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The *KISS1* metastasis suppressor: mechanistic insights and clinical utility

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Abstract

Melanoma is a highly metastatic cancer that accounts for the majority of skin cancer deaths. Unfortunately, very few improvements have been made during the last 20 years in the management of melanoma metastases, which is the major cause of melanoma deaths. Therefore, identification of molecular targets that can be exploited in the clinic to treat metastatic disease is desperately needed. The *KISS1* metastasis suppressor gene has emerged as a promising molecular target for the management of metastatic disease. This review compiles data regarding the molecular and biochemical properties of *KISS1* and its cognate receptor, focusing on the properties believed to be most pertinent to the use of *KISS1* in the clinical setting. In addition, clinical data that supports *KISS1* as having a dual role as a prognostic indicator and a therapeutic target for the management of metastatic disease will be highlighted.

Keywords

KISS1; Metastin; Kisspeptin; GPR54; Axor12; hOT7T175; Ligand; Receptor; G-Protein Coupled Receptor; Metastasis; Metastasis Suppressor Genes; Review

2. INTRODUCTION

Skin cancer is the most common of all cancers in the United States representing nearly 45% of all newly diagnosed invasive cancers (1). While melanoma only accounts for about 6% of all invasive skin cancer cases, it is the most lethal (1). Melanoma is responsible for 73% of all skin cancer deaths and the incidence of melanoma is increasing at a rate of 3% per year since 1981 (1). In 2005, 59,580 new cases of invasive melanoma are expected (1). Although there is an overall 90% five-year survival rate for melanoma patients, survival drops precipitously if there is evidence of metastatic disease (2). Most patients with metastatic disease confined to the subcutis and lymph nodes will survive for 12 months, while patients with visceral involvement have a median survival of 4–6 months (3). These grim statistics reflect the highly metastatic nature of melanoma and its ability to colonize multiple organs and tissues. This makes therapeutic intervention very challenging in melanoma patients with disseminated disease.

Despite all the recent advances in post-operative adjuvant therapies targeting metastatic disease, “there is no single treatment that significantly improves overall survival rates in melanoma patients (3).” As a result, there has not been a significant improvement in survival rates over the last 20 years (2). The lack of reduction of melanoma mortality rates strongly

indicates that there is a dire need for the identification of novel targets that can prevent or inhibit metastatic disease.

One set of promising targets for the prevention of metastatic disease are metastasis suppressors, which inhibit metastases while having little effect on primary tumor formation. Some of these proteins, most of which were discovered within the last 5–10 years, have also been shown to have an inverse correlation with tumor grade and the likelihood of metastatic disease in patients (reviewed in (4,5)). Unfortunately, little is known about their mechanism(s) of action or which step(s) in the metastatic cascade where these proteins exert their suppressive effects. In order to fully exploit metastasis suppressors as clinical targets for managing metastatic disease, more research is necessary. *KISS1* is one of these promising metastasis suppressors. We will review what is known about the *KISS1* melanoma metastasis suppressor, giving emphasis toward its potential as a clinical target for managing metastatic disease.

3. DISCOVERY OF *KISS1* AS A MELANOMA METASTASIS SUPPRESSOR

Translocations and/or deletions involving the long arm of human chromosome 6 (6q) have been found in greater than 80% of tumors representing late-stage, metastatic melanoma (6). Based upon this observation and in order to determine if a gene(s) on chromosome 6 is(are) capable of inhibiting metastases, an intact copy of human chromosome 6 was introduced into the metastatic C8161 human melanoma cell line (neo6/C8161) using microcell-mediated chromosomal transfer (7).

The introduction of chromosome 6 suppressed metastases to lung and lymph nodes (>95%), with no discernable effect on primary tumor formation. Subtractive hybridization was carried out on metastatic C8161 and neo6/C8161 cells and a novel cDNA transcript termed *KISS1* was isolated (8,9). *KISS1* expression was observed in primary melanocytes while its expression was inversely correlated with metastatic potential in a panel of melanoma cell lines (8,9). When *KISS1* expression was restored in C8161 cells, pulmonary metastases were inhibited by >95% following intravenous injection. Growth of orthotopic tumors was not blocked. These experiments identified *KISS1* as a potent melanoma metastasis suppressor gene with metastatic suppression similar to the levels observed with the introduction of chromosome 6. While *KISS1* was first suspected to be the metastasis suppressor encoded on chromosome 6, radiation hybrid mapping and fluorescence *in situ* hybridization showed that the *KISS1* gene mapped to the long arm of chromosome 1 existing as a single locus on 1q32. This result suggested that *KISS1* may be regulated by a gene on chromosome 6 (10).

4. REGULATION OF *KISS1*

Since an intact copy of *KISS1* (i.e., wild-type, not mutated) can be isolated from C8161 cells, it is likely that *KISS1* is regulated by a gene on chromosome 6 that is defective or lost in C8161 cells. Loss of heterozygosity (LOH) of 6q16.3-q23 in human melanoma metastases has been shown to correlate with the loss of *KISS1* expression (11). Miele *et al.* confirmed that this 40 cM region on chromosome 6 was important for metastasis suppression in C8161 cells (12).

