormones, growth factors and transcription factors such as LRH-1 and GCNF can either positively or negatively regulate cripto-1 expression (Table 2). Reciprocally, CR-1 can upregulate the expression of EMT regulated genes and EMT induced transcription factors as well as other growth factors receptors and novel angiogenic peptides and their cognate receptors such as apelin and adrenomedullin (see subsequent sections) as well as the NFkB-induced cytokines TNFα, IL-6 and IFNγ [86]. In colon cancer, CR-1 is found in the normal colonic crypt stem cells and is increased in colon cancer CSCs [87]. CR-1 knockdown inhibited colon cancer growth and metastasis *in vivo* [87].

Nodal is a member of the TGFβ superfamily and is expressed in all deuterostomes including hydra (Figure 3).

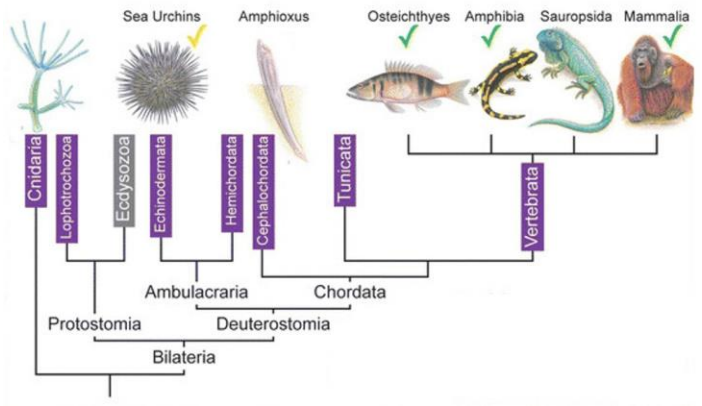

Figure 3: Phylogenetic conservation of Nodal [90].

Nodal is expressed as extended 347 amino acid prepro-protein (55kDa) that is cleaved to pro-Nodal which has 238 amino acids (37kDa) and eventually to mature Nodal which is a 110 amino acids (22kDa) monomeric form (Figure 1) which is heavily glycosylated [88-90]. Mature Nodal can homodimerize with itself or heterodimerize with GDF1 or GF3 [91]. Cleavage of these larger precursors is accomplished by proteins in the pro-protein convertase family such as Furin and Pace4. CR-1 can facilitate this intracellular processing by binding to these convertases and Nodal and can also enhance secretion of mature Nodal in early

endosomes where it becomes glycosylated and helps to stabilize the protein from degradation [92,93]. Nodal like CR-1 can function as a cell-associated autocrine growth factor or as a secreted paracrine factor. Nodal similar to CR-1 is important in regulating the expression through a Smad-dependent pathway expression of the pluripotency genes Nanog and Oct4 [74-76,92,93]. There are four distinct Nodal-related genes in Xenopus which has three related CR-1 genes (figure 3) and two Nodal genes are expressed in zebrafish namely squint, cyclops and southpaw that interact with the zebrafish version of CR-1 One-Eyed Pinhead (OEP) [94,95]. Nodal is not normally expressed in adult tissue which is similar to CR-1 except possibly adult tissue stem cell compartments such as the intestinal crypts, the embryonic day18 mammary mesenchyme and in the luminal progenitor cells in the adult early pregnancy mammary gland like CR-1 and in human trophoblast cells and endometrium. Nodal can upregulate its own expression, CR-1 and Lefty 1/2 through a Smad-dependent pathway. Lefty is an inhibitor that can bind to and block both Nodal and CR-1 activity [94,95]. Cerberus is another inhibitor of Nodal as wells as various Wnts and BMP. The Mir 15/16 family can block protein expression of CR-1 and the type-II Activin receptor by destabilizing their respective mRNAs resulting in their degradation [96-103].

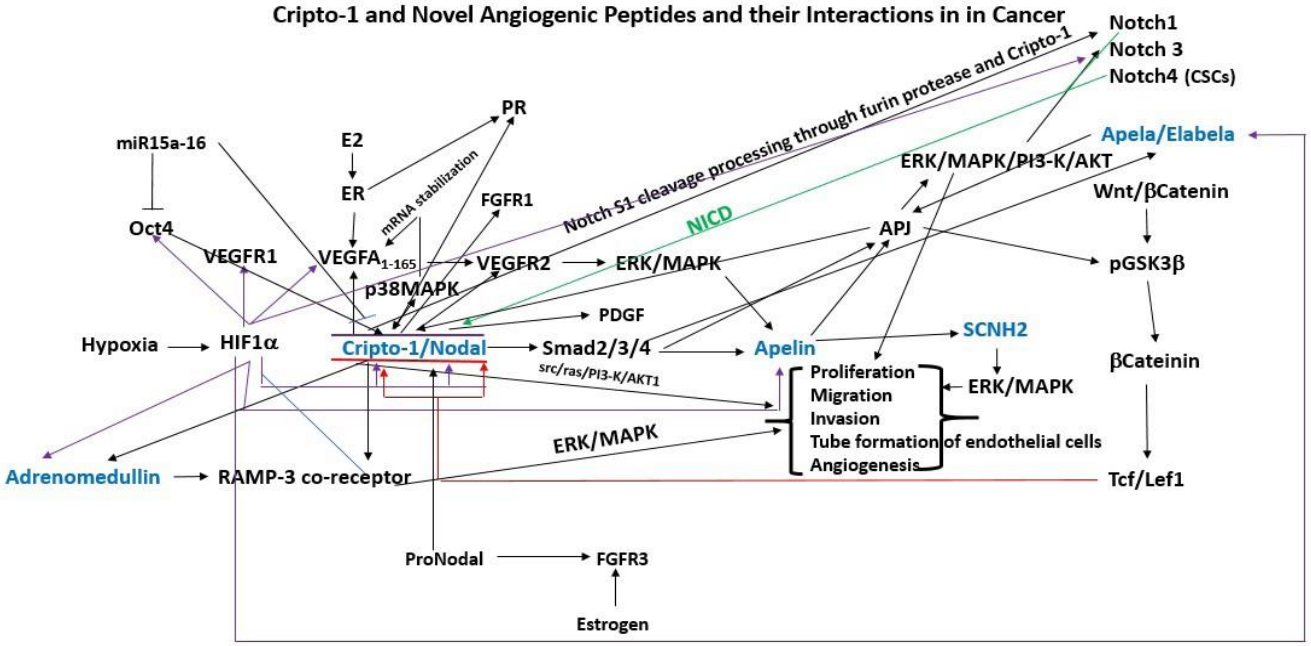

Figure 4: Molecular interactions between CR-I, Nodal, Adrenomedullin and Apelin/ SCNH2.

Similar to CR-1, Nodal can also be induced by hypoxia through HIF-1α in human MCF-7 and T47D breast cancer cells and in metastatic C81-61 human melanoma cells through HIF-1α which not only stabilizes the Nodal mRNA but upregulates both pro-Nodal and Nodal protein expression (Figure 4) [102,103]. HIF-1α can bind to the Nodal promoter through Nodal Dependent Enhancer (NDE) in the promoter region of the gene [99,100]. Hypoxia via HIF-1α can also enhance the expression of Oct4 and Notch receptors to promote a more dedifferentiated and aggressive phenotype in a variety of cancer cells probably by expanding the CSC population. Notch1 during early embryonic development and Notch4 in C81-61 cells can induce Nodal expression through the NDE. A similar effect was observed in T47 cells through the Intracellular Domain of Notch (NCID) and its binding to the NDE region following hypoxia treatment. Finally, hypoxia driven by HIF-1α can induce the invasion and migration of T47D cells and Conditioned Medium (CM) obtained from T47D cells could significantly enhance tube formation in HUVEC which was Nodal dependent as illustrated by shNodal knockdown in the T47 cells [100,101]. A similar observation relating to hypoxia and Nodal was observed in human A375 human melanoma cells [102]. In these cells, hypoxia can induce resistance to the cytotoxic drug dacarbazine. Hypoxia was able to induce Nodal expression through a HIF-1α–dependent pathway. Nodal expression was enriched in the CSC subpopulation of these cells as assessed by sphere formation and by the expression of stem cell related genes such as Oct4, Nanog Sox2, CD133 (prominin) and CD44 (hyaluronan receptor). Hypoxia induced both pro-Nodal and mature Nodal. In turn, Nodal was able to enhance the expression of the glucose-1 transporter gene and glucose uptake (Warburg effect), PDK-1 an upstream positive regulator of AKT1 action and cell survival. Nodal knockdown using a lentivirus transduced shRNA Nodal abrogated these effects. HIF-1α induced expression of Nodal also resulted in resistance to the cytotoxic effects of dacarbazine

