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Genetic status of KRAS influences Transforming Growth Factor-beta (TGF- β) signaling: an insight into Neuropilin-1 (NRP1) mediated tumorigenesis

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Abstract

Oncogenic RAS and deregulated transforming growth factor (TGF)- β signaling have been implicated in several cancers. So far, attempts to target either one of them therapeutically have been futile as both of them are involved in multiple fundamental cellular processes and the normal forms are expressed by almost all cells. Hence, their inhibition would disrupt several physiological processes. Besides, their downregulation stimulates the tumor cells to develop adaptive mechanisms and would most likely be ineffective as therapeutic targets. Furthermore, growing literature suggests that both of these signaling pathways converge to enhance tumor development. herefore, a lot of interest has been generated to explore the areas where these pathways interface that might identify new molecules that could potentially serve as novel therapeutic targets. In this review, we focus on such convergent signaling and cross-interaction that is mediated by neuropilin-1 (NRP1), a receptor that can interact with multiple growth factors including TGF- β for promoting tumorigenesis process.

Keywords

Pancreatic adenocarcinoma (PDAC); lung cancer; KRAS; Transforming Growth Factor-beta (TGF- β); Neuropilin-1 (NRP1); tumorigenesis

Introduction

The data from 'Centers for Disease Prevention and Control' place cancer as the second leading cause of death in the United States. KRAS, a proto-oncogene, is mutated in about 30% of cancers[1] specifically pancreatic ductal adenocarcinoma (PDAC) (>95%)[2], non-small cell lung cancer (~25%)[3], and colorectal cancer (~35–45%)[4]. The KRAS protein belongs to a family of small (about 21 kilodaltons), monomeric GTPases. There are over 150 members in the human RAS family. These proteins participate in several cellular processes

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like cell survival, differentiation, and proliferation [5]. They mediate their effects through various signaling pathways including the TGF- β signaling [6]. We will discuss synergism between KRAS and TGF- β signaling later in this review.

On the other hand, TGF- β family of proteins regulates several cellular processes like proliferation, differentiation, apoptosis, specification during development [7] and homeostasis maintenance in adult tissues [8]. This signaling cascade has been described in depth in a later section. In brief, the pathway is activated by binding of a ligand [TGF- β , activin, or bone morphogenetic proteins (BMP)] to one of the TGF- β receptors typically TGF- β receptor II (TGF- β RII). TGF- β RII then transphosphorylates and activates TGF- β receptor I (TGF- β RI) which then phosphorylates and activates a conserved group of proteins, SMADs. There are five receptor-regulated SMADs (R-SMADs; SMADs 1, 2, 3, 5 and 8), one common SMAD (SMAD 4), and two regulatory SMADS (SMADs 6 and 7) that act as negative regulators. SMADs are continually moving between the nucleus and the cytoplasm. Once the receptor-regulated SMADs are phosphorylated by the activated receptors, they form a complex with SMAD 4 and are transported to the nucleus where they regulate the transcription of their target genes. Once the SMADs form their heteromeric complex (R-SMADs and SMAD 4) their “rate of import” into the nucleus increases while their “rate of export” into the cytoplasm decreases, thus, in essence, favoring their presence in the nucleus. Hence, by promoting nuclear localization of the SMADs, ligand activation essentially regulates this process [9]. In many cancers, TGF- β signaling changes from anti to pro tumorigenic as cancer progresses [6]. This has been covered in subsequent sections. TGF- β signaling is a major pathway and with its involvement in cancer it has garnered loads of research interest.

Dr. Takagi and his colleagues discovered NRP1 in the *Xenopus* model in 1987. This transmembrane glycoprotein was initially characterized as a co-receptor for semaphorins [10]. Over the years, scientific contributions by various laboratories have established NRP1 as a co-receptor for several growth factors and their ligands including hepatocyte growth factor (HGF) and its receptor (cMet), fibroblast growth factors (FGFs) and vascular endothelial growth factor (VEGF) [11]. NRP1 has been established as a co-receptor for TGF- β that can mediate TGF- β signaling. Our group showed that upon TGF- β stimulation, NRP1 was able to regulate differential SMAD protein signaling and thereby, myofibroblast phenotypes [12]. NRP1 can also play dual roles in cancer. The recent findings of our lab provide an insight into the mechanisms regulating the pro/anti functioning of NRP1 in cancer and bring together a novel pathway binding KRAS and TGF- β signaling via NRP1 [13].