Using microarrays comparing neo6/C8161, C8161 with chromosome 6 containing a deletion of 6q16.3-q23 (neo6qdel/C8161) and parental C8161, Goldberg *et al.* identified TXNIP (thioredoxin interacting protein, also known as VDUP1, vitamin D up-regulated protein, TBP2, thioredoxin binding protein 2) to be consistently elevated in neo6/C8161 cells. Interestingly, like *KISS1*, VDUP1/TXNIP mapped to chromosome 1 and is capable of suppressing metastases in C8161. However, the regulatory gene on chromosome 6 was not identified until PCR-based karyotyping was performed on overlapping deletions of chromosome 6 in conjunction with their ability to suppress C8161 cells. Loss of the D6S457 marker on chromosome 6 was shown

to correlate with a gain of metastatic competence. CRSP3 (co-factor required for SP1 activity, also known as DRIP130, vitamin D receptor interacting protein) was the closest gene to D6S457. Like *KISS1*, CRSP3 expression was inversely correlated with melanoma progression as modeled by a panel of cell lines. Restoration of CRSP3 expression in C8161 cells inhibited metastases. In support of CRSP3 as a *KISS1* regulatory gene, cells overexpressing CRSP3 have elevated *KISS1* levels. Additionally, 20 patients undergoing melanoma surgery showed a 62.9% correlation between CRSP3 expression and *KISS1* expression (13). Therefore, CRSP3 is a strong candidate gene involved in the regulation of *KISS1* expression. Combined with data showing that TXNIP re-expression could increase *KISS1*, Goldberg hypothesized that there is a metastasis regulatory axis CRSP3 → TXNIP → *KISS1* (13).

5. KISS1 PROTEIN CHARACTERIZATION

5.1. KISS1 sequence analysis and processing

In 2001, three labs independently identified sites in the amino acid sequence of the *KISS1* protein that suggests it is processed and secreted (14–16). Careful examination of the sequence revealed that *KISS1* has characteristics in common with neuropeptides which include a secretion signal, several dibasic cleavage sites and a cleavage amidation site. The first 19 amino acids of *KISS1* comprise a secretion signal sequence which our laboratory has recently confirmed (unpublished). The remaining sequence of *KISS1* has two dibasic cleavage sites (R⁵⁶-K, R⁶⁶-R) followed by a cleavage amidation site (K¹²³-R) (Figure 1). These canonical cleavage sequences are purportedly recognized by furin or other prohormone convertases. Although, most precursors processed by furin in the constitutive pathway have the canonical sequence R-X-K/R-R or R-X-X-R, R-K and R-R are the predominant cleavage sites found in prohormones and proneuropeptides (17). Cleavage at dibasic sites R⁶⁶-R and K¹²³-R result in a 54-amino acid product termed metastin/kisspeptin-54 (KP54). Cleavage at R⁵⁶-K dibasic motifs occur more rarely and is consistent with the inability to identify kisspeptin-64 (KP64) (14–18). Despite the likelihood that *KISS1* is processed by prohormone convertases, it is still unknown which convertases(s) is(are) responsible for processing *KISS1*. In addition, the location in the cell where *KISS1* processing occurs has not yet been elucidated.

Takino *et al.* identified that *KISS1* as well as kisspeptins can be cleaved by matrix metalloproteinases (MMP) at G¹¹⁸-L¹¹⁹ (19). Interestingly, processing at this site abolishes kisspeptin-10's (KP10) ability to induce focal adhesions suggesting that MMP's may serve as a negative regulator of *KISS1*. In support of this, MMP expression has been shown to increase in tumors and has been shown to correlate with invasion and metastasis (20–22).

5.1.1. Proposed model for *KISS1* processing—There are three major steps envisioned for the processing of *KISS1* protein by prohormone convertases (Figure 1). First, endoproteolytic processing occurs at dibasic cleavage sites R-R and K-R and the newly exposed C-terminal basic residues arginine and lysine are removed by a carboxypeptidase that has a high specificity for basic residues. Finally, after the removal of the C-terminal basic residues, glycine undergoes conversion to an amide by a peptidyl-glycine- α -amidating monooxygenase (PAM) (17). Interestingly, amidation is important in enhancing the binding of neuroendocrine peptides to their cognate receptors and may play a role in inhibiting their degradation (17). The significance of amidation in mediating *KISS1*'s activity will be discussed below.

At this point it is worth mentioning that the term *metastin* is misused frequently in the literature to represent both unprocessed *KISS1* as well as the processed kisspeptins. Therefore, we strongly caution readers to be wary when reading the literature. We prefer the term *kisspeptins* to refer to the processed forms of *KISS1*, mostly because it retains reference to the

original name. We will refer to processed forms of KISS1 as kisspeptin (KP) followed by the size of the peptide (Figure 1).

5.2. KISS1 secretion

Horikoshi *et al.* identified KP54 in the plasma of men and women using a two-site enzyme immunoassay that recognizes both ends of the peptide (23). The levels of KP54 identified in both sexes were similar. However, when plasma levels were examined in pregnant women, KP54 was shown to increase in conjunction with prolactin, estrogen, and progesterone which indicates placental *KISS1* expression is not inhibited by increasing hormone levels. In the third trimester, KP54 levels in the plasma were ~7,000 times higher than in non-pregnant women and returned to baseline 5 days post-partum.

Immunohistochemical localization of KP54 to the outer syncytiotrophoblast compartment of the placenta suggests that *KISS1* may function as a placental derived hormone (18,23). It is plausible that *KISS1* may play a role in the regulation of placental invasion based on its localization in the syncytiotrophoblast compartment of the placenta. However, it is still unclear what *KISS1*'s function(s) is during pregnancy and what the source of KP-54 is in males.

Bilban *et al.* took this one step further by examining KISS1 secretion in isolated primary human trophoblast cells (18). They were able to identify full-length KISS1 in lysate but were unable to detect any processed forms of KISS1. This result is consistent with the proposed processing of KISS1 which may occur late in the secretory pathway or even at the cell surface.

Interestingly, detection of KP54 in the media of primary trophoblast cells could only be accomplished after reverse-phase HPLC and MALDI-TOF. The inability to detect KP54 in the media of cells in culture by western blot indicates that KISS1 secretion is likely to occur at low levels. Rapid degradation of KP54 is an unlikely explanation for low media levels since it could be readily detected in the serum of men and women albeit in the fmol/ml range. Although Bilban *et al.* also detected smaller peptides that have been termed kisspeptins-14, -13 and -10 in the media, there are no known cleavage sites in the KISS1 sequence that would suggest that these peptides could be formed. Spontaneous degradation or an artifact of sample preparation cannot be ruled out as a potential source of these peptides (14).