which could be restored in shRNA Nodal transduced A375 cells [102]. Impairment of Nodal expression by either shRNA Nodal transduction or by blocking Nodal signaling through Alk4 using the inhibitor SB431542 also reduced sphere formation and invasion *in vitro*. Primary breast carcinomas also express Nodal but not benign breast tissue biopsies. Nodal expression was directly correlated with the diagnosis and disease [103]. Out of 431 breast carcinomas, 66% were moderately or highly expressing Nodal. In addition, more poorly differentiated breast cancer were associated with higher levels of Nodal expression than more differentiated tumors. Likewise, higher tumor stage and lymph node positive patients presented with higher levels of Nodal expression. Breast cancer TNBC cell lines, MDA-MB-231 and MDA-MB-468 cells, Nodal was detected in ~39% and 22% of the cells in these two cell lines, respectively. Both cell-associated proNodal and the secreted mature Nodal protein were found in both cell lines. Treatment of either cell line with a Nodal neutralizing/blocking rabbit polyclonal antibody but not a control polyclonal IgG produced in a dose-dependent manner a 65-90 % decrease in the levels of the proNodal precursor with a concomitant decrease in the level of phosphorylated Smad2 levels. Cell proliferation in both cell lines was decreased in a dose-dependent manner following treatment with the Nodal blocking antibody but not with the control IgG [103]. Reciprocally, there was a dose-dependent increase in apoptosis following treatment with the Nodal blocking antibody but not with the control IgG. Finally, there was a dose-dependent inhibition of anchorage-independent growth in soft agar of both of these cell lines following treatment with the Nodal blocking antibody but not the control IgG. Similar inhibitory effects on cell proliferation, invasion and clonogenicity in soft agar were observed in MDA-MB-231 or MDA-MB-468 following transfection with an shNodal expression vector but not a control scrambled shRNA vector [100,101]. In those studies, tumor growth in nude mice of the Nodal knockdown cell lines was totally eliminated but not with a control shRNA vector. At the molecular level, Nodal knockdown repressed cyclin B1, cyclin D1 and c-myc expression but enhanced the expression of the negative cell cycle regulators p21 and p27 which explains the mechanism by which Nodal can modify cell proliferation.

Upstream CR-1 Regulators	Downstream CR-1 Targets
Wnt/β-catenin	VEGF
TGFβ1	VEGFR2
Activin A/B	Apelin
HIFα	APJ
BMP2/4 (-)	Adrenomedullin
Retinoic Acid (-)	Adrenomedullin RAMP-3 co-receptor
Germ Cell Nuclear Factor (GCNF)(-)	FGF2/4/8
Liver Receptor Hormone 1 (LRH-1)	FGFR1
Snail(-)	Snail
CR-1	ZEB-1
Nodal	E-Cadherin(-)
FGF4	Occulidins(-)
Progesterone	Claudins(-)
Nkx-2.5	N-cadherin
Msx1	Vimentin
	α6 integrin
	Fibronectin
	Netrin-1
	c-myc
	Cyclin D1
	RANKL
	NFkb
	Sox9
	Gata3

Table 2: Upstream CR-1 regulations, Downstream CR-1 targets.

(-) above designates negative regulation or down regulation of expression

MVD is well recognized in breast cancer as a reliable index of angiogenesis. In gliomas, Nodal can upregulate VEGF expression and MVD. Nodal expression in breast cancer is directly correlated to MVD in 83 primary breast carcinomas and can induce vascular recruitment *in vivo* as assessed by MVD and CD31 expression [64,66,104-109]. Similar to melanoma cells, Nodal can be secreted by breast cancer cells *in vitro* wherein the CM from these cells can stimulate HUVEC tube formation *in vitro*. Nodal also in breast cancer cells *in vitro* can upregulate VEGF and PDGF protein expression. In MDA-MB-468 cells transfected with a shNodal vector caused a decreased expression of VEGF and PDGF protein expression and secretion into the CM from these cells [104]. Knockdown of Nodal using an shNodal expression vector can decrease MVD and can induce necrosis within breast tumor cells. In MDA-MB-231 cells transfected an shNodal expression vector, CM from these cells significantly reduced HUVEC branching, migration and tube formation compared to CM obtained from control scrambled shRNA MDA-MB-231 cells which stimulated these responses in HUVEC [100,101,103,104]. Gain of function studies in T47D cells which express lower levels of endogenous Nodal like MCF-7 luminal breast cancer cells as compared to MDA-MD-231, MDA-MB-468 and Hs578t TNBC cells when transfected with a Nodal expression vector confirmed these results using CM from these Nodal transduced T47D cells on target HUVEC in which branching, migration and tube formation were enhanced. When cultured *in vivo* shNodalMDA-MB-231

cells reduced vascular recruitment as compared to shMDA-MB-231 cells. Reciprocally, T47D cells expressing ectopic Nodal enhanced angiogenesis as compared to control T47D cells. These results were also confirmed in a Chick Chorioallantoic Assay (CAM) with MDA-MB-231 Nodal knockdown cells where these cells were unable to form vascularized tumors as compared the shRNA MDA-MB-231cells tumors that developed. In nude mice. shNodalMDA-MB-231 tumor cells had a significant decrease in CD31 staining for innervating endothelial cells as compared to the shMDA-MB-231 tumor cells. In addition, tumor necrosis was elevated in the Nodal knockdown tumors [104].