1. KRAS

1.1 Structure and function of KRAS

The RAS proteins oscillate between ‘on’: GTP bound and ‘off’: GDP bound state. The association between guanine nucleotides and the RAS proteins is very tight in the presence of magnesium ions with the dissociation constant ranging between 10–100 picomolar. The intrinsic GTP hydrolysis rate of RAS proteins is 0.028 min^{-1} . Thus, it is quite low to be significant at the physiological levels [14]. This cycle is regulated by (a) RAS guanine

nucleotide exchange factors (GEFS) that help RAS exchange GDP to GTP thereby activating them and (b) GTPase-activating proteins (GAPS) that inactivate them by facilitating hydrolysis of GTP to GDP [15]. The G domain that forms the core of these RAS proteins is conserved in the three isoforms. This domain is involved in the binding and hydrolysis of GTP [5]. However, each isoform has a unique region called the 'hypervariable region' (HVR) in the C terminus. This distinct region is a site for posttranslational modifications that impact the association of the RAS proteins with the plasma membrane and thereby their cellular localization and signaling. For example, the alternate fourth exons utilized by the two KRAS splice variants differ in their HVR region by the presence (KRAS4A) and absence (KRAS4B) of a palmitoylation site[16].

1.2 Oncogenic KRAS and its influence on tumorigenesis

Most oncogenic KRAS have 'gain of function' mutations that are somatic point mutations, which impair the ability of the RAS GTPases to hydrolyze the GTP thereby locking it in a constitutively active form. The most common codons to be mutated are 12 (~80% of KRAS harbor mutations on this codon), 61(~60% of NRAS mutations occurs at this codon) and 13. HRAS doesn't seem to favor a particular hot spot for mutations. It is interesting that all the three isoforms share identical amino acid sequence where the mutations occur. In over 40% of the KRAS mutations, there is a transition in the second base of codons from G to in codons 12 or 13 that results in the substitution of glycine with aspartic acid (G12D) or (G13D). The next common mutation is a transversion in the same set of base codons that results in the substitution of glycine with valine (G12V). A less common mutation is a transversion in the first base of codons from G to T that results in cysteine (G12C; this is more common in lung cancers). Oncogenic NRAS have similar mutations at these codons but in a much lower frequency. Conversely, almost all HRAS harbor G12V mutations though they have a much higher rate of transversion mutations compared to transitions. Mutations at codon 61 also show similar variability [17]. Besides these hotspots, oncogenic KRAS mutations also hit codons 18, 117, and 146 albeit at smaller frequencies. It is interesting that some studies have correlated the type of mutation and the oncogenic outcome (physiological activities and transformation capabilities). For example, KRAS mutations in G12 protected murine NIH/3T3 cells from apoptosis and enhanced their anchorage-independent growth compared to G13 mutations. Similarly, KRAS mutations K117N and A146T resulted in relatively lower amounts of RAS that was bound to GTP than G12D mutations in HEK293FT cells [18]. Furthermore, adding strength to this on the clinical front were studies that showed that colorectal cancer patients with KRAS G13D mutations showed the better response to anti-EGFR therapy than patients with G12D mutations [18–20]. Furthermore, a study by Dr. Feng and colleagues showed that the rate of DNA damage and repair varied between the different mutations (both between the codons and the isoforms). They reported that exposing normal human bronchial epithelial (NHBE) and normal human fibroblast (NHF) cells to benzo[a]pyrene diol epoxide-induced DNA adducts specifically in KRAS and only at codons 12 and 14. The BPDE–DNA adducts were not observed in codons 13 and 61 of KRAS or at codons 12 and 14 in HRAS and NRAS. Further, they reported that the BPDE–DNA adducts in KRAS at codon 14 were repaired two times faster than the ones at codon 12. In HRAS and NRAS they did not observe any significant differences in the repair rates between the various codons [21]. Figure 1 is a representation of how oncogenic RAS

contributes to tumor growth. The differences between the mutations in the various isoforms can be attributed to a host of reasons including: the location of the RAS in the genome and the nucleus, epigenetic modifications, the primary DNA sequence, the structural and post-translational modifications and even exposure of a particular tissue to a carcinogen [17].