Despite strong evidence for *KISS1* as a secreted protein, it has not been determined if KISS1 secretion occurs in metastatic human cancer cell lines that are suppressed by *KISS1* (e.g. C8161.9, MelJuSo and MDA-MB-435). More importantly, it is also unclear if KISS1 secretion is required for metastasis suppression. These are important questions to address in the future in order to understand how to exploit metastasis suppression by *KISS1* in the clinic. If secretion is required for metastasis suppression, exogenous administration of a naturally occurring peptide will reduce the possibility of side effects and avoid the need for gene therapy.

6. MECHANISM OF *KISS1* METASTASIS SUPPRESSION

6.1. GPR54: a receptor for *KISS1*

GPR54 (also known as AXOR12 or hOT7T175) is an orphan G-protein coupled receptor that was identified in 1999 by Lee *et al.* (24). GPR54 is coupled to G proteins of the $G_{q/11}$ subfamily which, upon activation, result in the release of intracellular calcium stores and activation of phospholipase C- β (14,15,25). Interestingly, $G_{q/11}$ proteins have been implicated in regulating a diverse number of cellular functions which include secretory machinery (25). GPR54 bears structural similarities with other neuropeptide receptors and shares a 38% sequence similarity with galanin receptors. However, galanin is unable to bind or activate GPR54. Many neurotransmitters and neuromodulators terminate with the sequence Arg-Phe-NH₂ (RF-NH₂) but have variable amino-terminal sequences (26). KP54 has an RF-NH₂ C-terminus but it was

not discovered as a potential ligand to GPR54 until 2001 when three laboratories independently identified KP54 as a potent agonist to GPR54 (14–16). Stimulation of cells overexpressing human GPR54 with exogenous kisspeptins results in a robust intracellular Ca^{2+} release. The affinity of KP54 for GPR54 is low nanomolar which is well within any presumed physiological range. Taken together, these results support the notion that KP54 is a cognate ligand for GPR54. Ohtaki *et al.* also showed that amidation at the C-terminus of KP54 is essential for binding and activation of GPR54. Smaller amidated kisspeptins-10, 13 and 14 which are N-terminal truncations of KP54 are also capable of binding and activating GPR54. The last 10 amino acids of KP54 appear to be the most important for binding and activating GPR54 and are highly conserved among species (27,28). However, peptides representing regions N-terminal and C-terminal to KP54 were not able to bind or stimulate GPR54 (15). Therefore, only C-terminal amidated kisspeptins are capable of binding and signaling through GPR54. What role these peptides play in *KISS1*'s function is still a matter of speculation because it is still unclear if the interaction between GPR54 and kisspeptins is required for metastasis suppression. Since all of the studies mentioned above were performed in cells that had experimentally elevated GPR54 that were exposed to exogenous kisspeptins (at doses which could be either pharmacologic or physiologic), the relevance should not be misinterpreted.

6.2. GPR54/kisspeptin signaling

In addition to the release of intracellular calcium stores, kisspeptin signaling through GPR54 has been shown to activate the MAP kinase pathway. Several laboratories have consistently shown phosphorylation of MAP kinases ERK1 and ERK2 after exposure to kisspeptins. This occurs regardless of the cell line examined and is irrespective of whether GPR54 levels are endogenously high or artificially elevated (14,16,29,30).

Phosphorylation of p38 has been, however, less straightforward. p38 phosphorylation was observed after kisspeptin exposure in CHO cells overexpressing GPR54 as well as in PANC-1 pancreatic carcinoma cells with endogenously high levels of GPR54 (14,29,30). p38 phosphorylation was not observed in ARO81 cells, which have high endogenous levels of GPR54 (30). Therefore, p38 phosphorylation may be cell type specific or is dependent on the levels of GPR54 expressed. ERK1/2 signaling is likely to be the predominant GPR54 pathway activated by kisspeptins because of the consistency in its activation between cell lines. However, it is still unclear if GPR54 plays a role in metastasis suppression and how ERK1/2 signaling could accomplish this.

Although the MAPK pathway appears to be the predominant pathway activated by GPR54 following exogenous kisspeptin exposure, *KISS1* over-expression can activate pathways independent of MAPK. Boyd *et al.* found a decrease in MMP9 activity in HT-1080 cells overexpressing *KISS1* that is not mediated by the MAPK pathway (31). This effect was attributed to a reduction in $\text{NF}\kappa\text{B}$ binding to the *MMP9* promoter. The MAPK independent suppression of MMP9 may suggest alternative signaling pathways to GPR54. However, it is unclear if a decrease in MMP9 can also be observed after GPR54/kisspeptin signaling.

6.3. *In vitro* implications for GPR54/kisspeptin and metastasis

Several *in vitro* phenotypes have been observed in cells after the exposure of exogenous kisspeptins which support GPR54 as a potential regulator of metastasis (Table 1). The most extensive analysis of *in vitro* metastatic characteristics has been performed using CHO cells overexpressing GPR54 (CHO/GPR54). It is important to emphasize that CHO cells are non-metastatic. And while not ideal for functional assessment, they are extremely useful for biochemical characterization. When these cells are exposed to kisspeptins, a decrease in proliferation, motility, invasion, and soft agar colony formation have been observed with