Vasculogenic Mimicry (VM) that does not involve vascular endothelialcellscancompensateforalossofvascular(hemotogenous or lymphatic) angiogenesis in melanoma, prostate, ovarian, liver, lung carcinomas and gliomas [44,46,105]. Recently Nodal has been demonstrated to induce VM in metastatic melanoma cells. In primary breast cancers, high bot not low Nodal expression was correlated with lymph node metastasis, tumor stage, poor overall patient survival that was accompanied by a loss of differentiation and overexpression of a VM marker VE-cadherin [100,104,106]. VM frequency was found in 32% of the Nodal expressing tumors expressed VE-cadherin in the Nodal high tumors and in only 8% of the Nodal low tumors. Likewise, 73% of the Nodal high tumors while only 37% of the Nodal low tumors exhibited VM [103,104]. shNodal MDA-MB-MB-231 cells exhibited a reduction in VE-cadherin expression while T47D Nodal transduced cells exhibited an upregulation in VE-cadherin. In addition, shNodalMDA-MB-231 cells were impaired in their ability to form VM channel-like structures on or in Matrigel while the shMBA-MB-231 cells were able to form such structures that could be blocked by the Alk4 receptor inhibitor SB431542. Likewise, Nodal overexpression in MCF-7 cells promoted VM channel formation where VE-cadherin was found to be co-expressed with Nodal. Smad2/3 phosphorylation was inhibited in the shNodalMDA-MB-231 cells and reciprocally upregulated in the Nodal transduced MCF-7 cells. Using either the shNodal MDA-MB-231 cells or the T47D Nodal transduced cells, it was found that Nodal could enhance the expression of EMT as assessed by an increase in migration and invasion assays, expression of MMP2 and MMP9 and EMT-related genes such as N-cadherin, vimentin, Snail, Slug and c-myc, while E-cadherin expression was reduced [103,104]. In this respect, EMT has an important role in facilitating VM formation Finally, tumor growth in nude mice was found to be enhanced by Nodal overexpression and suppressed by Nodal inhibition with parallel changes in CD31 expression in both situations. Human glioma/glioblastoma cells express both CR-1 and Nodal [105]. Glioblastomas exhibit pronounced angiogenesis. Nodal expression correlates with invasive behavior and angiogenesis in these tumors and is more pronounced in glioblastomas than in anaplastic astrocytomas or normal brain tissue [105-107]. Likewise, there was a significant correlation between Nodal expression and VEGF expression in glioblastomas [107]. Knockdown of endogenous Nodal with an siRNA Nodal vector *in vitro* in U87MG human glioma cells decreased colony formation in soft agar and decreased VEGF secretion. Orthotopic intracranial injection of siRNA U87MG cells inhibited tumor growth of these cells and prolonged survival in SCID mice *in vivo*. Inhibition of Nodal expression also suppressed tumor vessel growth as assessed by CD31 analysis *in vivo* and inhibition of endogenous Nodal signaling in wild type U87MG cells using the Alk4 inhibitor SB431542 also produced a similar effect *in vivo*. Therefore, knockdown of Nodal suppresses angiogenesis *in vivo* in glioblastoma cells. Reciprocally, overexpression of Nodal in U87MG cells promoted *in vivo* the intracranial growth of these cells as tumors in SCID mice and enhanced angiogenesis as assessed by an increase in the number of CD31 positive endothelial cells within the tumors. Nodal overexpression increased HIF-1α expression which in turn upregulated VEGF secretion while knockdown of Nodal expression or by inhibiting Alk4 signaling suppressed HIF-1α expression and subsequent angiogenesis. Nodal could also enhance phosphorylation of ERK1/2 as this effect could be blocked by SB431542 through a CR-1/Alk4/Smad2/3-dependent signaling pathway. Interestingly, inhibition of ERK1/2 phosphorylation suppressed HIF-1α induction which subsequently impaired VEGF expression and angiogenesis. In U87MG, high levels of endogenous CR-1 are expressed in a small subpopulation of potential CSC [108]. Treatment of U87MG cells with exogenous CR-1 induces its own expression in a dose-dependent manner by activating a Nodal/Alk4/Smad2/3-dependent signaling pathway thereby engaging a feed-forward auto-regulatory loop by transcriptional activation by exogenous CR-1 through Smad Binding Elements (SBEs) within the promoter region of the endogenous CR-1 gene [108]. This same mechanism of autoregulation also been demonstrated in human embryonal carcinoma cells and in human colon cancer cell lines [55,56]. In addition, auto-induction of endogenous CR-1 by exogenous CR-1 was observed in MCF-7 breast cancer cells and in non-transformed human kidney HT-29 cells. Following treatment of the U87MG cells with recombinant CR-1, there was an expansion in the CSC subpopulation in U87MG cells as assessed by the expression of the CSC maker, Multi-Drug Resistant Transporter1 (MDR1) [108]. MDR1 is in the ABC transporter family of membrane pumps that utilizes ATP hydrolysis to efflux various chemotherapeutic drugs from CSCs which explains their drug resistance. CR-1 increased MDR1 expression from a basal level of 13% in the non-treated cells to 37% in the CR-1 treated cells. Therefore, a bimodal distribution and heterogeneous existence of both low CR-1 expressing U87MG cells and high CR-1 U87MG CSCs subpopulations might also exist in a dynamic equilibrium in other different types of tumors such as melanomas, embryonal carcinomas and potentially breast carcinomas and in in different tumor subpopulations [55,56,64,76, 86,97,100,102,108]. The percentage of such CR-1 positive low and high cells between different tumors and/or tumor subtypes may vary by several-fold depending upon the CSC niche properties and cell types within the niche such as macrophages and secreted growth factors and cytokines such as TGFβ, Wnt3, TNFα, IL-6 and BMP 2/4 from these different supportive niche cell types that might either enhance or repress CR-1 expression.

Regulatory Peptides That Function as Tumor Growth Factors Via Direct Trophic Effects or Indirectly Through Angiogenesis

A New Definition of Endocrine Organs

Fifty years ago, there were seven major anatomical tissue types (glands) that had been defined as endocrine in nature by virtue of their ability to produce and release peptide hormones into the blood stream and in turn, having distal effects on other organ function. Adrenal, parathyroid, thyroid, pituitary, pancreas, testis and ovary were considered classic endocrine glands. Today it is well recognized that almost every know cells type can produce and release bioactive peptides that have global effects on the host body. The heart, lung, GI tract, skin, adipose are only a few of the newly recognized tissue types having endocrine activity [109-114]. Anomalies in these newly defined endocrine organs can have detrimental effects on the host due to an abundant release of peptide hormones. For example, individuals with overactive adipose in obesity are known to have an increased risk for type-II diabetes, cardiovascular disease and cancer [115,116]. Adipocytokine secretions from excessive fat deposits in breast cancer patients can augment cancer cell proliferation, enlarge tumor stem cell populations, enhance angiogenesis/lymphoangiogenesis, drive pro-inflammatory environment and stimulate invasion [117-120]. Chronic inflammation is an established hallmark of cancer promotion primarily mediated by cytokines produced from infiltrating immune cells [121-125]. Interestingly, hematopoietic tumor infiltrates can also produce and respond to classic peptide hormones such as Vasoactive Intestinal Peptide (VIP) and Prolactin (PRL), transitioning breast cancer cells into a more aggressive phenotype by trans-activating EGFR/HER2, inducing neoplasm VEGF expression, enhancing microenvironment inflammation and augmenting metastasis [126-133].

Post-Translational Modifications of Regulatory Peptides and Resulting Biological Activity

All regulatory peptides are derived from larger precursor proteins denoted as the prepro-hormone comprised of the secretory signal peptide (specifying secretion) and the prohormone component which is further enzymatically processed into biologically active peptides. Several functional peptides can be produced from a single prohormone, the best example of which is Pro-Opiomelanocortin (POMC) hormones: ACTH, alpha-melanocyte-stimulating hormone (αMSH), gamma-melanocyte-stimulating hormone (γMSH), beta-endorphin (βEND) and beta-lipotropin hormone (βLPH) [134]. There are a multitude of post-translational modifications that can occur to proteins or peptides that include phosphorylation, acetylation, cysteinylation, farnesylation, glyosylaiton, sulfation, oxidation, palmitoylation, pyroglutamation, methylation, and amidation. When evaluating all of the structural alterations listed, it has been shown that only Carboxy-Terminal Amidation (CTA) exclusively tracks with bioactivity [135-137].

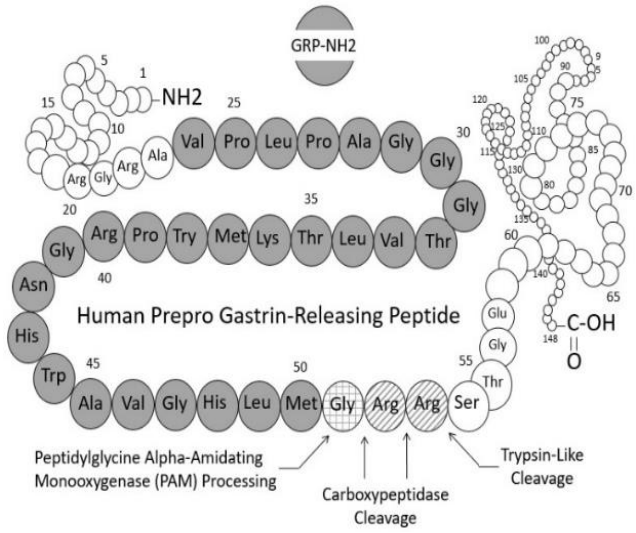

Figure 5: Complete AA sequence of pre-pro-human gastrin-releasing peptide (AA1-77). NCBI Accession number NP_002082.2. Identification of trypsin-like and carboxypeptidase cleavage sites at basic arginines. Targeted glycine-intermediate peptide that serves as a substrate for Peptidyglycine α-Amidating Monooxygenase (PAM) processing that converts the amine of glycine to the amide of it penultimate AA neighbor methionine resulting in a bioactive peptide. GRP (Met-amide, prepro GRP 24-50).

A distinct Amino Acid (AA) motif in the precursor protein codes for CTA to take place via a series of enzymatic processing steps [138-140]. Glycine is the only known AA to donate its amine to become the amide of its penultimate neighbor within the precursor's infrastructure [139]. Generally, CTA starts with a trypsin-like cleavage event occurring within the pro-hormone at a basic AA (arginine or lysine), followed by carboxypeptides activity to give rise to the glycine-intermediate moiety. This in turn is acted upon by an enzyme complex denoted as Peptidylglycine-α-Amidating Monooxygenase (PAM) which is composed of Peptidylglycine α-Hydroxylating Monooxygenase (PHM) that hydroxylates the α-carbon on glycine next to its acid residue and Peptidyl-α-Hydroxyglycine α-Amidating Lyase (PAL) that amidates the penultimate AA to glycine and forms glyoxylate as a by-product [140,141]. The prepro-human Gastrin-Releasing Peptide (GRP) diagram given in Figure 5 reveals the enzymatic processing steps necessary to generate a bioactive peptide amide from its precursor molecule.

