

Published in final edited form as:

Oncogene. 2012 April 5; 31(14): 1781–1793. doi:10.1038/onc.2011.376.

Mtss1 regulates epidermal growth factor signaling in head and neck squamous carcinoma cells

John C. Dawson^{*}, Paul Timpson, Gabriela Kalna, and Laura M. Machesky^{*}

Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, Scotland, UK

Abstract

Mtss1 is located within chromosomal region 8q23-24, which is one of the three most commonly amplified regions in HNSCC. Mtss1 is lost in metastatic cells but confusingly is commonly overexpressed in primary tumors. Here we address possible reasons why Mtss1 is positively selected for in primary tumors. We find that Mtss1 enhances the localization of the EGF receptor to the plasma membrane, prolonging EGF signaling and resulting in enhanced proliferation in HNSCC. Depletion of Mtss1 results in decreased EGF receptor levels and decreased phosphorylation of Erk1/2 and Akt. However, when cells are at high density and adherent to each other, analogous to conditions in a solid tumor, Mtss1 does not confer any growth advantage, either in basal conditions or following EGF stimulation. This could indicate why Mtss1 might be lost in metastases, but preserved in early primary tumors. This is supported by an organotypic assay showing that Mtss1 expressing cells display a less proliferative more epithelial-like morphology on top of a collagen matrix. Furthermore, xenograft tumors expressing Mtss1 initially grow more rapidly, but later show less proliferation and more differentiation. Mtss1 positively modulates EGF signaling at low cell densities to promote proliferation and, therefore, may be beneficial for the early stages of primary HNSCC tumor growth. However, at high cell densities, Mtss1 impacts negatively on EGF signaling and this suggests why it inhibits metastasis.

Keywords

Mtss1; HNSCC; epidermal growth factor receptor

Introduction

Regulation of growth factor signaling through receptor tyrosine kinases (RTK) is often disrupted in the development of cancer leading to uncontrolled proliferation, survival and metastasis. In head and neck squamous cell carcinoma (HNSCC), cells undergo numerous chromosomal deletions and amplifications that result in loss of key cellular regulatory proteins and amplification of oncogenic proteins (Cooper and Cohen, 2009; Kelley *et al.*, 2008). This often results in amplification of the EGF receptor (EGFR) and misregulation of EGF signaling, internalization and degradation making EGFR a strong target for chemotherapeutic strategies (Cooper and Cohen, 2009; Mosesson *et al.*, 2008). Therefore, understanding how signaling through EGFR is altered is of great interest and will be useful for the diagnosis and treatment of HNSCC.

^{*}Authors for correspondence j.dawson@beatson.gla.ac.uk l.machesky@beatson.gla.ac.uk.

No conflict of interest.

EGF binding to the EGFR results in internalization via clathrin-dependent and independent endocytosis routes (Goh *et al.*, 2010; Sigismund *et al.*, 2008). Once endocytosed, vesicles are trafficked to early endosomes for cargo sorting where the EGFR is either targeted for degradation in lysosomes or recycled back to the plasma membrane. During this translocation from the plasma membrane to the endosomal system the EGFR still signals to its downstream targets. Switching off EGFR signaling by degrading the receptor requires its targeting to lysosomes by receptor ubiquitination (Mosesson *et al.*, 2008; Sorkin and Goh, 2009). Critically, the disruption of this endocytic process in cancer results in reduced lysosomal targeting of receptors for degradation and prolonged signaling of classical kinase pathways such as Erk and Akt and leads to proliferation, increased survival and invasion.

During tumor progression, cancer cells undergo changes, including increased RTK signaling, that result in the ability to break off from the tumor mass, invade and metastasize to distant sites of the body (Mosesson *et al.*, 2008). This process is known as epithelial to mesenchymal transition (EMT) and involves the loss or mutation of the E-cadherin receptor, the major cadherin found in epithelia (Cheng *et al.*, 2007; Gan *et al.*, 2010; Yang *et al.*, 2008). Loss of E-cadherin results in the ability of cancer cells to break away from the tumor (Cheng *et al.*, 2007; Yang *et al.*, 2008). In addition to the mechanical role of physically anchoring cells in an epithelium, E-cadherin cell-cell junctions are also involved in negatively regulating RTK signaling (Bremm *et al.*, 2008; Qian *et al.*, 2004).

Mtss1 was originally identified as 'missing in metastasis' (MIM; MIM-B) in bladder cancer (Lee *et al.*, 2002) but also resides on 8q23-24, which is frequently amplified in HNSCC (Kelley *et al.*, 2008). Loss of Mtss1 expression in hepatocellular carcinoma, breast and gastric cancers is also associated with metastasis and correlates with poor patient survival (Callahan *et al.*, 2004; Liu *et al.*, 2010; Loberg *et al.*, 2005; Parr and Jiang, 2009). Interestingly, in hepatocellular carcinoma, Mtss1 is upregulated at the early stages of disease but missing in the later metastatic stages (Ma *et al.*, 2007). Similarly, Mtss1 has also been identified as a sonic hedgehog responsive gene (BEG4) in the development of basal cell carcinoma that, in concert with the transcription factor Gli, drives the proliferation and invasion of epidermal cells in regenerated skin assays (Callahan *et al.*, 2004). Why Mtss1 is overexpressed during the early development of cancer and then lost during the later metastatic stages is unclear.

Mtss1 is a scaffolding protein linking the actin cytoskeleton and the plasma membrane (Bompard *et al.*, 2005; Gonzalez-Quevedo *et al.*, 2005; Lee *et al.*, 2007; Lin *et al.*, 2005; Mattila *et al.*, 2007; Mattila *et al.*, 2003; Woodings *et al.*, 2003). Through this ability, Mtss1 antagonizes EGFR endocytosis during *Drosophila* oocyte border cell migration (Quinones *et al.*, 2010) and regulates signaling during ciliogenesis suggesting Mtss1 might influence EGFR signaling in cancer. We therefore decided to investigate the role of Mtss1 expression in HNSCC. We find that Mtss1 is differentially expressed in a panel of HNSCC cell lines and is upregulated in HNSCC primary tumor samples. We show that enhancing Mtss1 expression in HeLa or Scc9 cells, a HNSCC cell line with low expression of Mtss1, results in enhanced localization of the EGFR to the plasma membrane, enhancing EGF signaling and proliferation. We further show that depletion of Mtss1 in three HNSCC cell lines reduces the expression of the EGFR and decreases Erk1/2 and Akt phosphorylation. Finally, we find that Mtss1 enhances cell density dependent inhibition of EGF signaling, which requires cell-cell junctions. In organotypic invasion and mouse xenograft assays this results in reduced proliferation. These data suggest that Mtss1 regulates the EGFR and can enhance proliferation of early stage HNSCC cells/tumors. However, our data also suggests that Mtss1 inhibits EGFR signaling at high cell densities. This observation may explain why Mtss1 is overexpressed in early primary tumor development but is lost as disease progresses.

Materials and Methods

Western blotting and immunoprecipitation methods and a list of antibodies used are listed in Supplementary Methods.

All reagents were from Sigma-Aldrich (UK) unless otherwise stated. Cell culture reagents were from Gibco, Invitrogen. Mtss1 was cloned into the BamHI and EcoRI sites of pEGFP-N1 (Clontech) using standard molecular biology methods. Mtss1 siRNA was from Qiagen (Hs_Mtss1_5, target sequence, 5'-CCGACGGATGTTCCAAGCCAA-3'; E-cadherin, Hs_CDH1_13, target sequence, 5'-TCGGCCTGAAGTGAAGTCTCGTAA-3'; Hs_CDH12, target sequence, 5'-CTAGGTATTGTCTACTCTGAA-3'). Statistical significance was calculated using an unpaired T-test unless otherwise stated.