concomitant increases in focal adhesions and stress fiber formation (14,16). These characteristics are generally consistent with changes expected in cells which would have lower metastatic potential. Kisspeptin exposure has also consistently shown effects on migration in other cell lines with high endogenous or experimentally elevated levels of GPR54 (18,27,29, 30). However, a decrease in proliferation or invasiveness has not been seen in cells with high endogenous levels of GPR54 (18,29). Recently, Becker *et al.* found an induction of proapoptotic genes in MDA-MB-435S cells after prolonged exposure to micromolar levels of exogenous KP10 (32). Despite consistent findings for GPR54/kisspeptin signaling in the regulation of motility, the regulation of proliferation and invasion has been less straightforward. Interestingly, over-expression of *KISS1* in MDA-MB-435 human metastatic breast carcinoma cells had no effect on adhesion, motility, or invasion (33). This is in stark contrast with what was seen in CHO/GPR54 cells after exogenous exposure to kisspeptins. However, there was a significant decrease in soft agar colony formation as observed in CHO/GPR54 cells (33, 34). The inability of *KISS1* overexpression to inhibit motility of MDA-MB-435 cells may be due to suboptimal levels of kisspeptin secretion and/or inadequate levels of GPR54 at the cell surface. Of note, MDA-MB-435 metastases can still be suppressed by *KISS1* overexpression despite low levels of GPR54 mRNA (unpublished). Therefore, inhibition of *in vitro* motility may not be a relevant marker for examining the potential of *KISS1* metastatic suppression.

6.4. *In vivo* implications for GPR54/kisspeptin and metastasis

To the best of our knowledge, Ohtaki *et al.* is the only group so far to examine and publish the relationship between GPR54 and kisspeptin signaling in *in vivo* metastasis assays. B16-BL6 murine melanoma cells over-expressing human GPR54 (B16-BL6/GPR54) were injected for both experimental (intravenous injection) and spontaneous metastasis assays (subcutaneous injection). Exogenous KP10 was delivered through an osmotic pump and lung metastases were examined at the completion of the study. Interestingly, exogenous KP10 was only capable of suppressing B16-BL6/GPR54 pulmonary metastases arising from a subcutaneous injection (i.e., lung colonization was not inhibited if the tumor cells were inoculated directly into the venous circulation). This was an unexpected result because it had been previously shown that *KISS1* overexpression inhibits pulmonary metastases in both spontaneous and experimental assays (8,9,33). This may suggest that GPR54/kisspeptin signaling does not play a role in *KISS1* pulmonary metastasis suppression following intravenous injection and may point to the involvement of additional mechanisms by which *KISS1* can inhibit metastasis. However, it is unclear if the inability of exogenous administration of KP10 to inhibit pulmonary metastases after intravenous injection is due to 'trivial' experimental conditions such as pharmacokinetics (e.g., inadequate local concentrations of the peptide in the lungs). Interestingly, our laboratory has detected very low levels of GPR54 mRNA in C8161.9 cells despite a potent suppression of pulmonary metastases after intravenous injection when *KISS1* expression is restored (unpublished). Either low levels of GPR54 are sufficient for *KISS1* metastasis suppression or *KISS1* may mediate suppressive effects by interactions with other proteins within the cell or perhaps even signaling through an additional receptor(s).

7. WHICH STEP(S) OF THE METASTATIC CASCADE DOES *KISS1* SUPPRESS?

Most of the *in vitro* data to date implicate that *KISS1* metastasis suppression is mediated, in part, through its effects on growth, motility, and invasion of cancer cells. Most of these effects would suggest that the step in the metastatic cascade where *KISS1* is likely to exert its suppressive effects would be at the primary site where inhibition of growth, motility and invasion would be most effective. However, *in vitro* assays have provided limited mechanistic insight with regard to *KISS1* metastasis suppression. Therefore, the most unassailable way to

determine the step in the metastatic cascade where *KISS1* suppression occurs is through *in vivo* experimentation.

Since metastatic dissemination often occurs before clinical detection of the primary tumor, identification of molecular targets that block antecedent steps in the process will be less useful clinically. Molecular targets that prevent the proliferation of metastases once they have already disseminated will be most advantageous in the treatment of patients with metastatic disease. Goldberg, Harms *et al.* indicated that an intact copy of chromosome 6 suppressed pulmonary metastases of C8161 cells (neo6/C8161) at the final step of the metastatic cascade - colonization of the secondary site. Persistent single dormant neo6/C8161 cells were found in the lungs beyond 5 weeks while parental C8161 cells formed macroscopic metastases by 2–3 weeks and killed the host by ~4 wk (35). Since neo6/C8161 cells have elevated levels of *KISS1*, we asked whether *KISS1* expression is similarly capable of maintaining metastatic dormancy in the lung. Recently, our laboratory has addressed this question by restoring *KISS1* expression in green fluorescent protein-tagged C8161 cells. We found that *KISS1* expression does not affect initial seeding of metastatic cells to the lung, but prevents tumor cell proliferation after arrival (K. T. Nash and D. R. Welch, manuscript in preparation). Thus, we believe that *KISS1* is a promising molecular target for inhibiting growth of pre-symptomatic metastatic lesions. How *KISS1* maintains dormancy of disseminated cells at the secondary site is still unclear. In addition, it is still unknown whether *KISS1* will show efficacy in treating larger established metastatic lesions (masses >100–1000 cells). Recently, Palmieri and colleagues showed that restoration of Nm23 metastasis suppressor expression by treatment with methoxyprogesterone acetate (to induce a glucocorticoid transcription factor element in the Nm23 promoter) successfully reduced progression of established microscopic metastases (36). Their proof-of-principle studies are encouraging while answers to both questions will be important to resolve in order to understand the impact that *KISS1* may have in the clinic.

8. EVIDENCE FOR *KISS1* AS POTENTIAL CLINICAL TARGET

Progress toward understanding the mechanism of action of *KISS1* and relevant clinical correlates has been significantly hampered by the unavailability of reliable, validated antibodies to detect *KISS1* protein. All of the experimental evidence and clinical evidence highlighted below measured mRNA expression levels and not protein. Although this data does not detract from the potential of *KISS1*'s significance, development of reliable antibodies for determining protein levels in patient biopsy specimens in the future will be more pertinent. This issue is especially relevant since nascent *KISS1* would not appear to be the active form.