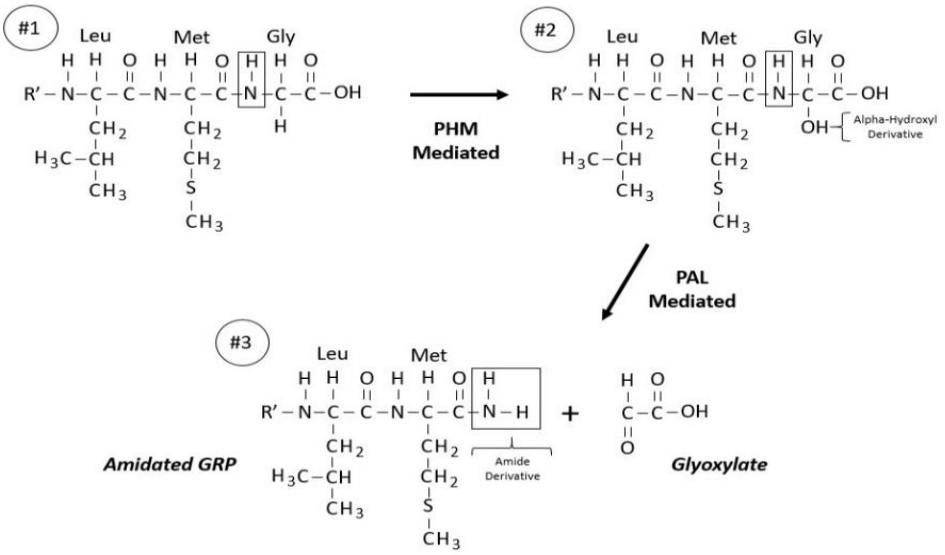

Figure 6: PAM enzymatic cascade that takes place on the glycine-extended intermediate substrate of GRP, ultimately results in the formation of the bioactive methionine amide derivative.

Step 1: Formation of the glycine-extended intermediate after two rounds of carboxypeptidease to remove adjacent basic arginines.

Step 2: Peptidylglycine α-Hydroxylating Monooxygenase" (PHM) hydroxylates the α-carbon on glycine.

Step 3: Peptidyl-α-Hydroxyglycine α-Amidating Lyase" (PAL) amidates glycine's penultimate neighbor AA methionine to for the fully functional bioactive peptide amide, plus generating glyoxylate as byproduct.

*Boxed area designates the site of amine to amide conversion.

(Figure 6) demonstrates the enzymatic cascade that takes place during the generation of the carboxy-terminal methionine-amide that is critical for receptor binding and biological activity of GRP.

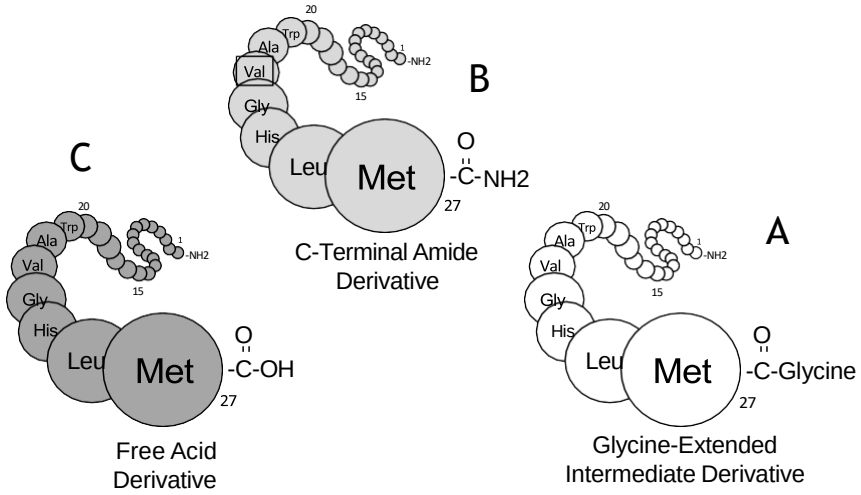

Figure 7: Bioactivity designation and structural comparison of inactive GRP glycine-extended intermediate **(A),** full functional bioactive amidated methionine derivative **(B)** and the methionine free-acid form **(C)** that is 100-1,000 times less potent than its amide counterpart.

(Figure 7) gives a structural comparison of GRP's glycine-extended intermediate (having no activity), its methionine amide (fully active) and the free-acid derivative (that is 100-1,000 times less potent than the amide). Peptide amides for all twenty commonly known AAs have been identified in nature having different signaling amidation motifs that flank the amide donor glycine [138].

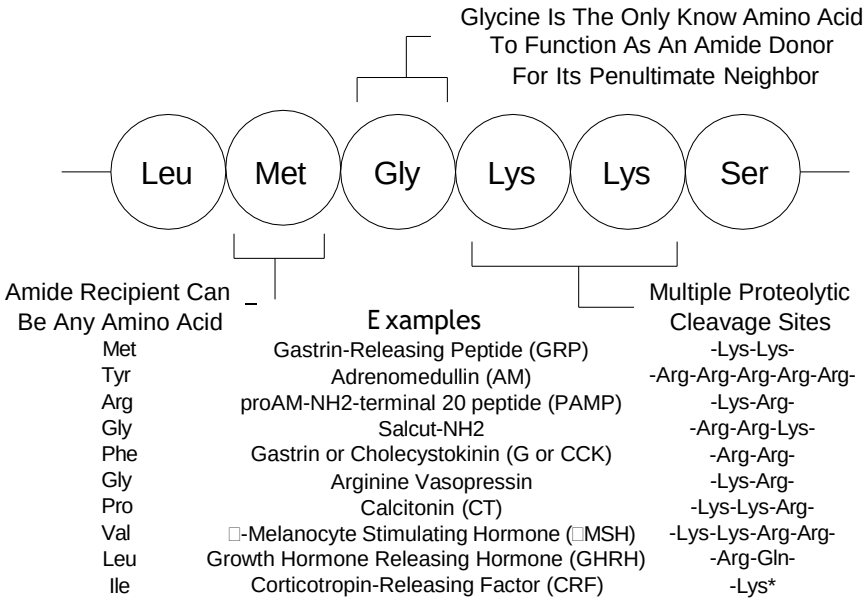

Figure 8: All twenty known AA have a naturally occurring peptide amide product found in nature. This diagram, utilizing the AA backbone of GRP, presents several examples of human peptide hormones with flanking basic AA cleavage sites identified, all using glycine as their amide donor AA, and giving various AA that can be enzymatically converted to their bioactive peptide amide.

(Figure 8) gives both the flanking basic AA motif and recipient AA amide for a few representative examples of bioactive human peptide hormones. PAM deficiency in mice is embryonic lethal, validating the critical role peptide amidation plays during fetal development as well as in wound repair, immunity and carcinogenesis [142-144]. CTA enzymes are highly conserved in evolution and are found in lower order invertebrates such as nematodes, confirming their importance in biological systems [145]. Interestingly, when assessing well established growth factors for the presence of amidation motifs in their respective precursor proteins, it becomes evident that there are many more possible bioactive components yet to be recognized given that pre-pro-epidermal growth factor has eleven possible peptide amides, the precursor of hepatocyte growth factor has six, preproendothelin-1 has five, intact transferrin has nine (is transferrin a prohormone unto itself?), and the precursor of Insulin-Like Factor-I (IGF-I) has two potential amide products [138]. We have actually generated an *in-silico* predicted peptide amide found in the alternatively spliced IGF1-B prohormone coined Tyr-23-Arg-NH2 (Y-23-R-NH2) and found it to be mitogenic for human tumor cells [146]. Y-23-R-NH2 did not mediate its cancer cell grow effects via the IGF1 receptor but through a unique high affinity binding site nor did Y-23-R-OH (free-acid) demonstrate any trophic ability or antagonize peptide amide binding [146]. Given that Nature has the benefit of protracted evolutionary time to develop biological control mechanisms via a Push/Pull or Ying/Yang phenomenology, it is not too surprising to find the existence of several deamidating enzymes that can convert amides to their free-acids counterparts thereby modulating bioactivity and having growth inhibitory effects in both the normal and malignant setting [147-150].

Unique peptide amides that augment tumor growth

For the remainder of this review we will be targeting three unique peptide amides that included Amphibian Bombesin (BN) and its mammalian counterpart GRP, Adrenomedullin (AM), and a split product from the apelin gene denoted as Selective Apelin-36 Cutting & Amidation peptide or Salcut-NH2 (SCNH2). These amidated mediators function as either direct autocrine/paracrine cancer cell growth factors or induce global angiogenic effects throughout the tumor microenvironment. As such, they have been investigated as potential targets for the clinical intervention of solid tumor growth or as imaging tools for the anatomical localization of cancers.