Cell lines and culture

The HNSCC cell lines (Scc9, FaDu and Detroit 562) were a kind gift of R. Daly (Garvan Institute, Sydney, Australia). HeLa cells were from ATCC. All cell lines were maintained as described on the ATCC website and were authenticated using the Amp/STR identifier kit and results were analyzed using the Genemapper software v4.0 (both from Applied Biosystems). Stable Scc9 cells expressing Mtss1-GFP were selected in 0.5 mg/ml G418 and cultured as a mixed population. Cells were transfected with DNA and siRNA using Polyfect and Hyperfect (Qiagen) following the manufacturers protocols. The organotypic invasion assay is described in (Edward *et al.*, 2005) and a brief description can be found in Supplementary Methods. For the clonogenic assay, 2000 cells/ well of a 6-well plate were seeded and cultured for 5 d. Cells were washed and labeled with coomassie to visualize the colonies and the area of colony growth was calculated in imageJ using the thresholding tool.

Animals and in vivo experiments

All animal procedures were carried out in accordance with UK Home Office regulations. Scc9 xenografts were made by injection of 2×10^6 cells in 100 μ l PBS/Matrigel (1:1) into the flanks of nude mice, using 6 animals per cohort. Tumors were measured with calipers at indicated times and animals were humanely sacrificed at 6 weeks where tumors were excised and processed for histology.

Surface biotinylation experiments

Cells were seeded at 3×10^5 cells per well of a 6-well plate and starved overnight in 0.1% normal growth media. Cells were stimulated with EGF and washed once with ice-cold PBS and incubated with 0.5 mg/ml EZ-Link Sulfo-NHS-Biotin (Pierce, cat. number 21326) in ice-cold PBS for 15 minutes to label the surface proteins. The reaction was quenched using 50 mM ammonium chloride/PBS and cells lysed in RIPA buffer containing Halt protease inhibitors (Pierce) and phosphatase inhibitors (Roche phosphatase inhibitor cocktail II). Debris and insoluble material was removed by centrifugation at $12,000 \times g$ for 10 minutes and the supernatants were diluted to equal concentrations and 200 μ g protein was incubated with 20 μ l of NeutrAvidin beads (Pierce) to capture biotinylated proteins for 2 hours. Beads were washed in lysis buffer 3 times and boiled in 2x SDS-PAGE sample buffer. Expression of EGFR and E-cadherin were analyzed in the surface and total cell lysate fractions.

FACS analysis

Scc9 and Mtss1-GFP expressing Scc9 cells were incubated with PBS/EDTA to remove them from the tissue culture dishes. 5×10^5 cells were centrifuged and resuspended in 100 μ l of 4% paraformaldehyde in PBS for 10 minutes to fix the cells. 1 ml of FACS buffer (5% goat serum in PBS) was then added to the cells followed by 2 washes in FACS buffer. Cells were then resuspended in 100 μ l of primary antibody (mouse anti-EGFR) diluted 1:1000 in FACS

buffer and incubated for 30 minutes. Cells washed three times before incubating with Alex-594 anti-mouse secondary antibody (1:500) for 30 minutes. Finally, the cells washed three times and resuspended in 1 ml of FACS buffer and analyzed using a BD FACSAria. Unlabelled cells were used as a reference and 10,000 cells were analyzed for each condition.

Analysis of *Mtss1* expression in primary HNSCC tumors

Affymetrix U133Plus2 CEL files of 71 HNSCCs and 14 normal epithelium samples were normalized and analyzed in Partek® Genomics Suite Software, version 6.5beta Copyright © 2009. RMA normalization and log₂ transformation of the data was followed by t-test, multiple test corrections of p-values (step-up) and Cox survival analysis. The cohort's clinical characteristics can be found in (Thurlow *et al.*, 2010).

Results

Mtss1 is overexpressed in HNSCC cell lines and primary tumors

Mtss1 is found within an amplicon on chromosome 8q23-24 that is commonly amplified in HNSCC (Kelley *et al.*, 2008). To confirm this observation we analyzed *Mtss1* expression in a microarray data set of primary HNSCC tumors (Figure 1A and B) (Thurlow *et al.*, 2010). We found *Mtss1* to be significantly overexpressed, by 1.3-1.5 fold, in primary HNSCC tumor samples (3 out of 4 probes from 71 HNSCC tumors Vs 14 normal tissue samples). We find that in a panel of HNSCC cell lines *Mtss1* expression is approximately 2.5 fold higher in FaDu and Detroit 562 HNSCC cell lines than Scc9, Scc15 or Scc25 (Figure 1C). We began by stably expressing *Mtss1*-GFP in Scc9 cells to approximately 3 fold over the endogenous level (Figure 1D) and comparable to that seen in FaDu and Detroit 562 cell lines.

Mtss1 alters the cellular localization of the EGFR and increases signaling in HNSCC

Mtss1 has previously been shown to regulate the surface dynamics of the EGFR in *Drosophila* and mouse fibroblasts (Quinones *et al.*, 2010). Therefore, we investigated whether *Mtss1* expression could influence EGFR dynamics in HNSCC cells. By FACS analysis, *Mtss1*-GFP expressing Scc9 cells had more cell surface EGFR compared to Scc9 cells alone (Figure 2A). Analysis of high and low *Mtss1*-GFP expressing Scc9 cells revealed that high expressing *Mtss1*-GFP cells displayed significantly more EGFR on the cell surface than low *Mtss1*-GFP expressing cells (Figure 2B and C).

To compare EGFR internalization rates in high and low *Mtss1* expressing cells, we biotinylated the cell surface proteins during a short time course of EGF stimulation and measured the surface expression versus the total EGFR expression (Figure 2D). In Scc9 cells, EGFR internalization commenced within minutes of EGF stimulation and by 30 minutes approximately 50% of the initial EGFR had been internalized. *Mtss1* expressing cells displayed approximately 1.4-fold increased surface expression of the EGFR at t=0 (Figure 2D). Upon EGF stimulation, surface EGFR levels decreased at a rate similar to controls (Figure 2D). We did not observe any change in surface expression of E-cadherin under these conditions (Figure S1). Thus, the increased surface level of EGFR in high *Mtss1* expressing cells is unlikely to be due to a decrease in ligand-induced internalization rates.

Mtss1 enhances EGF signaling in HNSCC cell lines

We asked whether increased surface levels of EGFR affected EGFR signaling in *Mtss1*-GFP expressing Scc9 cells (Figure 3A and B). EGF stimulation induced a rapid phosphorylation of the EGFR over a 45 minute period with markedly more sustained EGFR phosphorylation in *Mtss1*-GFP expressing Scc9 cells (Figure 3A). Following EGF stimulation, Erk1/2 phosphorylation in Scc9 cells peaked at 10 minutes and gradually decayed over the 240

minutes. High Mtss1 expressing cells showed increased Erk1/2 phosphorylation by 10 minutes after EGF stimulation and a subsequent slower decay was observed. In support of these experiments in Scc9 cells, we find that high expression of Mtss1 in HeLa cells also drives a decrease in EGFR degradation following ligand stimulation and a concurrent increase in Erk1/2 phosphorylation (Figure S2). Erk1/2 activity controls cell proliferation and high Mtss1 expression resulted in increased proliferation over 5d in culture of Scc9 cells in the presence of 10 ng/ml EGF in an MTT proliferation assay (Figure 3C). Finally we determined the effect of Mtss1-expression on the ability of Scc9 cells to form and grow as colonies in a clonogenic assay. Mtss1 expression in Scc9 cells gave a significant growth advantage over Scc9 cells alone (Figure 3D) demonstrating that Mtss1 can increase proliferation.

Depletion of Mtss1 reduces EGFR expression and downstream signaling in HNSCC

FaDu and Detroit 562 HNSCC cell lines, which have high endogenous Mtss1 (Figure 1C), exhibit high aberrant EGFR signaling (Timpson *et al.*, 2005; Timpson *et al.*, 2007). We therefore depleted Mtss1 to examine the connection with EGFR signaling (Figure 4). Depletion of Mtss1 in FaDu and Detroit 562 HNSCC cell lines reduced the basal levels of EGFR protein (Figure 4A). Erk1/2 and Akt phosphorylation were also reduced (Figure 4A). Interestingly, Scc9 cells responded similarly when treated with Mtss1 siRNA, displaying reduced EGFR protein and basal Erk1/2 and Akt phosphorylation (Figure 4A and B).