We were the first to show that the introduction of *KISS1* into highly metastatic human melanoma cell lines C8161 and MelJuSo suppressed metastases to the lung by >95% following intravenous or orthotopic injection (8,9,33). Interestingly, introduction of *KISS1* into a metastatic breast cancer cell line MDA-MB-435 also showed a >95% suppression of metastases to the lung following orthotopic injection (33). Those data strongly suggested *KISS1* metastasis suppression may be pertinent in tumors of widely different origins, a conclusion borne out in subsequent studies (11,30,37–40), albeit of varying quality and significance. In general, loss or reduction of *KISS1* expression in several different tumor types inversely correlates with tumor progression, metastatic potential and survival. The data summarized below highlights the potential value of *KISS1* as an important clinical target for the prognostication and treatment of metastatic disease.

8.1. Melanoma

Melanoma is the most lethal form of skin cancer that can affect adults of all ages including teenagers. Most melanomas have a period of superficial growth referred to as the radial growth phase in which the lesion increases in size but does not invade beyond the superficial dermal

layers. Melanomas caught at this stage are not metastatic and are associated with a good prognosis. Over time, growth of the melanoma continues until it enters the vertical growth phase in which cells invade into deeper dermal layers. In general, depth of invasion correlates with metastatic competence and poor prognosis (especially >0.75 mm). Fortunately, the majority of melanomas are caught early in the radial growth phase and full-thickness excisional biopsy is curative. If the biopsy reveals invasion, staging is required for determining treatment and prognosis. If metastatic disease is revealed, treatment is usually palliative in nature because metastatic melanoma is generally incurable.

The holy grail for pathologists is to unequivocally identify patients with tumors likely to metastasize so that aggressive adjuvant therapies can be administered. If accomplished, one can avoid unnecessary treatments for patients with tumors unlikely to have spread. Unfortunately, the subjectivity of pathologic prognosis has not reached that level of sophistication. Even when the world's most highly respected dermatopathologists read the same slides, there was >50% discordance amongst the group (41). Therefore, less subjective measures are needed. And it is in this situation where metastasis suppressors could provide enormous assistance (42).

KISS1 mRNA expression has been evaluated in human melanoma specimens from various stages of progression. Shirasaki *et al.* showed that *KISS1* expression is reduced by 50% when primary melanomas exceed > 4mm (11). Interestingly, a dramatic drop in *KISS1* expression coincides with the rapid drop in 5-year survival of patients with tumors of this size (43). In addition, Shirasaki *et al.* showed that there was no difference in *KISS1* expression between melanoma tumors > 4 mm deep and the metastatic lesions that were examined. This result was attributed to the evidence that deeply invasive vertical growth phase melanoma cells already contain numerous cytogenetic abnormalities and are metastasis competent (44). Therefore, like Herlyn and colleagues (45), Shirasaki *et al.* argued that no additional genetic alterations are necessary for metastatic progression. Collectively, the data indicate that there is a strong inverse correlation between loss of *KISS1* expression with gain of metastatic potential, suggesting that *KISS1* may serve to enhance the staging of melanoma. Coupled with the experimental evidence showing *KISS1* as a metastasis suppressor, the data suggests that *KISS1* shows promise as a target for the treatment of metastatic melanoma as well.

8.2. Thyroid cancer

Thyroid cancer is the most common malignancy of the endocrine system and shows a predisposition in females over males (~2:1). Although, thyroid neoplasms can arise from every cell type that populates the gland, papillary thyroid cancer accounts for the majority of slow growing, well-differentiated thyroid malignancies. While papillary tumors tend to be locally invasive and are associated with a good prognosis, follicular thyroid cancers are associated with a higher prevalence of metastatic disease and a poor prognosis (46).

KISS1 mRNA expression was evaluated in clinical samples of follicular carcinoma and papillary carcinoma. Consistent with a suppressor of metastasis (although not measuring it directly), Ringel *et al.* found that papillary carcinomas were more likely to express *KISS1* than follicular carcinoma (69% vs. 20%, $p < 0.05$) (30). Since information pertaining to the clinical stage of the specimens used was not provided, an evaluation of *KISS1* expression as a prognostic indicator in patients with well differentiated thyroid neoplasms cannot be inferred with certainty.

8.3. Bladder cancer

Bladder cancer is the fourth most common cancer in men and the tenth most common in women. Of the >60,000 new cases diagnosed in 2004, the vast majority (95%) were of transitional cell origin. The single most relevant prognostic indicator is colonization of lymph nodes, in which 5-year survival drops to 10–20%. Further precipitous decreases in survival occur if tumor cells colonize bone or other viscera.

KISS1 mRNA expression was evaluated in a cohort of superficial and invasive bladder neoplasms. Sanchez-Carbayo *et al.* found that *KISS1* expression was inversely correlated with tumor stage and overall survival (39). Interestingly, every bladder tumor that had developed distant metastases showed a complete loss of *KISS1* expression. Again, the results are consistent with a role of *KISS1* as a metastasis suppressor.

8.4. Esophageal squamous cell carcinoma

Squamous cell carcinoma and adenocarcinoma of the esophagus is extremely lethal and kills almost as many people a year as are diagnosed. Currently, esophagoscopy is required for staging and prognosis, while CT and endoscopic ultrasound are used to detect metastatic disease. Most current therapies are primarily palliative in nature, with fewer than 5% of patients alive after 5 years. Recent utilization of surgery followed by radiation and combination chemotherapy has shown some benefit, however.