Bombesin/Gastrin-Releasing Peptide (BN/GRP)

BN is tetra-decapeptide-amide (Pyr-Gln-Arg-Leu-Gly-Asn-Gln-Trp-Ala-Val-Gly-His-Leu-Met-NH2) initially isolated from the skin of the European frog *Bombina bombina* and shown to have biological function in mammalian systems [151]. There are two human homolog of BN, having the identical carboxyl-terminal heptapeptide amino acid sequences, initially isolated from pig stomach and spinal cord, and were termed GRP and Neuromedin-B (NMB) respectively [152,153]. BN-like immune-reactivity or mRNA has been found in the human brain, gastrointestinal tract, pancreas, lung, and breast, showing elevated expression in disease states [154-156]. BN or GRP have been shown to stimulate the growth of Swiss 3T3 cells, normal fetal lung, and cancers of the lung (SCLC/Non-SCLC), stomach, colon, head/neck, pancreas, prostate, and breast [157-166]. The cDNA of GRP, NMB and their respective receptor (GRPR/BB2, NMBR/BB1) have been cloned and shown to have a high degree of conservation in mammals [167-172]. Knockout mice for GRPR/BB2 and NMBR/BB1 have been generated and where shown not to be embryonic lethal but have resulting phenotypes involving locomotor activity and thermoregulation [173,174]. A third BN-like receptor denoted as BN receptor subtype-3 (BRS-3/BB3) has been identified, cloned and a knockout mouse generated that develops metabolic defects and obesity [172,175].

Several reports now exist that define a variety of BN/GRP/NMB regulated signal transduction pathways induced either directly through GRPR/BB2 and NMBR/BB1 or through the transactivation of the Epidermal Growth Factor Receptor (EGFR) that encompass cAMP, MAPK/ERK, PI3K and Akt [176-180]. When considering what factors upregulate GRP/GRPR expression, it has been demonstrated that cigarette smoke, oxidative stress or targeted pulmonary irradiation can elevate ligand/receptor levels in human lung epithelial cells, phorbol esters increase expression in small cell lung cancer cells and Type-I interferon and progesterone can enhance GRP levels in the ovine uterus [181-187].

Given the prevalent role that GRP/GRPR plays in neoplastic disease, multiple reagents have been developed to suppress GRP induced cancer cell growth by either masking the ligand with neutralizing monoclonal antibody, small molecule compounds preventing receptor interaction or peptide antagonists that bind receptor and sterically block ligand binding [171,188-191]. Such reagents have been shown to be effective in blocking GRP mediated growth of human breast cancer or lung cancer cells in the *in-vitro* or *in-vivo* settings either directly by suppressing tumor cell division or by blocking tumor induced angiogenesis or lymphoangiogenesis [161,162,165,191-194].

As previously discussed, chronic inflammation is a hallmark of cancer progression and infiltrating immune cells can make or respond to classic peptide hormones [121-133]. BN or GRP have been shown to regulate immune function by increasing lymphocyte or mast cell proliferation, augmenting macrophage, neutrophil, mast cell chemotaxis, enhancing cytokine release from activated macrophages, increasing vascular adhesion of monocytes, and performing a critical role in TLR4 mediated disease [195-203].

Recent preclinical studies have generated promising results for the use of labeled BN or GRP analogs to image human tumors with high GRPR expression that include cancers of the breast, lung, and prostate [204-209].

Adrenomedullin/Proadrenomdullin N-Terminal 20 Peptide (AM/PAMP)

AM is a 52 AA tyrosine amide peptide having a single disulfide bond in the mid portion of the molecule, initially isolated from a human pheochromocytoma, shown to stimulate cAMP activity, and elicit a dose-dependent hypotensive response in rats [210]. Human AM cDNA has been cloned and the prohormone was predicted to contain an additional bioactive peptide amide denoted as "proAM N-terminal 20 peptide" (PAMP) [211]. The predicted PAMP amidation motif and peptide is highly conserved in rat and porcine prepro hormones [212,213].

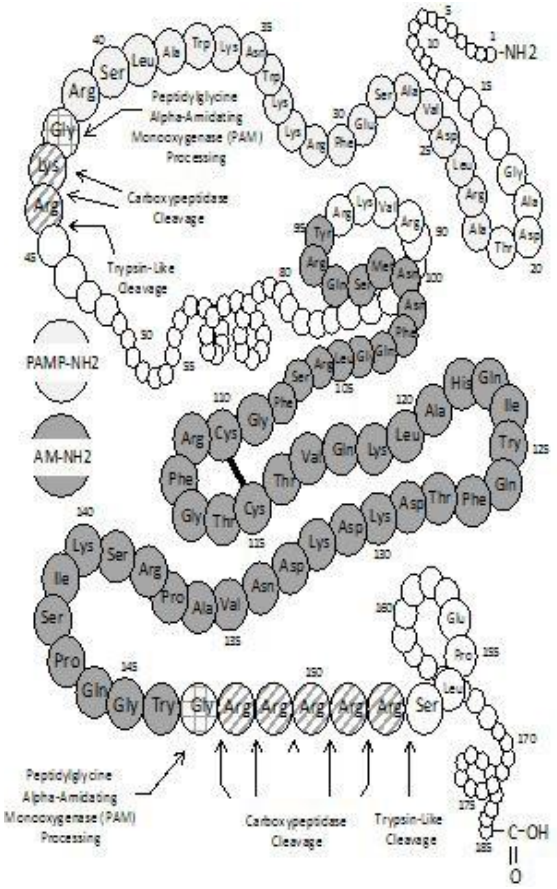

Figure 9: Complete AA sequence of prepro-human adrenomedullin (AA1-185) that identifies enzymatic processing sites for both AM (Try-amide, prepro AM 95-146) and PAMP (Arg-amide, prepro AM 22-41). GenBank Accession number BAA03589.1.

(Figure 9) illustrates the AA sequence of pre-pro-human AM and enzymatic processing site required for the generation of bioactive peptides. As was discussed previously in this text, the free acid and glycine-extended intermediates of peptide amides have diminished or completely absent bioactivity, representing a similar relationship that has been observed with AM [214, 215]. PAMP was proven to be a bioactive arginine amide that can function as a vasodilator in rats and a bronchodilator in guinea pigs [216,217]. AM mRNA and peptide have been shown to be expressed in a variety of animal tissues including normal adrenal, heart, lung, kidney, brain, vascular endothelium, liver, and intestine [211,218-220]. AM has been implicated to play a critical autocrine and/or paracrine role in mouse and rat embryogenesis and it is highly expressed in amniotic fluid and fetal tissue [221,222]. Genetically altered mice having non-functional AM die at mid-gestation with extreme hydrops fetalis and cardiovascular abnormalities [223].

Calcitonin Gene-Related Peptide (CGRP) and AM are related peptides with distinct pharmacological profiles [224]. The individual biological function for each peptide is mediated through a common primary receptor called the Calcitonin-Receptor-Like Receptor (CRLR/CLR), which is structurally modified to selectively respond to a given peptide by the interaction with co-receptors termed Receptor-Activity-Modifying-Proteins (RAMPs) [224]. The CRLR/RAMP1 complex initiates CGRP bioactivity while CRLR/RAMP2/RAMP3 controls AM function [224]. An AM-Binding Protein (AMBP-1) has been identified in plasma that is identical to complement factor H which augments AM induced breast cancer cell line growth without effecting ligand/receptor affinity. AM/AMBP-1 complexing has a negative effect on the complement cascade and suppresses AM's bacterial defensin capabilities [225]. Interestingly, CR-1 can upregulate RAMP2 expression in mammary epithelial cells (Table 3).