Mtss1 expression regulates HNSCC proliferation in organotypic invasion and mouse xenograft assays

Mtss1 has been proposed as a suppressor of metastasis, however our observations that Mtss1 can enhance EGF signaling seem puzzling in light of this. We therefore investigated how Mtss1 affected the ability of Scc9 cells to grow and invade in an organotypic invasion assay (Figure 5). After 10d of growth, we observed an increased epithelial-like morphology of Mtss1-GFP expressing cells on the top of the collagen matrix (Figure 5A, enlarged pictures, panels 1,3). However, we observed no significant difference in the invasion of Scc9 cells regardless of Mtss1-GFP levels (Figure 5A and B).

However, we noticed that the high Mtss1 expressing cells formed larger colonies of invaded cells in the collagen matrix (Figure 5A, panels 2 and 4, and 5C). As we have shown that Mtss1 expression increases proliferation through EGF signaling in Scc9 cells (Figure 3), we labeled the organotypic assays for Ki67, a marker of proliferation. For the colonies of invaded cells, controls and high Mtss1 expressing cells showed no difference (Figure 5D-F), but high Mtss1 expressing cells displayed significantly less Ki67 staining in the epithelial-like layer on top of the collagen (Figure 5 D-F). The Mtss1 expressing cells on top of the collagen also frequently formed more orderly layers than the Scc9 cells and appeared more epithelial. These results suggested that the cell density might affect EGF signaling and thus proliferation induced by Mtss1.

To determine the effect of high Mtss1 on Scc9 tumor formation in vivo, we used a subcutaneous transplant model in nude mice (Figure 6). Mtss1 expressing Scc9 tumors grew faster over the initial 12d period following implantation (Figure 6A), however, by 20d after injection, we observed no significant difference, suggesting that Mtss1 conferred a growth advantage during the initial stages of tumor formation but not at high densities as tumors grew (Figure 5). Immunohistochemical analysis revealed that Mtss1 tumors (5 of 5) had undergone a large degree of keratinisation (Figure 6B), while only (2 of 6) Scc9 tumors showed keratinisation. Ki67 labeling revealed that there was a significant reduction in proliferation in Mtss1 tumors compared to Scc9 alone (Figure 6C), confirming our previous observation in the organotypic assay (Figure 5). Thus, in vivo high Mtss1 expression may

confer a growth advantage at low densities during tumor initiation, but once a tumor is established, high Mtss1 rather leads to less proliferation in favour of differentiation in HNSCC tumors.

Mtss1-induced signaling is dependent on cell-cell junctions

Increasing cell density reduces RTK signaling through E-cadherin cell-cell junctions (Bremm *et al.*, 2008; Qian *et al.*, 2004) so we investigated whether Mtss1 expression increased susceptibility to cell density-induced inhibition of RTK signaling. Cells were grown to confluence over 1-3d and lysates were harvested each day to examine the activation of Erk1/2 and Akt (Figure S3 A and B). At day 1-2, Erk1/2 phosphorylation was significantly higher in Mtss1 expressing cells than Scc9, but by day 3 when cells were confluent the difference was not significant (Figure S3 A and B). Both Erk1/2 and Akt phosphorylation decreased in Mtss1 expressing and control cells as confluence increased (Figure S3A and B).

To test whether cell-cell junctions contributed to this change in EGF signaling, we analyzed EGF signaling in confluent cells with and without cell-cell junctions (Figure 7). Cell-cell junctions were disassembled by starving cells overnight in a low $[Ca^{2+}]$ medium (Qian *et al.*, 2004) or by prior treatment with E-cadherin siRNA (Figure S3C). In the presence of cell-cell junctions, 10 minutes of EGF stimulation induced a greater increase in EGFR phosphorylation in Scc9 cells compared to those expressing Mtss1 (Figure 7A). In the absence of cell-cell junctions, more EGFR phosphorylation was observed in Mtss1 expressing cells, supporting the hypothesis that in confluent cells making junctions high Mtss1 has a growth inhibitory effect, while at low densities or in the absence of junctions it can promote EGFR signaling and growth.

We next performed a longer time course of Erk1/2 and Akt signaling in cells with or without junctions in the presence or absence of high Mtss1 (Figure 7 B-E). Confluent cells with junctions expressing high Mtss1 showed a modestly but consistently lower level of ERK1/2 phosphorylation than Scc9 controls (Figure 7B). This effect was reversed in cells with disassembled cell-cell junctions or E-cadherin siRNA treatment, where Erk1/2 became rapidly phosphorylated in both Scc9 cells alone and Mtss1-GFP expressing cells (Figure 7C and Figure S3C). Confluent cells with disrupted junctions (Figure 7C) do not have as robust of an increase in pERK1/2 as subconfluent cells (Figure 3) suggesting that other factors likely contribute to the density dependent inhibition of signaling in addition to adherence junctions. Further, in the absence of cell-cell junctions, we observed a reduction of the total EGFR degraded in Mtss1-GFP expressing cells at both 90 and 120 minutes following EGF stimulation compared to Scc9 cells alone (Figure 7C) in agreement with our findings in HeLa cells (Figure S2).

We also measured the activation of Akt following EGF stimulation in the presence or absence of cell-cell junctions as Akt activation is thought to drive invasion and metastasis (Irie *et al.*, 2005). We observe reduced Akt phosphorylation in Mtss1 overexpressing cells with cell-cell junctions over the period of a 120-minute EGF stimulation (Figure 7D). In the absence of cell-cell junctions or E-cadherin siRNA, Akt phosphorylation is delayed (Figure 7E and Figure S3C). Therefore, high Mtss1 expressing cells are more sensitive to cell-cell junction induced-inhibition of EGF signaling. Finally, as we observed dramatic effects on Akt activation, a signaling pathway that controls survival, we investigated the link between Mtss1 expression in HNSCC and sensitivity to EGFR chemotherapy (Figure 7F). Mtss1 expression in Scc9 cells significantly increased the sensitivity to both Gefitinib and Erlotinib, two EGFR kinase inhibitors indicating a potential growth factor addiction in HNSCC overexpressing Mtss1.

Discussion

Numerous chromosomal alterations result in dysregulation of EGFR and other RTK signaling, which drives the development of HNSCC (Chung *et al.*, 2006). 8q23-24 contains *c-myc*, FAK, autotaxin and *Mtss1* (Canel *et al.*, 2006; Hsia *et al.*, 2003; Kelley *et al.*, 2008; Lin *et al.*, 2006) making it an important amplification during tumor development. *Mtss1* is a relatively uncharacterized member of this chromosomal region and while *Mtss1* has been widely shown to be lost during metastasis (Lee *et al.*, 2002; Lin *et al.*, 2005; Liu *et al.*, 2010; Loberg *et al.*, 2005; Parr and Jiang, 2009) it is also overexpressed in the early stages of hepatocellular carcinoma and basal cell carcinoma (Callahan *et al.*, 2004; Ma *et al.*, 2007).

Here we find here that *Mtss1* is overexpressed in primary HNSCC tumors and that *Mtss1* regulates EGF signaling in two distinct ways depending upon the cell density by regulating the localization of the EGFR to the plasma membrane. We show that this activity regulates cell proliferation and is beneficial for the initial growth of a tumor but rapidly becomes detrimental to EGF signaling.

Mtss1 enhances EGF signaling at low cell density and in the absence of cell-cell junctions

We found that *Mtss1* overexpression in HeLa and Scc9 HNSCC cells resulted in enhanced EGF signaling via elevated Erk1/2 phosphorylation to increase cell proliferation. In agreement with this, *Mtss1* is a sonic hedgehog responsive gene expressed during the development of basal cell carcinoma and enhances epidermal proliferation and invasive ingrowths (Callahan *et al.*, 2004).