KISS1 mRNA expression was evaluated in esophageal squamous cell carcinoma (ESCC). Ikeguchi *et al.* found that *KISS1* expression was lost in 38% of ESCC tumors and was not correlated with tumor size or degree of tumor invasion (38). Like melanoma and bladder cancer, *KISS1* expression was lost late in the progression of ESCC and was directly correlated with the likelihood of lymph node metastases and a subsequent poor prognosis. Loss of *KISS1* expression in ESCC may serve to augment current staging as well as improve current prognostication.

8.5. Gastric cancer

Despite a slow, steady decline in incidence, gastric cancer in the United States still accounted for 11,000 deaths in 2004. The disease is significantly more common in Asia. The majority of gastric cancers are adenocarcinomas and endoscopic biopsies are required for staging and prognosis. Five-year survival rates are 90% when the cancer is limited to the mucosa but survival drops to 3% once metastatic disease has been detected. Surgical resection is possible in only a third of patients but, in those patients, a prolongation of survival and a reduction in the recurrence rates can be observed. However, in patients with advanced gastric carcinoma, partial responses have only been observed in 30–50% of cases after the use of combination chemotherapy while radiation therapy is predominantly palliative in nature.

Dhar *et al.* found that gastric cancers with low *KISS1* mRNA had frequent venous invasion, distant metastases, tumor recurrence and a significantly worse overall and disease-free survival (37). To date, *KISS1* appears to be the strongest independent prognostic indicator for gastric cancer, suggesting that determining *KISS1* expression in surgical biopsies should be considered. Although the effect of *KISS1* restoration in gastric cancer metastases has not been examined, its potential as a molecular target for the treatment of metastatic disease in patients with gastric cancer should not be overlooked.

8.6. Hepatocellular carcinoma

Globally, hepatocellular carcinoma (HCC) is one of the most common tumors and is the third most common cause of cancer deaths. Staging and clinical course is based on the Okuda system

which is determined by tumor size, presence of ascites, bilirubin levels, and albumin levels. Surgery is the best form of treatment before symptoms appear. But after symptoms are apparent, survival drops precipitously and current treatments offer little improvement.

KISS1 mRNA expression was evaluated in HCC. Ikeguchi *et al.* did not find any significant changes in *KISS1* expression between normal liver and HCC samples (47). The lack of correlation with disease cannot be determined because the authors did not include the stage of the clinical specimens examined nor did they report on liver metastases.

8.7. Breast Cancer

Breast cancer is the second most common cause of cancer death in women. Tumors arise primarily from the epithelial cells lining the ducts or lobules. The most important prognostic indicator is lymph node involvement after biopsy. Once metastatic disease is detected, 5-year survival rates drop from greater than 90% to 14%. Nearly half of all breast cancer patients treated for localized disease develop metastatic disease and combinations of local therapy and systemic therapy are not curative.

In a single small study, *KISS1* mRNA expression was significantly reduced in all breast cancer brain metastases examined when compared to primary tumor expression levels (40). The results, while promising, are inconclusive with regard to overall prognostic utility of *KISS1*.

9. GPR54/KISSPEPTIN IN NORMAL PHYSIOLOGY

Although the above studies suggest the possibility that GPR54/kisspeptin signaling could be important in metastasis suppression in human cancers, nothing about their function points to a mechanism *a priori*. Some recent data showing a role for both molecules in normal physiology may provide important insights into the mechanism of *KISS1* in cancer metastasis. However, questions still persist regarding how this receptor-ligand pair, whose physiologic function is to control the hypothalamic-pituitary axis in puberty and pregnancy, regulates metastasis.

Co-expression of human *KISS1* and *GPR54* mRNAs is greatest in the placenta with a wide distribution throughout the brain including the hypothalamus and basal ganglia (15). Co-expression was also seen, but at lower levels, in the pancreas, kidney, liver, lung, prostate and small intestine (8,15,16). The predilection for co-expression of *KISS1* and *GPR54* mRNA in neuroendocrine tissues such as placenta, pancreas, and hypothalamus is consistent with sequence similarities between *KISS1* and other neuropeptides. These similarities to neuropeptides, as well as its tissue localization, may prove to be invaluable in determining the mechanism of *KISS1* metastasis suppression. We will review what is known about the physiological relationship of *KISS1* and *GPR54* signaling in the placenta and brain and how this may pertain to metastasis suppression.

Since the identification of high *GPR54* and *KISS1* expression in the placenta, several papers implied that GPR54/kisspeptin signaling may regulate placental invasion. Horikoshi *et al.* identified elevated levels of KP54 in the serum of pregnant women suggesting that KP54 may be a placental derived hormone (23). Janneau *et al.* identified that *KISS1* and *GPR54* expression levels can be detected in trophoblast cells, increase in early pregnancy and are lost in choriocarcinoma (48). These data were the first to implicate *KISS1*/kisspeptins and *GPR54* in the potential regulation of the invasive properties of trophoblast cells. Bilban *et al.* identified higher *KISS1* and *GPR54* expression in first trimester (high invasiveness) trophoblasts than in term placentae (no/low invasiveness). Both *KISS1* and *GPR54* mRNA and protein were found in the non-invasive syncytiotrophoblast compartment of the placenta; whereas, *GPR54* was also found in the invasive extravillous cytotrophoblast (18). The tissue compartmentalization

in placenta could suggest autocrine, paracrine or endocrine interactions. Although, this data does not directly address GPR54/kisspeptin signaling in placental invasion, the proximity and compartmentalization is alluring. Since placental invasion is often considered to parallel metastasis it is plausible that GPR54/KISS1 signaling may be an important regulator of metastasis through its anti-invasive effects. However, the data are confusing with regard to a role in tumor cell invasion. In experimental systems, expression of KISS1 did not suppress invasion, while metastasis was suppressed (8,16,33,38).