Growth Factors/Cytokines/Hormones	Fold Change
IL-4	4.6
Oncostatin M	5.5
TNF alpha	2.0
FGF-4	2.6
TGFβ1	3.4
Growth and Differentiation Factor-3	4.8
BMP-4	3.0
Adrenomedullin	2.3
Gastrin	3.0
Gastric Inhibitory Peptide	2.8
Mitogen-Regulated Protein/Proliferin	3.4
LH β subunit	4.4
Persephin	2.6
Receptors/Co-receptors	
Frizzled-3	1.8
TNF alpha	3.0
IL-6 beta chain (gp130)	2.6
BMP2/BMP4 R1 A (Alk-3)	3.9
Integrin α5(fibronectin receptor)	3.6
Integrin β1 (fibronectin receptor)	2.4
Endoglin TGF beta type III (CD 105)	5.0
Neuropeptide Y/Y6	5.5
Leukotriene B4	4.0
Thromboxane A2	3.3
β3-Adrenergic	3.1
G Protein-Coupled	5.6
Adrenomedullin RAMP-3 co-receptor	3.1
Syndecan-4 (Ryudocan)	6.0
Transcription Factors	
H2.0-like Homeobox	2.4
Tbx-2	3.2
Sox-18 HMG box	3.9
Vax-1 Homeobox	3.5
Ikaros	2.0
NFAT-alpha 1	3.4
Msx-2 Homeobox (Hox8)	3.0
Slug	3.0
Kruppel-like factor 13	4.0
Ets-like factor Pet-1	3.8
Ets factor Sp1	2.4
Pontin 52 (β-catenin co-activator)	3.2
C/EBP	2.2
ALY co-activator (Lef-1/Tcf-1 co-transcription)	3.9
Proteases and Inhibitors	
Mast Cell protease 4 (MMCP-4)	2.9
Kallikrein 8 (neuropsin/ovasin)	3.8
MMP-14	2.3
tPA	3.5
PA	2.8
Calpain 7	3.1
ATP-dependent protease	4.5
Alpha-1 antitrypsin 1	5.0
PAI-1	2.8
Phosphatidylethanolamine-binding protein	4.1

Enzymes	Fold Change
MEK-2	1.9
Jak-3	4.6
PKC theta	3.2
Prostaglandin D synthase	2.4
17β-HSD	3.4
Alpha-1 GST	5.9
	4.7
Alpha enolase	6.2
LDH-B	5.1
Cu-Zn Superoxide Dismutase	3.7
ATPase	3.6
RNA polymerase II	6.3
Homeobox interacting protein kinase 2	-4.7
CYP2C39	4.6
CYP2C38	3.2
CD39/ecto-ATP diphosphohydrolase (ATPDase)	5.4
Cdc42 GTPase	2.8
Others	
MAdCAM	2.6
Tiam-1 GDP-GTP exchanger (Rac-GEF)	3.9
Cyclin G2	4.0
Bax	2.8
Bcl-x	2.1
TDAG51 (FAS-mediated)	3.2
GAP-III Rho GTPase activating protein	4.1
β-Actin	3.6
Actin-related protein (Arp)	3.6
Translation initiation factor 3 p47 (eIF3/p47)	3.0
DNA replication factor	4.4
Keratin 6	9.9
Keratin 5	13.7
Ferritin L subunit	9.0
Ferritin H subunit	7.2
JNK binding protein	3.0
HSP90	3.6
Ribosomal Protein L19	5.0
CD20	7.7
Alpha fetoprotein	3.1
Clusterin (SGP-2)	1.9
Glucose-regulated stress protein 78	2.2

RED= motility, migration and/or metastasis
BLUE= β-catenin/Tcf/*wnt* pathway
LIGHT BLUE= apoptosis and involution
GREEN= sex steroid metabolism
YELLOW=inflammation

(Cumulative data obtained from assessment of 12,600 mouse genes from Affymetrix Oligo Array; from the 588 Mouse cDNA Gene Atlas Clontech Filter array and from RTPCR of 75 different embryonic genes. Expression of 90 gene found to be significantly altered in 2-3 separate assays)

Table 3: Gene profiling in CR-1 transduced EpH-4 mouse mammary epithelial cells.

AM orchestrates a major impact on species survival given its evolutionary conservation, broad spectrum regulatory role on animal physiology, and its critical involvement in normal cell proliferation during embryogenesis and wound repair [223,226-228].

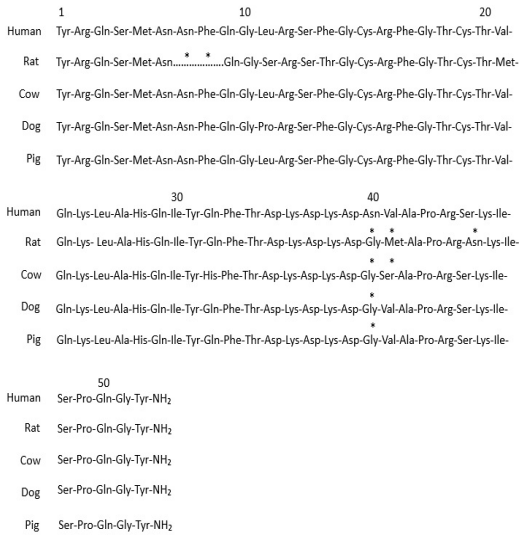

Figure 10: Identification the high degree of evolutionary conservation found for AM in mammalian species when comparing the AA sequence of the peptide amide derived from diverse species of human (Accession# BBA03589.1), rat (Accession# AAB60519.1), cow (Accession# AA123827.1), dog (Accession# AAD09957.1) and pig (Accession# BAA03590.1). Note all products contain conserved disulfide bond regions and identical carboxyl-terminal hepta peptide amide AA sequence that has been proven critical for bioactivity [214,215]. AA differing from the human AA sequence is indicated by "*". Interesting that both dog and pig AM differ by one AA from the human sequence in a biologically irrelevant region of the peptide.

(Figure 10) compares the AA structure among diverse mammals, it is interesting to note that there is only a single AA difference between human, dog and pig AM. AM-like peptides are also expressed in non-mammalian vertebrates (cartilaginous and bony fish, amphibians, reptiles, and birds), found in invertebrates (starfish), and has recently been demonstrated to have growth promoting qualities in plants [229-231].

Mitogenic events associated with embryogenesis, wound repair and carcinogenesis all share common growth potentiating pathways that are corrupted in disease states [117,232,233]. We have previously discussed the involvement of AM in embryogenesis and wound healing and will now present a historic overview of the peptide's participation in human tumor development, growth, invasion and metastasis. AM was initially shown to function as a trophic factor for murine Swiss 3T3 fibroblast cells by elevation intracellular cAMP [234]. Follow up studies demonstrated that AM was expressed in a variety of human lung cancer cell lines (carcinoids, large cell CA, adeno CA, and squamous CA) and that the peptide amide could mediate autocrine and/or paracrine tumor cell growth [118,235]. Over the years, numerous solid human neoplasms have been shown to produce AM, including tumors of the brain, colon, stomach, kidney, skin, pancreas, adrenals, prostate, uterus, ovary, and breast [236-250]. Several reports have implicated AM to underpinning cancer progression via the regulation of tumor cell proliferation, migration, angiogenesis, and inflammation, which are modulating events that ultimately enhances the malignant process [241,244,250-254]. Multiple reagents have been developed (neutralizing antibodies, small molecule inhibitors, shRNA, peptide receptor antagonists) that block AM bioactivity and have been shown to suppress *in-vivo* cancer cell growth, offering promising therapeutic application potential in the clinic [189,235,255-259]. Cumulative scientific evidence has reported that AM can induce a wide range of internal cell signaling which include initiating Ca2+ mobilization, elevating intracellular cAMP and activating MAPK/PKA/PKC/PI3K/Akt/eNOS signal transduction pathways [234,239,251,260-262]. Some of the reported factors that can upregulate AM expression are hypoxia, phorbol ester, retinoic acid, Interleukin-1beta (IL-1β), Tumor Necrosis Factor-Alpha (TNFα), Interferon-Gamma (INF-γ), Lipopolysaccharide (LPS), EGF, FGF, PDGF, CR-1 and cigarette smoke [263-268].

AM represents yet another example of a peptide amide functioning as a modulator of the immune response expressing first line defensin capabilities as an antimicrobial compound and regulating the activity of tissue infiltrating leukocytes. Defensin-like compounds are small protein primitive immune products of both plants and animals that lyse bacteria by intercalating into the outer cell wall of pathogens and disrupt the selective permeability barrier allowing the free movement of water and causing a lytic event in the invading organism, qualities shared by both AM and PAMP [269-271]. Several members of the leukocyte family, include macrophages, mast cells, basophils and lymphocytes, have been shown to express AM, CRLR, RAMPs and play a role in regulating immune function [272-278]. Recent evidence has shown that tumor mast cell infiltrates in breast cancer patients augment tumor progression and track with disease severity [279-281]. Mast cell mobility exhibits a biphasic response to AM gradients produced by cancer cells in that they migrate up from lower to higher concentrations of the peptide amide and once within the tumor infrastructure they stop their movement and de-granulate releasing angiogenic factors such as VEGF, MCP-1, basic FGF and AM, and can thereby enhance malignant progression and metastasis [274]. Recent studies have revealed that tumor-expressed AM accelerates breast cancer bone metastasis and that small molecule AM inhibitors can effectively block this process in an animal model [282].