The mechanism by which *Mtss1* controls EGFR localization on the cell surface is not understood. *Mtss1* is a member of the IMD family of proteins (also known as I-BAR) and the BAR domain family are critical regulators of membrane curvature in endocytosis (Machesky and Johnston, 2007). Some parallels may exist with the metastasis suppressor CD82, a tetraspannin, which regulates the mobility of the EGFR on the plasma membrane and increases its recruitment to clathrin-coated pits for internalization (Danglot *et al.*, 2010; Miranti, 2009). However, we did not find a significant change in the rate of internalization of biotinylated EGFR in *Mtss1* overexpressing cells (Figure 2) suggesting that altered receptor internalization is unlikely to explain our observations.

High levels of *Mtss1* increases surface EGFR levels and alter EGFR signaling. The EGFR signals only briefly from the plasma membrane, during the initial 10 minutes following EGF stimulation, before it is internalized via clathrin-mediated endocytosis (Sigismund *et al.*, 2008). We observe an enhancement of EGF-induced Erk1/2 phosphorylation at 10 minutes when the majority of the receptor is still on the plasma membrane (Sigismund *et al.*, 2008); this is in contrast to overexpression of cortactin in HNSCC, which act further downstream to prolong endosomal signaling (28, 29). Further, high *Mtss1* expressing cells in the absence of cell-cell junctions show a delayed phosphorylation of Akt (Figure 7), which predominantly occurs on signaling endosomes (Mosesson *et al.*, 2008). Thus, at low densities, high *Mtss1* expressing cells have upregulated EGFR signaling to Erk1/2 but delayed activation of Akt.

High cell density and cell-cell junctions inhibit Mtss1-mediated EGF signaling

In contrast to the enhancement of EGF signaling that *Mtss1* confers at low cell densities, *Mtss1* increases the inhibition of EGFR signaling at high cell densities when cells are making junctions (Figure 7). E-cadherin can recruit the EGFR to form a complex following the formation of cell-cell junctions (Bremm *et al.*, 2008; Qian *et al.*, 2004). The E-cadherin/EGFR complex inhibits EGF signaling and mutation or loss of E-cadherin results in reactivation of RTK signaling (Bremm *et al.*, 2008). We have shown that *Mtss1* enhances EGFR plasma membrane localization (Figure 2). Therefore, the EGFR may be sequestered

away from Mtss1 by E-cadherin following the establishment of cell-cell junctions (Qian *et al.*, 2004). Interestingly, a mutant of E-cadherin lacking exon 8, $\Delta 8$ E-cadherin, that can still bind to EGFR but has reduced cell-cell adhesion has enhanced localization of the EGFR to the cell surface and this also enhances EGF signaling (Bremm *et al.*, 2008).

Other metastasis suppressors, such as Nm23-H1 enhance cell-cell junctions to inhibit downstream targets of RTK signaling (Boissan *et al.*, 2010). Tiam1, a Rac specific guanine nucleotide exchange factor, important for the formation of cell-cell junctions, enhances primary tumor formation and inhibits metastasis (Uhlenbrock *et al.*, 2004; Woodcock *et al.*, 2009). Lastly, EGFR signaling regulates cell-cell junction formation, suggesting the possibility that Mtss1-driven EGF signaling might directly enhance cell-cell junction formation (Betson *et al.*, 2002). High expression of Mtss1 enhances the formation of an epithelial-like layer in our organotypic assay which is less proliferative than lower Mtss1 expressing Scc9 controls further supporting the idea that high Mtss1 can enhance junction formation, epithelialization and quiescence at high cell densities. Finally, in vivo, high Mtss1 expression in xenograft tumors leads to increased keratinisation and decreased proliferation in established tumors, supporting an inhibitory role of Mtss1 for proliferation of cells at high densities (Figures 5 and 6).

Mtss1 as a marker of primary tumors for targeted chemotherapy

The majority of studies involving Mtss1 have focused on metastatic cancer cells where expression is reduced or lost (Lee *et al.*, 2002; Lin *et al.*, 2005; Liu *et al.*, 2010; Loberg *et al.*, 2005; Parr and Jiang, 2009). However, our data suggest that Mtss1 promotes the growth of HNSCC through enhanced EGF signaling, suggesting Mtss1 may have a significant role in proliferation during the early stages of tumor development. Scc9, FaDu and Detroit 562 cells are well-differentiated HNSCC and express epithelial markers (E-cadherin and plakoglobin) but not mesenchymal markers (vimentin or N-cadherin) (Basu *et al.*, 2010; Yang *et al.*, 2008). Chemotherapy of Scc9-xenografts leads to repopulation of the tumors with mesenchymal-like vimentin-positive Scc9 cells that are more resistant to chemotherapy due to a decrease in surface EGFR expression (Basu *et al.*, 2010).

We observe a more epithelial-like morphology of Scc9 cells over expressing Mtss1, and an increase in the EGFR localization to the plasma membrane, which increases their sensitivity to the EGFR inhibitors Gefitinib and Erlotinib (Figure 7F). Therefore, Mtss1 expression may serve as a useful patient stratification marker for tumors that are more susceptible to EGFR chemotherapy. However, Mtss1 is upregulated in FaDu and Detroit 562 HNSCC cell lines, which harbor several gene amplifications that enhance their proliferation, survival and resistance to chemotherapy (Timpson *et al.*, 2005; Timpson *et al.*, 2007). For example, amplification of the c-Met receptor results in an alternative signaling pathway for Akt activation (Donev *et al.*, 2011). Under these circumstances treatment with PI3K inhibitors in combination with EGFR inhibitors is required to overcome c-Met amplification. We observe a poor induction of Akt phosphorylation in Mtss1 expressing cells (Figure 7) and potentially making Gefitinib or Erlotinib chemotherapy more effective (Figure 7E). Therefore, Mtss1 amplification in isolation may control EGF signaling differently compared to when it is co-amplified with other genes and further investigation is required.

Mtss1 as a regulator of metastasis

Akt signaling, specifically Akt2, is required for EMT and invasion (Cheng *et al.*, 2007; Irie *et al.*, 2005). Akt phosphorylation is either delayed or reduced in response to EGF stimulation when Mtss1 is present (Figure 7) and This is consistent with an anti-metastatic role for Mtss1. Interestingly, in prostate cancer cells Erk-inhibition is required for EGFR degradation while Akt is required for EGF-mediated EMT and migration (Gan *et al.*, 2010).

High Mtss1 expression confers enhanced Erk1/2 activation and reduced EGFR degradation (Figure 3 and 7), but a reduced/delayed Akt activation. Basal cell carcinomas rarely metastasize but Mtss1 expression in this situation can drive proliferation at the tumor periphery and local invasion of the surrounding tissue (Callahan *et al.*, 2004) much like we observe here with Scc9 cells (Figure 5 and 6). We propose that Mtss1 is selectively beneficial for primary tumor initiation and may drive local invasion through proliferation in early tumor formation, but we saw no evidence of increased invasion in our Scc9 xenografts expressing Mtss1.

In conclusion, we have shown that Mtss1 enhances the surface expression of the EGFR, which results in enhanced Erk signaling and proliferation. However, with increasing cell density, we show that Mtss1 dampens EGF signaling, which inhibits proliferation in epithelial-like layers but not, critically in small colonies of invading cells. We propose a simple model where Mtss1 is upregulated during the early stages of HNSCC (and hepatocellular carcinoma and basal cell carcinoma; Callahan *et al.*, 2004; Ma *et al.*, 2007) and is, mostly likely, driving proliferation through enhancing EGF signaling, though we cannot rule out the possible involvement of other RTK. Mtss1-driven proliferation, however, becomes inhibited as the cell density increases through the establishment of cell-cell junctions in a large primary tumor. Mtss1 is lost in metastatic cancers as it enhances cell-cell junction inhibition of EGF signaling that is required for cell motility and invasion through Akt.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

JD was funded by an AICR grant to LMM (07-0438). LMM received funding from an MRC Senior Research fellowship (G117/569) and a CRUK core grant. We thank Roger Daly for HNSCC cell lines and Heather Spence for technical help.