While the data strongly support that KISS1 is a ligand for GPR54, the mechanism(s) by which KISS1 suppresses metastasis may vary depending upon which cell expresses either (or both) molecule. It is unclear whether low levels of GPR54 are sufficient to support KISS1 metastasis suppression via autocrine or intracrine signaling (Figure 2). Both C8161 and MDA-MB-435 express extremely low levels of GPR54 yet are suppressed by KISS1 in an expression-dependent manner. This concern is mitigated by the relative potency of other G-protein coupled receptors despite their modest expression.

One cannot rule out the possibility that KISS1 suppression can also be mediated by paracrine or endocrine interactions with GPR54 on the surface of stromal cells (Figure 2). It is also plausible that KISS1 is capable of inhibiting metastases through receptor interactions other than GPR54 since RF-NH₂ amides are common peptide motifs found ubiquitously throughout the animal kingdom (49).

Since GPR54 and KISS1 expression overlap in many areas throughout the brain it was only a matter of time before these two genes were implicated in a physiological function. In 2003, GPR54 was implicated as an important regulator of puberty in both mice and humans (50–52). Mutations in GPR54 led to isolated idiopathic hypogonadism in which patients lack pubertal development. These same effects were observed in GPR54 knockout mice (51,52). Over the last two years, a surge of papers have emerged suggesting that the interaction of GPR54 and KISS1 play a pivotal role in the onset of puberty. Kisspeptin administration has been shown to induce LH and FSH surges in rodents and primates (53–61). These effects are seen despite the mode of injection (intravenous, intraperitoneal, intracerebroventricular). The LH and FSH surge following kisspeptin exposure can be abolished with a GnRH antagonist, suggesting that kisspeptin's activity is mediated by GnRH neurons. GnRH neurons have been shown to express the GPR54 receptor and kisspeptin administration induces c-fos activity (54,55). Interestingly, KISS1 expression is localized in areas of the hypothalamus that play a role in the regulation of neuroendocrine secretion (62). Lessons learned from kisspeptin/GPR54 signaling in the brain would support the possibility that kisspeptins binding to receptors on stromal cells in the microenvironment may regulate the secretion of peptides in a similar fashion as is seen in GnRH neurons (Figure 2). These stromal peptides could then exert an anti-proliferative effect on metastatic cells rendering them in a state of dormancy. Secreted kisspeptins or stromal peptides could also be deposited in the matrix of the microenvironment. These peptides could provide cues to metastatic cells in the vicinity rendering them in a dormant state (Figure 2). It is also possible that restoration of KISS1 expression in metastatic cells may blunt the response to growth factors released in the microenvironment rendering them incapable of proliferating to form metastatic lesions. Whatever the mechanism(s) is(are) required for *KISS1* metastasis suppression, the interplay between *KISS1* and the microenvironment is likely to be important.

10. PERSPECTIVE

Despite the advances that have been made since KISS1 was discovered 9 years ago, a mechanistic understanding of KISS1 metastasis suppression is still elusive. Several important

questions remain to be addressed in order to determine how KISS1 metastasis suppression may be exploited in the clinic and the types of patients that may receive a benefit.

10.1. Is KISS1 secretion required for metastasis suppression?

If secretion is required, exogenous administration of naturally occurring kisspeptins could be envisioned as a potential therapy for the treatment of metastatic disease avoiding the complications associated with gene therapy. Additionally, research can be focused on evaluating kisspeptin signaling through GPR54 as well as identify other novel receptors that may play a role in the prevention and treatment of metastatic disease. This research will provide a greater mechanistic understanding of KISS1 metastasis suppression that may lead to the identification of additional downstream targets that can be exploited for metastatic therapy.

10.2. Is KISS1 metastasis suppression organ and tissue specific?

Currently, KISS1 has only been investigated as a lung and lymph node metastasis suppressor for melanoma and breast cancer. It will be interesting to determine if KISS1 is a universal metastasis suppressor capable of inhibiting metastases to multiple organs throughout the body regardless of the cancer origin. Since metastatic disease can be observed in multiple organs throughout the body, an ideal molecular target will prevent metastatic growth in any location regardless of the cancer origin. The clinical utility of KISS1 will depend on the number of organs sites where metastases can be suppressed as well as the number of cancer types this suppression is limited to.

10.3. Will KISS1 suppress progression of already disseminated cells?

If KISS1 can inhibit the proliferation of larger established metastatic lesions as well as occult metastases (less than a few millimeters), its clinical potential will be more substantial. Since restoration of KISS1 expression can maintain single disseminated metastatic cells in a state of dormancy, it is possible that adjuvant KISS1 treatment in patients with a high likelihood of occult metastatic disease may prove beneficial. The effects of restoring KISS1 expression in larger metastatic lesions has not been addressed experimentally. Therefore, the possible benefit of using KISS1 treatment in patients with large clinically detectable metastases is uncertain.

Despite many of the unresolved questions, KISS1 remains a promising molecular target for the treatment of metastatic disease and has shown great promise as a prognostic indicator for several cancers. However, greater efforts need to be made in the characterization of KISS1 metastasis suppression before its clinical value can be determined.