Another interesting phenomenon through which RAS modulates tumor growth and development is through the induction of senescence. Senescence typically marks the permanent and irreversible exit of a cell from the cell cycle. These cells are flat and have an enlarged morphology [22]. Several studies have shown that oncogenic RAS can induce senescence. This phenomenon is called “oncogene-induced senescence.” It differs from the typical senescence by aberrant signaling of oncogenes, loss of tumor suppressors and not trailing regular cell differentiation [23]. Oncogenic RAS-induced senescence typically involves either the loss of tumor suppressor genes {examples: accumulation of p53 and p16 [24]; retinoblastoma mediated senescence [25] or cooperation from other oncogenes {for instance up-regulation of TBX2 [26]}. The reasons why an oncogene will invoke a mechanism that will impede tumor growth are not clear. Some of the speculated causes are activation of certain downstream effectors/pathways that trigger senescence like p53/p19 [22] and as a response to drug therapy when apoptosis is inhibited [23]. Besides, the anti-proliferative response evoked by RAS seems to be cell-type dependent. Rb, p53, p21 pathways have been observed in primary fibroblasts [27]. Future in-depth studies are required to fully understand whether this senescence acts a brake on tumor growth or if it helps tumor cells survive toxicity from chemotherapy by inhibiting apoptosis [23].

1.3 RAS as a therapeutic target

In the light of RAS being an important oncogene its potential as a therapeutic target is being investigated on a very large scale. In 2013, the National Cancer Institute launched the RAS initiative to look into new ways of targeting RAS proteins [28]. Several reasons have undermined this possibility. Figure 2 represents some of the reasons why RAS therapy has been unsuccessful so far. First, the drugs need to target the mutant RAS proteins specifically. It is postulated that drugs targeting all the RAS isoforms would be very toxic to the cells [28]. Recently, compounds that specifically bind to KRAS G12C mutants have been identified. Both of these compounds bind to KRAS G12C and render it inactive by inducing covalent modifications on the cysteine residue at 12 [29] or by binding to an allosteric regulatory site and shifting the preference of RAS binding to GDP over GTP and impairing the interactions between the regulatory and effector proteins [30]. However, compounds specifically targeting other mutant alleles are yet to be reported. Second, new pockets that can serve as potential targets need to be identified. The high cellular GTP and GDP concentrations along with the slow off-rate for the nucleotide that is bound to the RAS make the GTP/GDP binding domain non-targetable [31]. Another manner to impede RAS activation was to inhibit its farnesylation by using farnesyltransferase inhibitors (FTIs). This attempt wasn't successful as NRAS and KRAS4B could be prenylated by geranylgeranyl transferase as well. HRAS is the only isoform against which farnesyl transferase inhibitors were effective. However, this isoform is the least mutated isoform in cancers [32–34]. About six FTIs have been or are currently in clinical trials. Some examples are BMS-214662, L778123 tipifarnib, lonafarnib, FTI-277 and L744832 [35].

An approach that has shown promising results in *in-vivo* studies is the use of targeted siRNA delivery. One study demonstrated that inhibition of KRAS using siRNA impaired the growth of colorectal cancer in xenograft models. The efficiency of this was enhanced by simultaneous inhibition of RAF or PI3K [36]. Another publication reported that in orthotopic pancreatic cancer mice models targeted siRNA delivery against KRASG12D via Local Drug EluteR system slowed down the tumor growth and increased the mice lifespan [37]. However, the potential of this method is yet to be tested in clinical trials. An alternative way to impede the oncogenic RAS signaling would be to target one of its chaperone proteins. A study showed that inhibiting prenyl-binding protein PDE-Delta with a small molecule impaired *in-vitro* and *in-vivo* proliferation of pancreatic cancer cells. PDE-Delta helps RAS proteins in their localization and signaling by aiding their diffusion in the cytoplasm [38]. This avenue is relatively new and needs to be examined in depth. Similarly, another area that is drawing attention but needs to be investigated is the possibility of immune therapy. A clinical trial study reported that Anti-CTLA-4 treatment increased the survival of patients with melanoma through the activation of cells [39]. Given that oncogenic BRAF and NRAS mutations are observed in melanoma, it would be interesting to test to see if the immune system could be programmed to destroy oncogenic RAS.

A major concern about targeting RAS is the possibility of the tumor cells acquiring drug resistance. Furthermore, two studies have reported that YAP protein, which is an effector for the Hippo pathway, can help the cells overcome the inhibition of RAF/MEK pathway [40] and KRAS suppression[41]. Besides these, another potential risk is the deregulation of the pathways upstream of KRAS. Studies have shown that downregulation of KRAS has led to aberrant activation of EGFR signaling [42]. Similarly, the blocking of MEK pathway up-regulated the phosphatidylinositol 3-kinase (PI3K) pathway [43]. Thus, it is essential to understand and explore new pathways through which RAS functions to find new targets.