References

- Basu D, Nguyen TT, Montone KT, Zhang G, Wang LP, Diehl JA, et al. Evidence for mesenchymal-like sub-populations within squamous cell carcinomas possessing chemoresistance and phenotypic plasticity. *Oncogene*. 2010; 29:4170–82. [PubMed: 20498638]
- Betson M, Lozano E, Zhang J, Braga VM. Rac activation upon cell-cell contact formation is dependent on signaling from the epidermal growth factor receptor. *J Biol Chem*. 2002; 277:36962–9. [PubMed: 12147707]
- Boissan M, De Wever O, Lizarraga F, Wendum D, Poincloux R, Chignard N, et al. Implication of Metastasis Suppressor NM23-H1 in Maintaining Adherens Junctions and Limiting the Invasive Potential of Human Cancer Cells. *Cancer Res*. 2010; 70:7710–7722. [PubMed: 20841469]
- Bompard G, Sharp SJ, Freiss G, Machesky LM. Involvement of Rac in actin cytoskeleton rearrangements induced by MIM-B. *J Cell Sci*. 2005; 118:5393–403. [PubMed: 16280553]
- Bremm A, Walch A, Fuchs M, Mages J, Duyster J, Keller G, et al. Enhanced activation of epidermal growth factor receptor caused by tumor-derived E-cadherin mutations. *Cancer Res*. 2008; 68:707–14. [PubMed: 18245470]
- Callahan CA, Ofstad T, Horng L, Wang JK, Zhen HH, Coulombe PA, et al. MIM/BEG4, a Sonic hedgehog-responsive gene that potentiates Gli-dependent transcription. *Genes Dev*. 2004; 18:2724–9. [PubMed: 15545630]
- Canel M, Secades P, Rodrigo JP, Cabanillas R, Herrero A, Suarez C, et al. Overexpression of focal adhesion kinase in head and neck squamous cell carcinoma is independent of fak gene copy number. *Clin Cancer Res*. 2006; 12:3272–9. [PubMed: 16740747]

- Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, Wang LH. Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. *Cancer Res.* 2007; 67:1979–87. [PubMed: 17332325]
- Chung CH, Ely K, McGavran L, Varella-Garcia M, Parker J, Parker N, et al. Increased epidermal growth factor receptor gene copy number is associated with poor prognosis in head and neck squamous cell carcinomas. *J Clin Oncol.* 2006; 24:4170–6. [PubMed: 16943533]
- Cooper JB, Cohen EE. Mechanisms of resistance to EGFR inhibitors in head and neck cancer. *Head Neck.* 2009; 31:1086–94. [PubMed: 19378324]
- Danglot L, Chaineau M, Dahan M, Gendron MC, Boggetto N, Perez F, et al. Role of TI-VAMP and CD82 in EGFR cell-surface dynamics and signaling. *J Cell Sci.* 2010; 123:723–35. [PubMed: 20144992]
- Donev IS, Wang W, Yamada T, Li Q, Takeuchi S, Matsumoto K, et al. Transient PI3K inhibition induces apoptosis and overcomes HGF-mediated resistance to EGFR-TKIs in EGFR mutant lung cancer. *Clin Cancer Res.* 2011; 17:2260–9. [PubMed: 21220474]
- Edward M, Gillan C, Micha D, Tammi RH. Tumour regulation of fibroblast hyaluronan expression: a mechanism to facilitate tumour growth and invasion. *Carcinogenesis.* 2005; 26:1215–23. [PubMed: 15746159]
- Gan Y, Shi C, Inge L, Hibner M, Balducci J, Huang Y. Differential roles of ERK and Akt pathways in regulation of EGFR-mediated signaling and motility in prostate cancer cells. *Oncogene.* 2010; 29:4947–58. [PubMed: 20562913]
- Goh LK, Huang F, Kim W, Gygi S, Sorkin A. Multiple mechanisms collectively regulate clathrin-mediated endocytosis of the epidermal growth factor receptor. *J Cell Biol.* 2010; 189:871–83. [PubMed: 20513767]
- Gonzalez-Quevedo R, Shoffer M, Horng L, Oro AE. Receptor tyrosine phosphatase-dependent cytoskeletal remodeling by the hedgehog-responsive gene MIM/BEG4. *J Cell Biol.* 2005; 168:453–63. [PubMed: 15684034]
- Hsia DA, Mitra SK, Hauck CR, Streblov DN, Nelson JA, Ilic D, et al. Differential regulation of cell motility and invasion by FAK. *J Cell Biol.* 2003; 160:753–67. [PubMed: 12615911]
- Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, et al. Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. *J Cell Biol.* 2005; 171:1023–34. [PubMed: 16365168]
- Kelley LC, Shahab S, Weed SA. Actin cytoskeletal mediators of motility and invasion amplified and overexpressed in head and neck cancer. *Clin Exp Metastasis.* 2008; 25:289–304. [PubMed: 18324357]
- Lee SH, Kerff F, Chereau D, Ferron F, Klug A, Dominguez R. Structural basis for the actin-binding function of missing-in-metastasis. *Structure.* 2007; 15:145–55. [PubMed: 17292833]
- Lee YG, Macoska JA, Korenchuk S, Pienta KJ. MIM, a potential metastasis suppressor gene in bladder cancer. *Neoplasia.* 2002; 4:291–4. [PubMed: 12082544]
- Lin J, Liu J, Wang Y, Zhu J, Zhou K, Smith N, et al. Differential regulation of cortactin and N-WASP-mediated actin polymerization by missing in metastasis (MIM) protein. *Oncogene.* 2005; 24:2059–66. [PubMed: 15688017]
- Lin M, Smith LT, Smiraglia DJ, Kazhiyur-Mannar R, Lang JC, Schuller DE, et al. DNA copy number gains in head and neck squamous cell carcinoma. *Oncogene.* 2006; 25:1424–33. [PubMed: 16247453]
- Liu K, Wang G, Ding H, Chen Y, Yu G, Wang J. Downregulation of metastasis suppressor 1 (MTSS1) is associated with nodal metastasis and poor outcome in Chinese patients with gastric cancer. *BMC Cancer.* 2010; 10:428. [PubMed: 20712855]
- Loberg RD, Neeley CK, Adam-Day LL, Fridman Y, St John LN, Nixdorf S, et al. Differential expression analysis of MIM (MTSS1) splice variants and a functional role of MIM in prostate cancer cell biology. *Int J Oncol.* 2005; 26:1699–705. [PubMed: 15870888]
- Ma S, Guan XY, Lee TK, Chan KW. Clinicopathological significance of missing in metastasis B expression in hepatocellular carcinoma. *Hum Pathol.* 2007; 38:1201–6. [PubMed: 17442377]
- Machesky LM, Johnston SA. MIM: a multifunctional scaffold protein. *J Mol Med.* 2007; 85:569–76. [PubMed: 17497115]