Stromal fibroblasts of tumors, also known as Cancer Associated Fibroblast (CAFs), are phenotypically different from normal tissue fibroblasts in that they have been usurped by the tumor cells to produce a large array of growth promoting substances that cross-talks to adjacent cancer cells augmenting tumor progression [283]. In breast cancer, CAFs have been shown to promote tumor cell growth and convey therapy resistance,

hence are actively being pursued as a new drug target for clinical intervention of disease [284]. Along this same theme, new evidence has been revealed that CAFs produce large quantities of AM in breast cancer that contribute to the regional expansion of tumor blood vessel formation driving neoplasm progression and that such events can be blocked with anti-AM, anti-AM receptor antibodies or the peptide antagonist AM22-52 [285]. Given the collective cellular components encompassing the tumor microenvironment that include cancer cells, infiltrating leukocytes (macrophages, mast cells, basophils, and lymphocytes), endothelial cells and stromal fibroblasts, all of which can produce and release AM, it is relatively easy to see how critical a role this neuropeptide plays in the progression process of carcinogenesis.

Lastly, given that this review article is targeting novel angiogenic factors of breast cancer, it should be noted that AM is expressed in luminal epithelium of small/large ducts and in terminal end buds during normal mammary gland development, is elevated during lactation and AM/AMBP-1 are found in milk as beneficial supplements that participate in gut maturation of the neonate [286-288]. Hence, under normal circumstances this peptide amide is already present in the ductal epithelium of the mammary gland and when malignant transformation takes place, all of AM's previously discussed growth promoting biological assets can contribute to breast cancer progression.

Apelin/APELA/ELABELA/ELA/Toddler/APJ/AR/APLNR/SalcutNH2

The natural ligand for the orphan G protein-coupled receptor APJ was initially isolated from bovine stomach tissue extracts, shown to bind APJ at high affinity, and stimulate APJ cell signaling. The ligand was produced from a 77AA prepro-molecule based on human cDNA cloning data, and named "apelin" as a truncation of APJ endogenous ligand [289]. Further analysis of the predicted precursor protein showed that several synthetic carboxy-terminal peptide derivatives can activate APJ signal transduction with apelin-13 (last 13 AA of the prepro-hormone) and its pyroglutamate homolog being the most potent ligands [289].

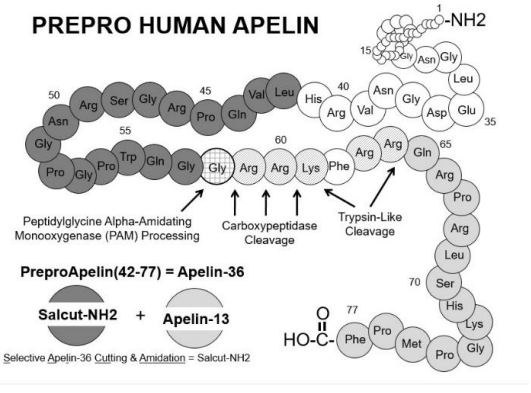

Figure 11: Complete AA sequence of prepro-human apelin (AA1-77) that identifies enzymatic processing sites for apelin-36 (AA42-77)/apelin-13 (AA65-77) and a potentially new peptide amide found within the apelin precursor. UniProKB/Swiss-Prot Accession No. Q9ULZ1.1.

(Figure 11) represents the human prepro-apelin precursor molecule along with proven and predicted enzymatic processing sites. Later on in this text we will discuss in detail a putative peptide amide derived from apelin-36 and its implications in both normal and malignant cell growth.

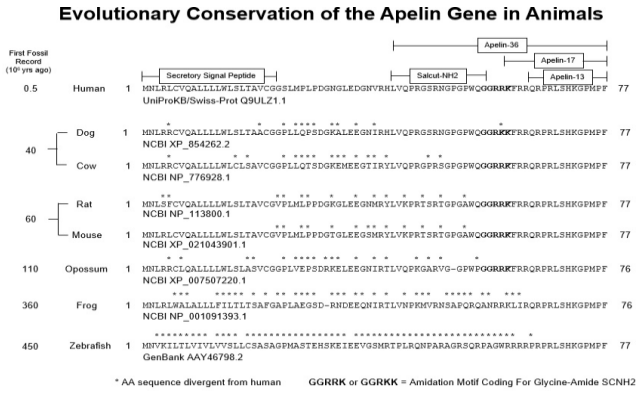

Figure 12: Conservation of apelin-13 structural integrity is maintained over 450 million years of evolutionary adaptation with a single AA substitution at the amino-terminus of the peptide (Gln- > Pro) occurring when going from humans to bony fish. It is interesting to note that the putative amidation motif within apelin-36 (indicated by the highlighted GGRRK or GGRKK) that coded for SCNH2 is consistently carried in all mammals, maintained in marsupials representing transitionary animals going from egg laying to live birth, and lost in lower vertebrates like frogs and fish. UniProKB/Swiss-Prot, NCBI or GenBank accession numbers are given below each animal prepro-hormone AA sequence. Diagram is a modification of the figure presented in Fang C et al.

(Figure 12) demonstrates that the conservation of apelin-13 structural integrity is maintained over 450 million years of evolutionary adaptation with a single AA substitution at the amino-terminus of the peptide (Gln- > Pro) occurring when going from humans to bony fish. It is interesting to note that the putative amidation motif within apelin-36 is consistently carried in all mammals, maintained in marsupials representing transitionary animals going from egg laying to live birth, and lost in lower vertebrates like frogs and fish.

A recent review by Shin K, et al. provides a comprehensive overview of the human apelinergic system and its relationship with normal and disease states [290]. Apelin/APJ have been shown to be ubiquitously expressed in brain, vascular endothelium, heart, lung, gut, pancreas, adipose, liver, kidney, adrenal gland, testis, ovary, thyroid, and bone [290-294]. A variety of human disease are attributed to anomalies in apelin/APJ expression and include cardiovascular disorders, obesity, type-II diabetes and cancer [295]. Hypoxic insult causes a marked elevation of apelin/APJ expression followed by activation of the MAPK and PI3K/Akt pathways which leads to enhanced proliferation of endothelial progenitor cells [296,297]. Interestingly, in two independent *in vivo* studies on oxygen deprivation disease involving stroke and acute lung injury in rats, therapeutic intervention with exogenous apelin-13 reduced disease severity, enhanced vascular stability

and suppressed inflammation induced tissue damage while APJ antagonist enhanced these processes [298,299]. The expression of apelin or APJ are elevated in cancers of the lung, liver, breast, kidney, colon, prostate and brain [295]. Apelin can promote lymphangiogenesis and lymph node metastasis in a mouse model of melanoma [300]. Human cholangiocarcinoma cells express APJ receptor and their growth was markedly diminished by the APJ antagonist ML221 both *in vitro* and *in vivo* [301]. In cancer patients, apelin blood levels track with disease stage representing a possible risk marker for cancer progression [302]. *In vitro* studies with MCF-7 cells have demonstrated that exogenous addition of Apelin-13 can augment breast cancer cell proliferation and gel matrix invasion in a dose-dependent manner through the p44/42 MAPK signaling pathway [303]. Western blot analysis of patient colon cancer tissue showed that apelin, APJ and Notch 3 were elevated in these tumors but not in patient matched normal tissue [304]. Follow-up studies by the same group, using the human colon cancer cell line LS180, demonstrated that these cell actively produce and secret apelin, that the peptide can function as an autocrine growth factor that increases cell proliferation via a Notch 3 signaling pathway and that inhibition of either apelin or Notch 3 could block the mitogenic process [196].

Two independent studies have identified the existence of a second peptide that binds the APJ receptor termed ELABELA/ELA/Toddler. ELABELA/ELA was present in human embryonic stem cells and shown to a secreted 37 AA peptide that regulated cardiovascular system development in zebrafish, with null mutations being lethal [305]. Toddler, identical to ELABELA/ELA, was shown to be important initiating an early signal that promotes gastrulation movement during the embryogenesis of zebrafish [306]. ELABELA/ELA/Toddler are now referred to as apelin receptor early endogenous ligand or apela based on UniProtKB/Swiss-Prot: PODMC3 universal nomenclature.