1.4 Signaling pathways engaged by KRAS

RAS proteins engage several pathways by associating with many effectors. Most literature suggests that RAS proteins signal at the cell surface predominantly [32]. Some of the most frequently employed pathways are discussed here briefly.

One of the best-characterized RAS pathways is the MAP/ERK pathway. RAF-1, the first identified RAS effector, comes to the membrane upon binding with RAS-GTP. Here it phosphorylates the serine residue and activates MAP-kinase extracellular signal-regulated kinase (MEK). Activated MEK can phosphorylate both tyrosine and threonine residues. It phosphorylates and activates its targets mitogen-activated protein kinases p44 extracellular signal-regulated kinase (ERK1) and p42 ERK2. Phosphorylated ERK, in turn, activate their effectors like Ets-2, C/EBP, SOS and MEK through phosphorylation. Activated MAP/ERK pathway is implicated in the induction of transformation and differentiation in many cell types [27]. Furthermore, wild-type and mutant RAS can modulate different facets of the same pathway. The wild-type isoforms regulate the mitogen-activated protein kinase (MAPK) pathway signaling in response to a growth factor stimulation while the oncogenic RAS take charge of the basal level signaling [42].

Another well-studied pathway is that of Phosphatidylinositol 3-kinase (PI3K). PI3K phosphorylates the phosphoinositides present at the third position in an inositol ring. This lipid kinase has a regulatory subunit, p85, and a catalytic subunit p110. Each of the subunits has five isoforms. GTP bound RAS can bind two isoforms of p110- alpha and beta and can control the p110 activity. RAS can also synergize with p85 subunit and activate PI3K [27]. This pathway is known to promote cell survival signaling [17]. The different isoforms can signal through different pathways. For example, KRAS shows a higher preference for RAF and RAC while HRAS shows stronger ability to activate PI3K pathway [17].

RAS can also bind to Ral-GDP dissociation stimulator (Ral-GDS) upon stimulation from growth factors. This binding results in GDP/GTP exchange for Ral. Besides Ral-GDS, RAS also binds proteins related to it- Rlf, Rgl, and Rgl2. RAS proteins can additionally signal along with RhoGTPases. Proteins like p190 Rho-GAP (that associates with p120 Ras-GAP) and Ras-binding protein (RIP) could facilitate these interactions. Certain RAS effectors like SOS have Dbl (DH) domain that can help Rho GTPases exchange GDP/GTP [17].

RAS proteins are known to be involved in cell cycle. RAS can regulate the expression of Cyclins A, D1, D3 and E. RAS controls the phosphorylation of Retinoblastoma. RAS can induce the expression of p15, p16, and p21 [27]. Wang and colleagues demonstrated that oncogenic KRAS contributes to oncogenesis by suppressing the non-canonical Wnt/Ca²⁺ signaling. It binds to calmodulin and downregulates the activity of Ca²⁺/calmodulin-dependent protein kinase II. This pathway deregulation, in turn, results in the reduced expression of Fzd8 causing continual downregulation of Wnt/Ca²⁺ signaling and thereby promoting tumor growth. Furthermore, the authors showed that this was specific for KRAS4B [44]. Growing literature indicates that RAS and transforming growth factor (TGF)- β signaling converge in cancer. TGF- β signaling is covered in the next section.

2. TGF- β signaling

2.1 TGF- β receptors and its signaling

The canonical TGF- β signaling involves binding of the TGF- β ligand to the constitutively active type II receptor which then complexes with and trans phosphorylates type I receptor. The TGF- β ligand belongs to a family of 33 growth factors that have six conserved cysteine residues. Various ligands evoke differing cellular responses. The different ligands share similarity in their structure and some of their sequence. The activated ligand is typically in a dimeric form. Hydrophobic interactions and a disulfide bond between the subunits give stability to the structure. Each monomer has a 'cysteine knot': beta-strands connected by disulfide bonds. Proteins called 'ligand traps' control the contact of the ligands with their receptors [7]. Both the receptors are serine-threonine kinases and share structural similarity: comprise of about 500 amino acids and consist of an ectodomain that is glycosylated and rich in disulfide bonds, a single transmembrane domain and a cytoplasmic domain that has kinase activity. The receptors can phosphorylate tyrosine residues in addition to serine and threonine. The type I receptors are also known as 'activin-receptor like kinases' (ALKs). There are seven type receptors. Some of them function in a cell type-specific manner. For example, ALK1 facilitates TGF- β signaling in endothelial cells while ALK5 can propagate the signaling in all mammalian cells [8]. The type receptors possess a glycine-serine-rich

‘SGSGSG’ domain close to the kinase domain. It is here where the type II receptors phosphorylate them. There are five type II receptors [7, 8].