- Mattila PK, Pykalainen A, Saarikangas J, Paavilainen VO, Vihinen H, Jokitalo E, et al. Missing-in-metastasis and IRSp53 deform PI(4,5)P₂-rich membranes by an inverse BAR domain-like mechanism. *J Cell Biol.* 2007; 176:953–64. [PubMed: 17371834]
- Mattila PK, Salminen M, Yamashiro T, Lappalainen P. Mouse MIM, a tissue-specific regulator of cytoskeletal dynamics, interacts with ATP-actin monomers through its C-terminal WH2 domain. *J Biol Chem.* 2003; 278:8452–9. [PubMed: 12482861]
- Miranti CK. Controlling cell surface dynamics and signaling: how CD82/KAI1 suppresses metastasis. *Cell Signal.* 2009; 21:196–211. [PubMed: 18822372]
- Mosesson Y, Mills GB, Yarden Y. Derailed endocytosis: an emerging feature of cancer. *Nat Rev Cancer.* 2008; 8:835–50. [PubMed: 18948996]
- Parr C, Jiang WG. Metastasis suppressor 1 (MTSS1) demonstrates prognostic value and anti-metastatic properties in breast cancer. *Eur J Cancer.* 2009; 45:1673–83. [PubMed: 19328678]
- Qian X, Karpova T, Sheppard AM, McNally J, Lowy DR. E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *EMBO J.* 2004; 23:1739–48. [PubMed: 15057284]
- Quinones GA, Jin J, Oro AE. I-BAR protein antagonism of endocytosis mediates directional sensing during guided cell migration. *J Cell Biol.* 2010; 189:353–67. [PubMed: 20385776]
- Sigismund S, Argenzio E, Tosoni D, Cavallaro E, Polo S, Di Fiore PP. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev Cell.* 2008; 15:209–19. [PubMed: 18694561]
- Sorkin A, Goh LK. Endocytosis and intracellular trafficking of ErbBs. *Exp Cell Res.* 2009; 315:683–96. [PubMed: 19278030]
- Thurlow JK, Pena Murillo CL, Hunter KD, Buffa FM, Patiar S, Betts G, et al. Spectral clustering of microarray data elucidates the roles of microenvironment remodeling and immune responses in survival of head and neck squamous cell carcinoma. *J Clin Oncol.* 2010; 28:2881–8. [PubMed: 20458058]
- Timpson P, Lynch DK, Schramek D, Walker F, Daly RJ. Cortactin overexpression inhibits ligand-induced down-regulation of the epidermal growth factor receptor. *Cancer Res.* 2005; 65:3273–80. [PubMed: 15833860]
- Timpson P, Wilson AS, Lehrbach GM, Sutherland RL, Musgrove EA, Daly RJ. Aberrant expression of cortactin in head and neck squamous cell carcinoma cells is associated with enhanced cell proliferation and resistance to the epidermal growth factor receptor inhibitor gefitinib. *Cancer Res.* 2007; 67:9304–14. [PubMed: 17909038]
- Uhlenbrock K, Eberth A, Herbrand U, Daryab N, Stege P, Meier F, et al. The RacGEF Tiam1 inhibits migration and invasion of metastatic melanoma via a novel adhesive mechanism. *J Cell Sci.* 2004; 117:4863–71. [PubMed: 15340013]
- Woodcock SA, Rooney C, Liontos M, Connolly Y, Zoumpourlis V, Whetton AD, et al. SRC-induced disassembly of adherens junctions requires localized phosphorylation and degradation of the rac activator tiam1. *Mol Cell.* 2009; 33:639–53. [PubMed: 19285946]
- Woodings JA, Sharp SJ, Machesky LM. MIM-B, a putative metastasis suppressor protein, binds to actin and to protein tyrosine phosphatase delta. *Biochem J.* 2003; 371:463–71. [PubMed: 12570871]
- Yang MH, Wu MZ, Chiou SH, Chen PM, Chang SY, Liu CJ, et al. Direct regulation of TWIST by HIF-1 α promotes metastasis. *Nat Cell Biol.* 2008; 10:295–305. [PubMed: 18297062]

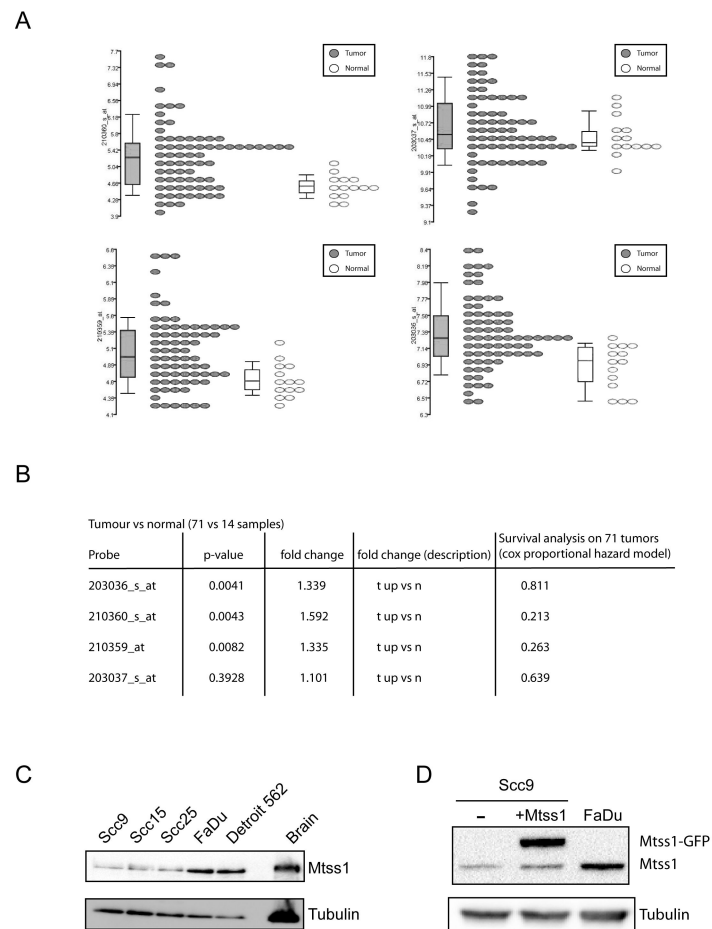


Figure 1. Analysis of *Mtss1* expression in primary HNSCC tissue samples and cell lines
Mtss1 expression was analyzed in 71 HNSCCs tumors, consisting of 61 primaries, 8 recurrences and 2 second primaries. Out of 14 normal tumors, 11 were normal contralateral. (A) Box-plots of *Mtss1* expression from 4 different *Mtss1* probes; 210360_s_at, 203037_s_at, 210359_at, 203036_s_at. (B) Statistical analysis, as described in methods, of *Mtss1* expression and survival analysis. (C) Analysis of *Mtss1* protein expression in HNSCC cell lines by western blotting. *Mtss1* migrates as a single band at approximately 100 kDa. (D) We generated stable Scc9 cells with GFP-*Mtss1* expressed at approximately 3-fold of endogenous levels. Western blots are representative of at least three experiments.

