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Suppression of Tumor and Metastasis Progression through the Scaffolding Functions of SSeCKS/Gravin/AKAP12

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Abstract

Scaffolding proteins such as SSeCKS/Gravin/AKAP12 (“AKAP12”) are thought to control oncogenic signaling pathways by regulating key mediators in a spatiotemporal manner. The downregulation of AKAP12 in many human cancers, often associated with promoter hypermethylation, or the loss of its locus at 6q24-25.2, correlates with progression to malignancy and metastasis. The forced re-expression of AKAP12 in cancer cell lines suppresses *in vitro* parameters of oncogenic growth, invasiveness and cell motility through its ability to scaffold protein kinase C (PKC), F-actin, cyclins, Src and phosphoinositides, and possibly through additional scaffolding domains for PKA, calmodulin, β 1,4-galactosyltransferase-polypeptide-1, β 2-adrenergic receptors and cAMP-specific 3',5'-cyclic phosphodiesterase 4D. Moreover, AKAP12 re-expression in tumor models results in metastasis suppression through the inhibition of Src-regulated, VEGF-mediated neovascularization at distal sites. The current review will describe the emerging understanding of how AKAP12 regulates cellular senescence and oncogenic progression at the level of tumor cells and tumor-associated microenvironment via its multiple scaffolding functions.

Keywords

SSeCKS/AKAP12; metastasis; neovascularization; microenvironment; Src; PKC; PKA; cyclin

SSeCKS/Gravin/AKAP12 (“AKAP12”) was identified in the mid-90s by four labs independently screening for novel autoantigens in myasthenia gravis, antagonists of Src-induced oncogenic transformation, phosphatidylserine-dependent binding partners of protein kinase C (PKC), and binding partners of PKA [1]. Besides its activity as a tumor- and metastasis-suppressor, which is the focus of this review, there is clear evidence that AKAP12 also facilitates resensitization of β 2-adrenergic receptors, regulates neuronal cell injury, helps establish the blood-brain and blood-retinal barriers, and contributes to long-term memory- all of which are reviewed elsewhere [2, 3, 4].

AKAP12 was originally identified as a minor autoantigen, Gravin [5], correlating with poor prognosis in myasthenia gravis [6], and then shown to be orthologous to a rodent protein, SSeCKS (pronounced *essex*), a major PKC substrate and binding protein whose expression is downregulated in Src- and Ras-transformed cells [7, 8]. These orthologues were subsequently named AKAP250, and now AKAP12 (for A kinase anchoring protein 12), based on their ability to bind RII isoforms of PKA [9]. A current view is that AKAP12 functions as a scaffolding protein, which controls mitogenic signaling and cytoskeletal remodeling by binding key signaling mediators such as PKC, PKA, calmodulin, F-actin, cyclins, Src and phospholipids in a spatiotemporal manner [1] (Fig. 1). AKAP12 also fulfills other typical scaffolding functions required to coordinate signal transduction pathways [10,

11]. For example, AKAP12 crosslinks several cellular compartments, namely, the actin cytoskeleton and the plasma membrane, thereby facilitating signal transfer from the cell surface to cytoplasmic and even perinuclear complexes. This is mediated by an F-actin-binding domain [12], and by N-terminal myristylation [7] and phospholipid binding domains [13]. AKAP12 translocates to new cellular compartments after mitogenic stimulation (from the plasma membrane to the perinucleus [7]), thereby affecting signaling by transporting bound proteins to sites of action or inaction. AKAP12 multimerizes as a homomultimer or as a heteromultimer with other scaffolding proteins such as AKAP5, a function thought to amplify scaffolding activity at specific cellular sites [14, 15]. Finally, AKAP12 exhibits differential scaffolding activity during cell cycle progression, tracking with changes in its own phosphorylation level. Specifically, mitogen-induced serine phosphorylation of AKAP12 in or near scaffolding sites for PKC or cyclins correlates with decreased binding activity, resulting in increased PKC kinase activity and PKC-induced cytoskeletal remodeling, and in increased nuclear translocation of cyclin D [16, 12, 17]. The FAK-dependent tyrosine phosphorylation of AKAP12 induced by EGF antagonizes its binding to F-actin, correlating with cytoskeletal remodeling and increased cell motility [12].

All vertebrate species analyzed including fish (Danio, Tetraodon and Fugu) have AKAP12 orthologs sharing >40% homology at the protein level, however invertebrates (e.g., insects, fungi, or worms) lack credible orthologs. Vertebrates encode a single AKAP12 protein family, and although mice deficient in AKAP12 develop normally [18], albeit with minor defects reflecting pre-cancerous conditions (see below), there is new evidence that AKAP12 deficiency in Danio results in defects to vascular integrity [19].