Activated type I receptors then mediate the signaling through SMAD proteins. Type I receptors can directly phosphorylate SMADs 2 and 3. SMADs 2 and 3 are essentially activated in response to TGF- β ligands while SMADs 1, 5 and 8 can be stimulated by BMPs (non-canonical TGF- β signaling pathway) [7]. An accessory protein betaglycan was shown to function like TGF- β receptor III. It doesn't have a signaling domain of its own. It has been shown to bind TGF- β ligands and facilitate their association with type II receptors. This particularly enhanced TGF- β 2 binding to the type II receptors. Studies later showed that it could bind inhibin A, member of the TGF- β superfamily [45]. Another protein, endoglin, can also act like TGF- β receptor III. Endoglin can bind to type II receptors and TGF- β 1 and TGF- β 3 through this interaction resulting in the activation of the type I receptors and subsequently the signaling cascade [46].

2.2 Dual role of TGF- β signaling in tumor growth

TGF- β signaling can act both as a tumor suppressor and a tumor promoter. It has been reported that during the early stages of tumor development TGF- β signaling acts as a tumor suppressor by promoting cell cycle arrest and apoptosis [47]. TGF- β signaling can evoke a cytostatic response by inducing the expression of cyclin-dependent kinase inhibitors p21Cip1 and p15Ink4b and repressing the expression of proliferation promoters like c-Myc. This has been observed in epithelial cells and hematopoietic progenitor cells [48]. Another mechanism through which TGF- β signaling checks tumor development is by inducing apoptosis. It could be in a SMAD dependent (example up-regulation of SH2-domain-containing inositol-5-phosphatase in hematopoietic cells) or in a SMAD independent (example increased expression death-associated protein kinase in hepatoma cells) manner [48]. The idea of TGF- β signaling being a tumor suppressor is augmented by several reports that have described the inactivation of TGF- β signaling in cancer either through mutations or due to the allelic loss of heterozygosity (LOH) of one its key signaling components. For instance, TGF- β receptor II is mutated in several cancers including colorectal [49], endometrial cancer [50], and laryngeal squamous cell carcinomas [51]. Similarly, in over fifty percent of pancreatic ductal adenocarcinomas, SMAD 4/DPC4 is rendered inactive either by mutations or through the LOH of 18q [48].

However, in the later stages of tumor growth and progression, TGF- β signaling assumes the role of a tumor promoter. This alteration is called “TGF- β paradox” [52]. In several cancers, including non-small cell lung carcinoma (NSCLC) and prostate cancer increased TGF- β 1 expression corresponds with increased tumor progression. Similarly, in breast and colorectal cancers, stronger TGF- β staining correlated with more metastasis [48]. One study showed differential expression of TGF- β signaling components during early vs. late hepatocellular carcinoma. They reported that the “early TGF- β signature”-low levels of TGF- β , SMAD 7 and high transcriptional SMAD 3 activity were replaced by high levels of TGF- β , SMAD 7 and decreased SMAD 3 signaling in “late TGF- β signature” [53, 54]. There are several aspects of cancer progression to which TGF- β signaling is reported to contribute: epithelial-to-mesenchymal transition (EMT), cell stemness, motility, invasion [54], and escape from

the immune system [48]. A few ways through which TGF- β signaling promotes tumor are discussed here. Some tumors selectively inhibit only the tumor-suppressing wing of the TGF- β signaling. For example, in glioma, P15INK4B deletion and FOXO inhibition have been observed. Likewise, in breast cancer, C/EBP β inhibition and a switch in ID1 response have been reported [48]. TGF- β signaling can negatively regulate T lymphocytes and help natural killer cells and neutrophils escape the immune system. It could also impede the functioning of antigen-presenting cells, subsequently inhibiting the activation of cells [48]. TGF- β signaling promotes EMT through SMAD dependent (transcriptional regulated) or SMAD independent (impairing cell junction complexes) mechanisms. TGF- β can induce the expression of transcription factors Snail and Slug that can downregulate the expression of E-cadherin (a cell adhesion receptor). TGF- β receptor II can facilitate Par6 phosphorylation that in turn promotes the loss of tight junctions [55]. Another interesting hypothesis that is evidenced by some findings yet needs more thorough investigation is that besides EMT, this signaling gives the cancer cells 'stem cell-like properties' like the ability to self-renew [48], initiate tumor, and chemoresistance [52].