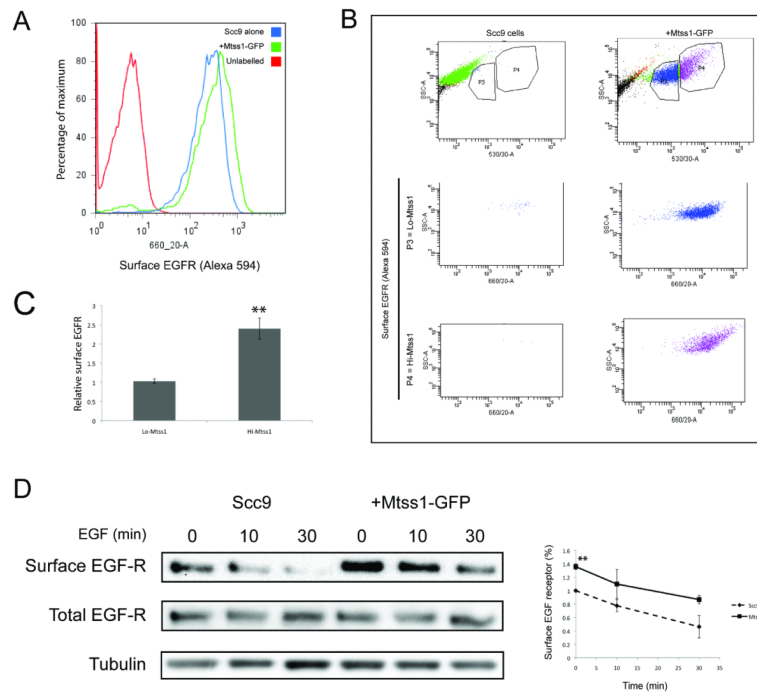


Figure 2. Mtss1 regulates the expression of the EGFR on the plasma membrane
 FACS analysis of surface EGFR expression in Scc9 cells and Mtss1-GFP expressing Scc9 cells were performed using 10,000 cells per experiment. (A) Histogram of surface EGFR labeling from a representative of 3 experiments. (B) Analysis of EGFR surface expression in low (P3) and high (P4) expressing Mtss1-GFP cells. (C) Mean relative EGFR surface intensity in high and low expressing Mtss1-GFP Scc9 cells. \pm S.E.M. is shown from 3 independent experiments. (D) Starved cells were stimulated with 25 ng/ml EGF for 0, 10 and 30 minutes and then proteins on the cell surface were biotinylated. Surface and total lysates were probed for EGFR and tubulin. Mean intensity of western blot bands (\pm S.D.) of surface EGFR from 3 independent experiments relative to Scc9 cells is shown in the graph to the right. For C and D, * = $p < 0.05$, ** = $p < 0.01$.

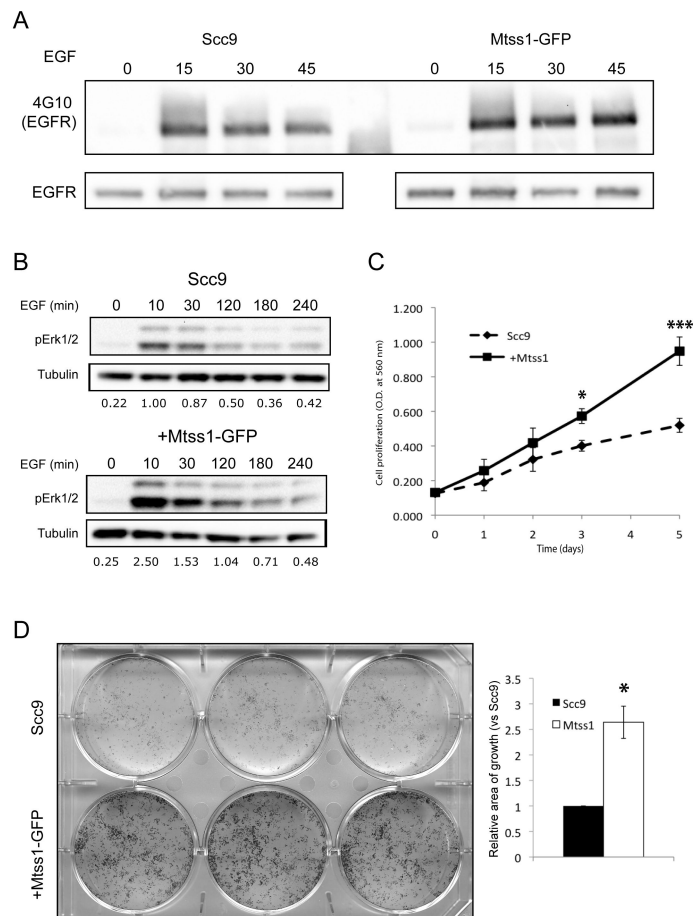


Figure 3. Mtss1 enhances EGF signaling in HNSCC cell lines

(A) EGFR was immunoprecipitated following EGF stimulation for the indicated times and blotted for total EGFR and tyrosine phosphorylation (4G10). (B) Western blot for phosphorylated Erk1/2 following stimulation with 25 ng/ml EGF. Numbers under blots show relative Erk1/2 phosphorylation compared to Scc9 cells stimulated at 10 minutes. Western blot is representative of at least three experiments. (C) MTT growth assay. Cells were grown in the presence of 10 ng/ml EGF for the indicated times. Mean optical density (560 nm) is shown \pm S.E.M. from 4 independent experiments. (D) Colony forming assay. 2000 cells per well of a 6 well plate were cultured for 5d. Area of colony growth was calculated using imageJ and is representative of 3 experiments performed in triplicate. For C and D * = $p < 0.05$, *** = $p < 0.001$.

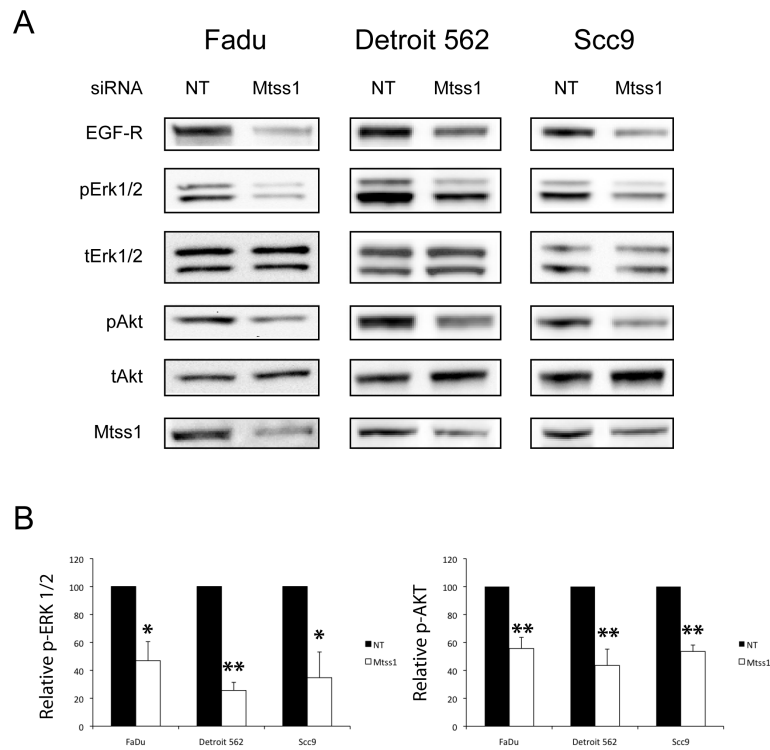


Figure 4. Mtss1 influences EGFR protein levels and signaling

(A) siRNA-mediated knockdown of Mtss1 in sub-confluent FaDu, Detroit 562 and Scc9 cells under basal conditions. Cells were transfected with either non-targeting or Mtss1 siRNA. Lysates were probed for EGFR expression and downstream signaling was analyzed using phospho-Erk1/2 (pErk1/2) and Akt (pAkt) antibodies. Western blots representative of at least three experiments. (B) Quantification of phosphorylated Akt and Erk1/2 relative to their total protein level is shown from 3 independent experiments for each cell line. Mean \pm S.E.M. is shown. ** $p < 0.01$, * $p < 0.05$.