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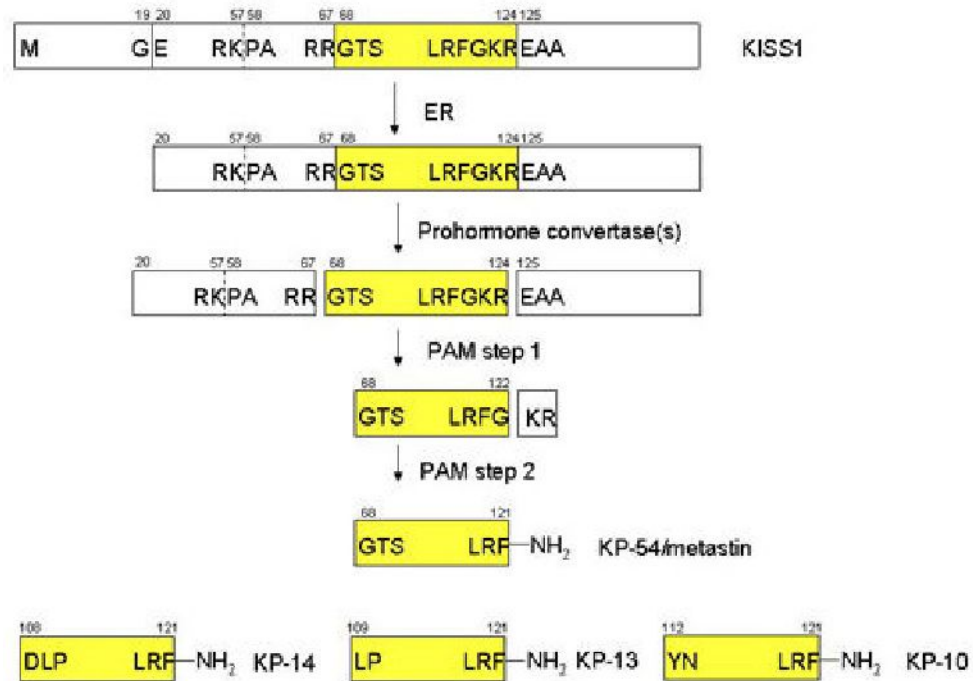


Figure 1.

Proposed model for KISS1 protein processing by prohormone convertases. KISS1 is initially targeted to the ER by the secretion signal sequence. Once KISS1 leaves the trans Golgi, the R⁶⁶-R and GK¹²³-R cleavage sites are likely to be recognized and processed in acidic secretory vesicles by furin (family) or other prohormone convertases. K⁵⁶-R is not likely to be recognized and processed because cleavage at K-R dibasic motifs rarely occurs. Peptidyl-glycine- α -amidating monooxygenase (PAM) removes the newly exposed C-terminal basic residues K-R¹²⁴ and converts the C-terminal G¹²² to an amide producing metastin/KP54 (KP54). The production of kisspeptins 14 (KP14), 13 (KP13), and 10 (KP10) do not have any known processing sites that would lead to their creation. Whether these smaller kisspeptins occur from processing by an unknown enzyme or are a byproduct of spontaneous degradation remains to be seen.

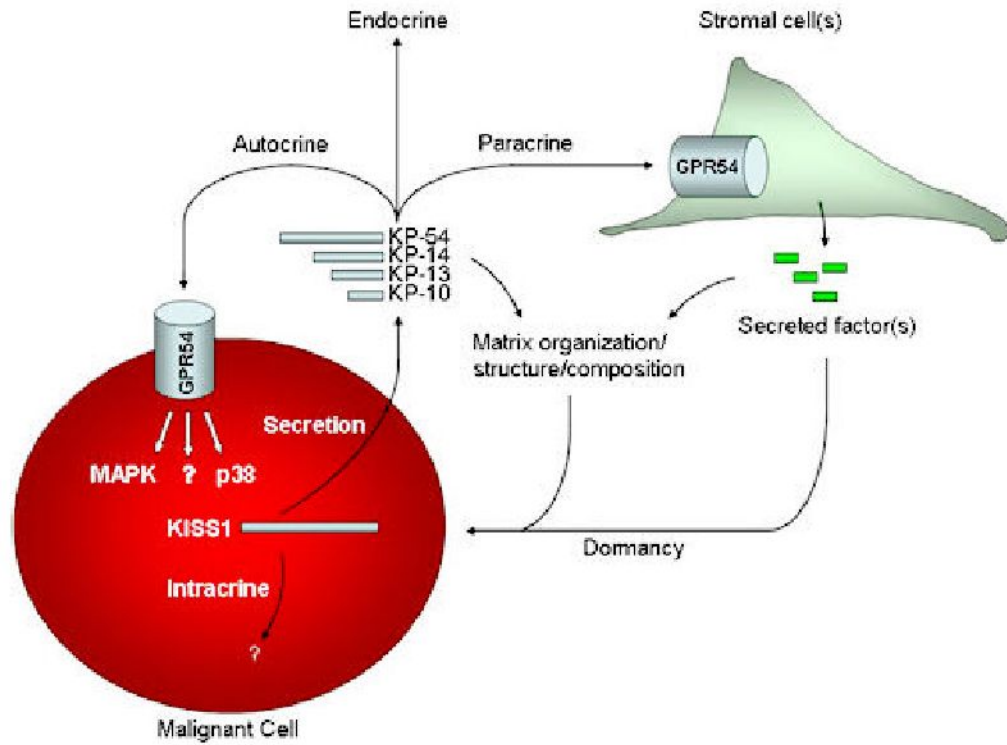


Figure 2. Schematic representation of the potential mechanism(s) that *KISS1* may employ to suppress metastatic proliferation at the secondary site. Experimental evidence to date suggests that *KISS1* may accomplish this in several ways. First, unprocessed KISS1 may interact with other proteins in the cell using an intracrine mechanism. Second, secreted kisspeptins could signal back to the metastatic cell through its interaction with GPR54 in an autocrine loop. Third, secreted kisspeptins could interact with other unknown cells and organs distant from the metastatic cells in an endocrine fashion that in turn may inhibit metastatic proliferation. Fourth, secreted kisspeptins could interact with GPR54 on the surface of stromal cells (e.g. fibroblasts, macrophages, dendritic cells, epithelial cells, *etc.*) regulating the secretion of peptides that have an anti-proliferative effect on metastatic cells. Finally, secreted kisspeptins or stromal peptides induced by kisspeptins may deposit in the matrix altering its organization, structure, or composition in such a way that provides an anti-proliferative signal to metastatic cells in the vicinity.