In addition to the above-listed reasons, TGF- β signaling promotes tumor growth and development by modulating the tumor microenvironment by impacting several cell types including stromal and hematopoietic cells besides the epithelial cells. It has been shown in pancreatic cancer that it modifies the tumor microenvironment to promote fibrosis and escape the immune system [56]. One effect of this change is the deregulation of microRNAs; example up-regulation of -chemokine ligand 22 in hepatocellular carcinoma cells [57]. TGF- β signaling also helps remodel the tumor microenvironment. It reduces the production of matrix depleting proteins like matrix metalloproteinases. This results in an imbalance between matrix protein synthesis and matrix proteinase activity causing 'desmoplasia', a fibrotic response. This mainly occurs in breast, colon and pancreatic cancers. The desmoplastic microenvironment brings changes in the gene expression due to the increased compressive physical forces inside the tumor and hinders drug delivery by generating a barrier [58]. Several reasons are attributed to the dual nature of TGF- β signaling: genetic heterogeneity, tumor stage and type and other mutations [54]. Many published literature suggest that oncogenic KRAS and TGF- β signaling converge in cancer.

3. KRAS and TGF- β signaling crosstalk in tumor

3.1 KRAS and TGF- β signaling crosstalk and variances

Oncogenic KRAS and TGF- β signaling can crosstalk in three ways. One, TGF- β family receptors can activate the RAS pathway by phosphorylating its members [6]. For example, TGF- β receptor I can phosphorylate ShcA proteins on their tyrosine and serine residues. This activation of ShcA results in its associating with Grb2 and Sos and thereby stimulating the ERK/MAPK pathway [59]. Another study demonstrated that p38MAPK and SMAD pathways together regulated the expression of tropomyosins which was necessary for TGF- β mediated induction of stress fibers [60]. The second way in which RAS and TGF- β pathways interact is via the phosphorylation of SMADs through kinases controlled by RAS that enables RAS to control the localization (nuclear vs. cytoplasmic) and activity of the SMAD proteins [6]. For instance, in NMuMG mammary epithelial cell line, LY294002, an

inhibitor of the p110 catalytic subunit of PI3K inhibited SMAD 2 phosphorylation and further blocked the transcriptional responses stimulated by TGF- β pathway [61]. Kretzschmar and colleagues showed that oncogenic RAS phosphorylated SMADs 2 and 3 in the region between their DNA-binding domain and transcriptional activation domain through the ERK/MAPK pathway. This phosphorylation blocked their nuclear accumulation and thereby transcription that they would have induced [62]. The third manner how RAS and SMAD signaling pathways converge is regulating gene transcription together [6]. For instance, in pancreatic adenocarcinoma, oncogenic RAS and TGF- β pathways together induced Snail that downregulates the transcription of E-cadherin. Furthermore, this was via a mechanism independent of SMADs and/or SMAD linker region phosphorylation [63]. A study in breast cancer showed that mutant p53 which was activated by RAS associated with SMADs to overpower p63 and promote invasion, migration, and metastasis [64].

Several questions still remain unresolved: what causes the TGF- β signaling to switch from anti to pro tumorigenic? Targeting either RAS or TGF- β signaling pathways have remained unsuccessful owing to the drug toxicity and the biological disparities that would accompany the inhibition of a fundamental pathway [6].

4. NRP1: a receptor of several growth factors and cytokines

4.1 NRP1 in cancer

The involvement of NRP1 with several pathways has inevitably linked it to cancer. Several studies showed that NRP1 promotes tumor albeit through diverse mechanisms. Our lab showed that NRP1 promoted VEGF induced cell migration in endothelial cells [65]. In agreement with our finding, other studies also showed that NRP1 transduces VEGF induced pro-survival signaling in cancer models [66, 67]. Some studies reported that NRP1 promoted cell migration and dispersal through autocrine Semaphorin 3A (Sema 3A) signaling [68]. Likewise, other studies have shown that NRP1 promotes tumor growth through HGF/SF autocrine pathway [69]. NRP1 has been demonstrated to modulate tumor microenvironment to enhance tumor growth. Yaqoob and group proved that NRP1 facilitates association between myofibroblasts and soluble fibronectin, which in turn helps $\alpha 5 \beta 1$ integrin-dependent fibronectin fibril assembly and promotes matrix stiffness, and hence tumor growth [70]. Our lab has shown that NRP1 promotes dedifferentiation and propagation phenotypes in renal carcinoma cells [71]. It has been shown to enable cancer stem cells to retain their properties and help their signaling [72]. NRP1 complexes with PLEXIN A4 and Sema 3A and stimulates an immunoinhibitory signaling. It has been shown that mice cells with mutant *Nrp1* that cannot bind Sema 3A have an immuno-aggressive phenotype [11, 73]. NRP1 has been shown to enhance epithelial-to-mesenchymal transition (EMT) and thereby, cell migration and invasion, which contribute to cancer metastasis [74].