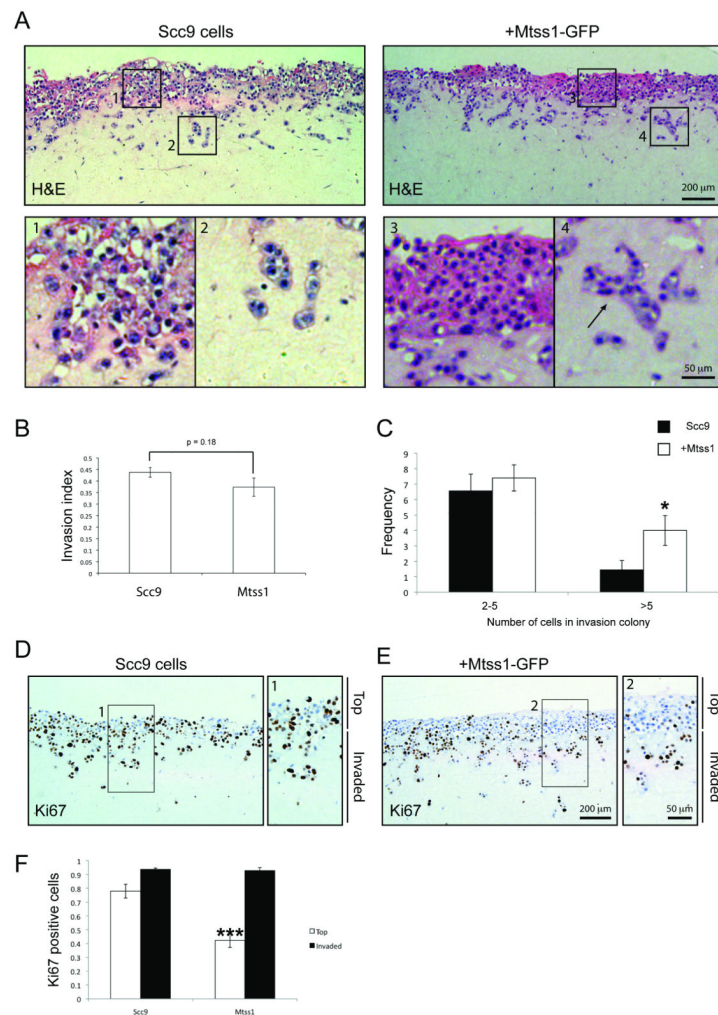


Figure 5. Mtss1 expression induces epithelial-like morphology of Scc9 cells in an organotypic assay
 Scc9 cells and Mtss1-GFP expressing Scc9 cells were cultured as described in methods. (A) Haematoxylin and eosin (H&E) stained cross sections of 10d organotypic invasion assay. Boxed regions 1-4 are enlarged below the main images. Arrow, invasion colony. (B and C) Quantification of invasion and the number of cells found in invasion colonies. Mean \pm S.D. is shown from 2 experiments. (D and E) Ki67 stained cross section of 10d organotypic invasion assay. Boxed regions are shown enlarged to the right of the main image. (F) Quantification of Ki67 staining in organotypic assays for the distribution of Ki67 staining. Mean \pm S.E.M. is shown from two experiments. For C and F, * = $p < 0.05$, *** = $P < 0.001$.

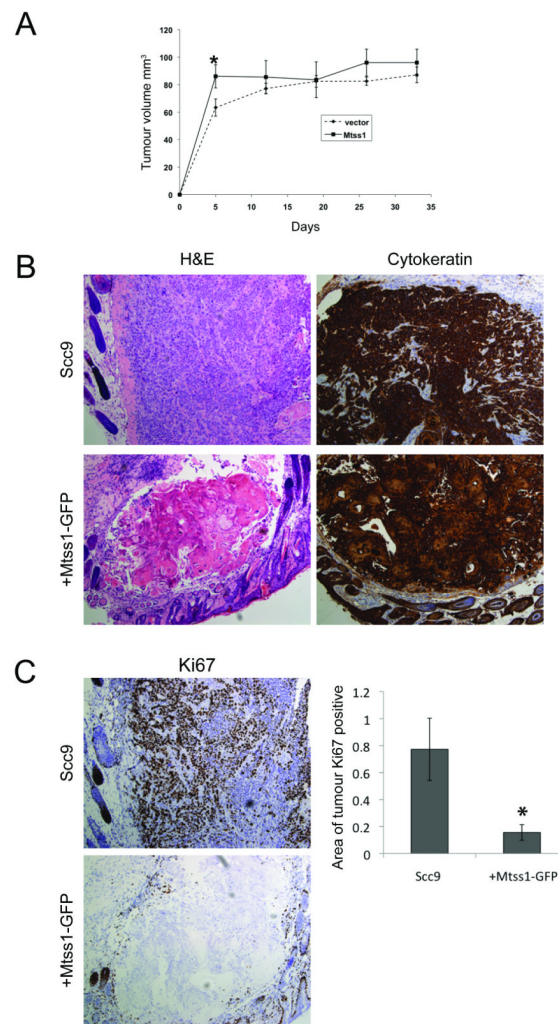


Figure 6. Mtss1 regulates proliferation in a mouse xenograft model

(A) The volume of the tumors was measured each week over a 6 week period following injection of cells. Graph represents mean volume \pm SEM from at least 6 animals. (B) Immunohistochemical labeling of tumor sections at day 35. H&E and pan-cytokeratin show the tumor areas. (C) Ki67 labeling of tumor sections and quantification of the Ki67 positive area of the tumor. Mean \pm SEM from 6 mice per group is shown. For A and C, * = $p < 0.05$.

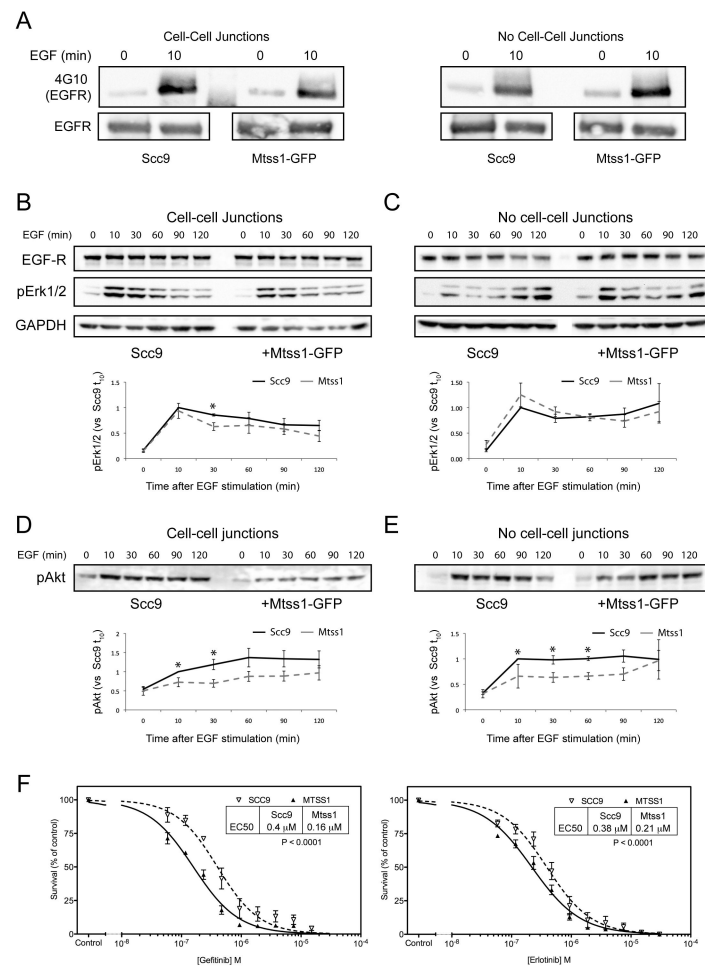


Figure 7. Cell-cell adherence junctions at least partly account for the density dependent effects of Mtss1 on EGFR signaling

Scc9 cells were grown to confluence, starved overnight in DMEM or to disassemble cell-cell junctions in Ca^{2+} -free DMEM before EGF stimulation (25 ng/ml). (A) EGFR was immunoprecipitated and western blotted for total EGFR and EGFR tyrosine phosphorylation (4G10). Lysates were also prepared from a longer EGF stimulation time course and analyzed for Erk1/2 phosphorylation (pErk1/2), total EGFR and GAPDH (B and C) and Akt activation (pAkt) (D and E) and typical blots are shown. Below each western blot in (B-E) is quantification of Erk1/2 or Akt activation showing fold-increase relative to Scc9 cells at 10 minutes EGF stimulation as determined by densitometry from 4 independent experiments. (F) Effect of Mtss1 expression in Scc9 cells on Gefitinib and Erlotinib treatment. Cells were assayed after 5d growth with increasing drug concentrations and values were normalized to solvent control. Experiments were performed at least 3 times. For B-E, For A and C, * = $p < 0.05$.