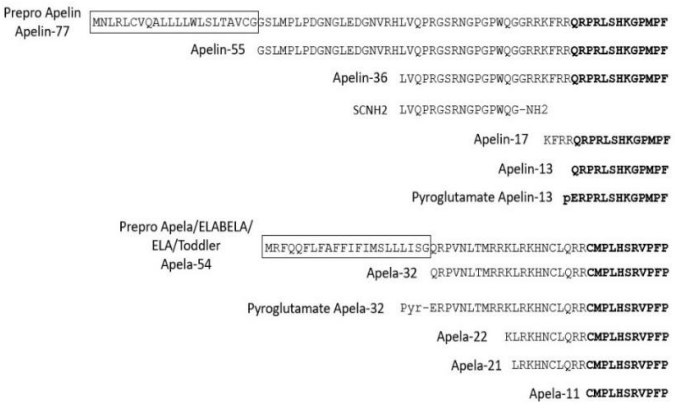

Figure 13: Compares AA sequence of human apelin (UniProKB/Swiss-Prot Q9ULZ1.1) verses apela (GenBank AHW47894.1) prepro-hormones and their reported processed peptide derivatives. Boxed area indicates "secretory signal sequence" for each precursor peptide.

Note: Pyroglutamate ("Pry-E" designation) is derived from the amino-terminal glutamine ("Q" designation). Highlighted AA represents the bioactive sequence dictating receptor recognition and bioactivity for each truncated in isoforms of apelin or apela. Diagram represents a modification of the figure from Chapman NA, et al. [307].

(Figure 13) compares the AA sequence of human apelin to that of apela, defines the reported processing peptides from the prepro-hormone and identifies the APJ binding region of each peptide [307]. Loss of apela expression in mice has been shown to induce low penetrance embryonic lethality and effects early mesodermal development [308]. Down regulation of apela expression can lead to pulmonary arterial hypertension in human and rats, a disease condition that can be compensated for by treatment with exogenous peptide in animal models [309]. Various histological types of human ovarian cancer express apela and experimental manipulation of the peptide in ovarian cell lines tracks with increased or decreased cell growth and migration in a p53-dependent manner [310]. Recent experimental studies of apela mutants in developing zebrafish showed mesodermal cell migration under peptide control that was downstream of Nodal/CR-1 signaling and indirectly modulating endodermal cell movement by the Cxcr4a pathway [311].

Analysis of the human prepro-apelin protein as presented in Figure 11 reveals the possibility of a secondary bioactive peptide derived from the carboxy-terminal end of apelin-36. As previously discussed in this text, peptide amidation consistently tracks with bioactivity and is defined by a distinct AA sequence motif comprised of a glycine followed by basic amino acids (lysine or arginine) [137,138]. Within the mid-portion internal AA structure of apelin-36, we have identified a bona fide AA sequence (gly-gly-arg-arg-lys) that would potentially dictate enzymatic processing events that could lead to the formation of a bioactive glycinie-amide peptide (leu-val-gln-pro-arg-gly-ser-arg-asn-gly-pro-gly-pro-trp-gln-gly-NH2) [312]. As previously discussed, this predicted peptide amide was named Salcut-NH2 (SCNH2) [312]. Although SCNH2 was derived from apelin-36, it had a totally different AA sequence than apelin-13 and was shown to interact with a different receptor than APJ. There is historic precedence to show that a peptide amide can be derived from another non-peptide amide hormone as seen with ACTH and its internal partner αMSH [141,313]. We have previously demonstrated that *in-silco* analysis of prepro-hormones can be a viable approach to identify new bioactive peptide amides as was shown with Y-23-R-NH2 derived from the IGF-1B precursor and PAMP identified in the prepro-AM molecule [38,211,216,217]. To validate the importance of CTA in the predicted glycine-amide SCNH2 peptide, the following peptide isoforms where synthesized and evaluated for bioactivity: SCNH2 (peptide amide), SC-OH (free-acid derivative) and SC-Gly (glycine intermediate peptide). These peptide isomers were evaluated in a variety of bioassays and only the SCNH2 (peptide amide) proved effective in inducing a biological response. Initial proliferation studies with SCNH2 were performed using APTlite one-step firefly luciferase assay and the peptide amide was shown to give a dose-dependent mitogenic

response with statistical differences over negative controls at 1nM concentrations using primary Human Umbilical Vein Cells (HUVEC), Human Microvascular Endothelial Cells (HMEC-1), Porcine Endothelial Cells (PAE), Mouse Mammary Epithelial Cells (NMuMG), human breast cancer cells (MCF-7/T47D), human fibrosarcoma cells (HT-1080), human leiomyosarcoma cells (SK-LMS-1) and human mast cells (HMC-1). SCNH2 was shown to be a potent angiogenic factor which was able to induce, in a dose-dependent manner, HUVEC tube formation with a statistical significant response at the 10pM range. Similar angiogenic effects were seen using the rat aortic ring assay or using chick embryo chorioallantoic membrane analysis. SCNH2 was shown to activate p44/42 MAPK, PI3K/Akt, p38 MAPK, CXCR4 signal transduction pathways and enhance migration and invasion of human melanoma cells (MB435). MB435 cells bound biotinylated SCNH2 at high affinity and this interaction was not blocked SC-OH (free-acid), SC-Gly (glycine intermediate), apelin-13 nor other glycine amide peptides (luteinizing hormone-releasing hormone, arginine vasopressin, or oxytocin). Interestingly, SCNH2 induced phosphorylation of p44/42 MAPK or PI3K/Akt on MB435 cells was resistant to pertussis toxin treatment but sensitive to cholera toxin, the complete opposite of what was seen with apelin-13 activating the APJ receptor [314]. Given the combined data obtained from the two previous MB435 studies, it becomes abundantly clear that SCNH2 interacts with a yet to be determined novel G-protein coupled receptor that is independent of APJ.

A series of interesting findings has been recently reported that link apelin/APJ with Nodal/TGFβ/CR-1 downstream signaling which modulates stem cells activity in both embryonic cardiogenesis and early carcinogenesis [315,316]. As apelin/APJ are known to modulate angiogenesis, the precise mechanism how vascular elongation actually occurs has remained elusive. Independent studies by Del Toro R et al and Palm MM et al have identified an anatomical separation of apelin and APJ at the leading edge of the sprouting blood vessel where tip cells express apelin and stalk cells express the APJ receptor, thus defining a biological push/pull ligand/receptor relationship that drives microvasculature advancement [317,318]. A relatively new study done by the National Cancer Institute demonstrates that APJ activates JAK1 which regulates tumor responses to INF-γ and that immunotherapies of adoptive cell transfer in mouse models are reduced in efficiency when APJ is lost or mutated leading to a reduction in therapeutic immunomodulation [319]. Finally, glioblastoma is a rapidly advancing brain tumor associated with poor clinical outcome and having a 50% mortality rate 15 months after diagnosis. Ongoing studies by Team SOAP (University of Nantes, France) have demonstrated a novel apelin/APJ nexus point between endothelial cells (ligand donor) and glioblastoma stem cells (receptor expressing recipient) that drives tumor proliferation and augments plasticity, progressionary events that can be blocked by the APJ antagonist MM54 inducing tumor regression and increased survival in animal models [320,321].

Summary

The consideration that single modality therapy in cancer treatment including breast cancer has proven to be virtually ineffective. Therefore, novel combinatorial therapeutics and new targets are needed to treat primary cancer initiation, tumor growth, metastasis and angiogenesis at early stages in each of these biological processes. We have attempted to elucidate a number of novel proteins and peptides that have proven to be involved in these different stages of cancer progression. CR-1, Nodal, GRP, AM, Apelin and SCNH2 have been shown to interact at a biological level in cancer progression and CR-1 has been demonstrated to directly regulate AM, RAMP-3, Apelin, APJ and Apela expression through a Nodal and Smad-dependent signaling pathway. Moreover, each of these proteins/peptides can directly regulate the expression of more classic angiogenic peptides such as VEGF, FGF, angiopoietin and some of their cognate receptors which can further be modified by micro-environmental agents such as hypoxia, inflammatory cytokines, cancer stem cell transcription factors, regulatory signaling pathways and systemic hormones (Figure 4 and Table 3). In conclusion this new class of angiogenic peptides that have multiple biological activities may represent novel targets in cancer for therapeutic intervention.

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