Fueled by the prevalent view that NRP1 is a tumor promoter, several studies were conducted to test NRP1 as a therapeutic target. However, effective targeting of NRP1 has not yet been accomplished. A study showed that monoclonal antibody (mAb) targeting the b1/b2 domains of NRP1 showed only low efficiency in delaying tumor growth when used alone. The authors found that combining anti-VEGF targeting with it improved the efficacy [75]. However, in clinical trials combination of bevacizumab, an inhibitor to block the neuropilin

pathway, with sunitinib resulted in patients with renal cell carcinoma developing hypertension and vascular and hematologic toxicities [76]. Another major issue with using an antibody against NRP1 is that it is expressed in several adult tissues besides the tumor resulting in NRP1 antibody (MNRP1685A) to be cleared out very fast, impeding its uptake by the tumors [77]. Besides, NRP1 inhibition in normal tissues may deregulate the physiological functions it is involved in [78]. Thus, it has become crucial to unraveling the molecular pathways of how NRP1 contributes to tumor growth and development to identify new molecules that could potentially become new therapeutic targets.

Then, differing from the popular opinion that NRP1 is pro-tumor, Gray and colleagues published that in pancreatic adenocarcinoma cell line PANC-1, NRP1 played the role of a tumor suppressor. They reported that NRP1 overexpression suppressed tumorigenic characteristics like anchorage-independent cell growth and migration *in-vitro* and lowered tumor incidence and volume *in-vivo* [79]. Thus, the role of NRP1 in tumor growth and development is inconsistent, and the underlying molecular mechanisms governing these divergent roles of NRP1 are not fully understood. Besides, given this strong association of NRP1 with cancer, it has been invariably linked to TGF- β signaling and RAS.

4.2 NRP1 as a TGF- β receptor

NRP1 has been shown to function as TGF- β receptor III. NRP1 shares structural similarity with TGF- β RIII: β -glycan and its homolog endoglin [12]. The structure of NRP1 is shown in Figure 3. NRP1 has three extracellular domains: a1/a2 - two cubilin (CUB) homology domains; b1/b2 - two coagulation factor V/VIII homology domains and a C domain ccc-meprin/A5-protein/PTPmu (MAM) domain. The intracellular domain is very small, about 40 amino acids, and lacks intrinsic kinase domain. However, it has a PSD-95/Disc-large/ZO-1 (PDZ) binding motif that makes it similar to β -glycan and endoglin [12]. The b1 domain of NRP1 has a negatively charged cleft [80] which gives a plausible explanation of how several unrelated ligands might bind NRP1. TGF- β binds to the b1 domain [81]. Moreover, NRP1 can bind latency-associated peptide (LAP)-TGF- β 1 as well as active TGF- β 1. Furthermore, in breast cancer cells, NRP1 can bind and activate (LAP)-TGF- β 1 [81].

In addition to TGF- β receptor III, NRP1 can bind TGF- β receptors and II as well [82]. Our lab has shown that, in stromal fibroblast cell lines, NRP1 downregulation decreases SMAD2/3 phosphorylation and increases SMAD1/5 phosphorylation upon TGF- β stimulation. These changes were reflected in the corresponding downstream targets with an increased inhibitor of differentiation (Id-1) and decreased α -smooth muscle actin (α -SMA) expression [12]. These findings were augmented by the reports of another group. Glinka and colleagues also reported that NRP1 supported the SMAD2/3 signaling [82].

It is postulated that the negative regulation of human T cells by NRP1 is also influenced by TGF- β [11]. Nrp1 serves as a marker for the majority of the regulatory cells (Treg) in murine models and a subpopulation of activated Treg cells in humans. Nrp1 is thought to increase the association time between Treg cells and antigen during the antigen presentation by immature dendritic cells. This interaction is important as its duration influences how many MHC class II-peptide complexes can be engaged by the synapse and thereby the initiation of the immune response. As naive helper T (Th) cells do not express Nrp1, this is

an advantage that Treg cells have as Nr1 lengthens their interaction time with the immature dendritic cells. Thus, in a situation where an antigen can be recognized by both Treg and Th cells, Treg will likely dominate and in the absence of “danger signals” cause suppression of immune responses [83]. Another group used a tissue-specific Nr1 deletion mice model and reported that the loss of Nr1 in CD4⁺ T cells increased ‘helper cells’ lineage, experimental autoimmune encephalitis, and decreased Treg cell functioning. This group further showed that TGF- β inhibition could impede Nr1 mediated suppression of T effector cell proliferation and cytokine production [84].

4.3 NRP1 and RAS

This glycoprotein lacks intrinsic kinase activity and needs an effector molecule. GAIP interacting protein terminus (GIPC) is a cytoplasmic protein that can bind to the PDZ-binding domain of about twenty-five receptors including NRP1 [85]. Several studies have shown GIPC can transduce NRP1 mediated signaling [86, 87]. Our group demonstrated that in the VEGFR receptors lacking renal carcinoma cell line, 786-O, NRP1 was able to activate RAS on being stimulated with VEGF. We observed an increased phosphorylation of downstream RAS targets: ERK1/2 and AK. We further showed that this RAS activation was dependent on RAS GEFs SOS1/2 [88]. Based on this new finding that RAS could act as an effector for NRP1 our group hypothesized that the genetic status of KRAS might be modulating the role of NRP1 in tumorigenesis. We examined the published literature and found out that in all the tumors where NRP1 acted as a tumor promoter, the cells had wild-type KRAS: breast cancer [89], osteosarcoma [90], renal cancer [71], non-small cell lung cancer [91], neuroblastoma [92] and hepatocellular carcinoma [93] among others. Conversely, the cell line where NRP1 was promoting tumor, PANC-1 [79], had oncogenic KRAS [37]. We then went ahead to confirm our hypothesis in pancreatic and lung cancer models that in the presence of oncogenic KRAS lack of NRP1 promoted tumorigenesis while the absence of NRP1 in cells with wild-type KRAS inhibited tumor growth. We further defined a role for TGF- β signaling pathway in the NRP1/KRAS mediated tumorigenesis axis. We found that in pancreatic cancer cells with mutant KRAS, reduced NRP1 expression results in decreased phosphorylation. Likewise, down-regulation of SMAD2 in pancreatic cancer cells with oncogenic KRAS resulted in increased tumor growth. Furthermore, another interesting finding we had was that TGF- β cytokine down-regulated the expression of NRP1 in both mRNA and protein levels only in cells with oncogenic KRAS. It is intriguing that TGF- β specifically reduces NRP1 expression only in cells with mutant KRAS. Thus, our study gave an insight into how oncogenic KRAS might be influencing TGF- β signaling through one of its receptors NRP1 [13].

5. Conclusions

Oncogenic KRAS, TGF- β signaling, and NRP1 are key players in several cancers. So far, therapeutically targeting any of them has not been successful as their inhibition impacts plethora of physiological functions and toxicity issues. Therefore, it is critical to identify new mechanisms through which they influence tumor growth and progression so that we can uncover new molecular targets for developing new therapies. Our recent results showing that in cells with oncogenic KRAS, NRP1 downregulation mediates tumor growth by

suppressing SMAD2 phosphorylation elucidates a novel mechanism where all the three players seem to converge. In cells with wild-type KRAS, decreased NRP1 expression suppressed tumor growth [13]. Nevertheless, more in-depth investigations are needed to unravel the complete underlying mechanisms and test the findings in other cancer models. Furthermore, these findings illustrate the importance of determining a patient's molecular signature and highlight the necessity for individualized cancer therapies.

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Figure 1.
 The GTPase activity of wild-type RAS is regulated by GEF and GAP proteins. Oncogenic RAS is 'dominant active' and cannot hydrolyze GTP to GDP. It is constitutively active, thereby promoting aberrant cell growth. The most common mutations are indicated by the arrow.

Figure 2.
Challenges of targeting RAS for therapy

Figure 3. Structure of Neuropilin-1

a1/a2: cubilin (CUB) homology domains

b1/b2: coagulation factor V/VIII homology domains

c: Meprin/A5-protein/PTPmu (MAM) domain

TMD: Transmembrane domain

PDZ: PSD-95/Disc-large/ZO-1