Multiple lines of evidence bolster the concept that AKAP12 functions as a suppressor of oncogenesis. In brief, these include i) mapping of human AKAP12 to 6q24-25.2, a deletion hotspot in advanced prostate, breast, and ovarian cancer [20], ii) downregulation of AKAP12 by specific oncogenes, in cancer cell lines and human cancer tissues compared to normal controls, iii) upregulation of AKAP12 by treatments such as retinoids that cause cancer cell growth arrest or differentiation, iv) suppression of oncogenic growth *in vitro* and *in vivo*, especially metastasis formation, following the forced re-expression of AKAP12, v) induction of a tumor- or metastasis-prone condition in mice lacking AKAP12.

AKAP12 downregulation by oncogenes and in cancer

An early suspicion of potential cancer suppressive activity for AKAP12 was based on its downregulation in fibroblasts and epithelial cells transformed with specific, but not all oncogenes [1], strongly suggesting that AKAP12 antagonizes specific oncogenic pathways. For example, AKAP12 is downregulated by oncogenic Src, Ras, Myc, Jun, Fos, Wnt1 and Dnmt1, but not by Raf, Mos or Neu. AKAP12 levels also track with transformation status, i.e.- they are restored in non-oncogenic revertants and then decrease in retransformants exhibiting anchorage-independent growth [21]. Recent data indicate that *Akap12*-null mouse embryo fibroblasts (MEF) are prone to oncogenic transformation (anchorage-independent growth) after expression of only a single oncogene [22], strengthening the notion that loss of the AKAP12 tumor suppressor function serves as one of the two required genetic events to drive oncogenic progression [23].

Scores of differential gene expression and copy number variation (CNV) studies demonstrate AKAP12 downregulation in many cancer types in human tumors and cancer cell lines (reviewed in [1]). Importantly, there is a building literature identifying decreased AKAP12 transcript or gene copy levels in metastases compared to matched primary-site colon, prostate, breast or endocrine cancers, or as a predictive marker for bladder and pancreatic cancer metastasis (reviewed in [1]). For example, loss of AKAP12 staining

correlates strongly with Gleason sum >6 ([24], Ray M, Zheng S, Nochajski P, Davis W, Mohler JL, Marshall J, Gelman IH. Loss of SSeCKS/Gravin/AKAP12 expression in prostate cancer tissues correlates with disease progression, *submitted for publication*). However, 40% of more aggressive AKAP12-deficient lesions (Gleason sum 7 to 10) [24] also exhibited gene deletion, as determined by laser capture microdissection followed by PCR analysis using AKAP12 and GAPDH control primers (I.H. Gelman, unpublished data). The correlation of AKAP12 gene loss to prostate cancer metastasis was demonstrated by Lapointe et al. [25], who showed that whereas three CNV subtypes of primary prostate cancers show deletions in the 6q13-22 region, lymph node metastases exhibit an additional deletion of the AKAP12 locus at 6q24-25.2 (Fig. 2).

Validated losses of AKAP12 expression (>5-fold over normal tissue controls) have been reported in human and rat prostate cancer cell lines, pulmonary adenocarcinomas, leiomyoma, chronic and acute myeloid leukemias and myelodysplastic syndromes, multiple myeloma, papillary thyroid carcinoma, pediatric acute lymphoblastic leukemia, gastric cancer, non-small cell lung carcinoma, osteosarcomas, melanomas, retinoblastomas, colon and pancreatic cancer, fibrosarcomas, and squamous cell lung carcinoma (reviewed in [1]). Additional microarray and *in silico* analyses deposited in Entrez GEO (Gene Expression Omnibus; www.ncbi.nlm.nih.gov/geo), Oncomine (www.oncomine.org), the Cancer Genome Anatomy Project SAGE site (<http://cgap.nci.nih.gov/SAGE>), or The Cancer Genome Atlas site (<http://cancergenome.nih.gov>) identify AKAP12 downregulation (3- to 10-fold) in breast, prostate, lung, liver, thyroid and ovarian cancers and in gliomas (reviewed in [1]). Importantly, though, AKAP12 loss correlates with increased metastatic potential of human sarcoma, melanoma, endocrine, bladder and breast cancers [1](Fig. 3) and of spontaneous liver and kidney metastases compared to either normal prostates or primary-site prostate cancers in the “TRansgenic Adenocarcinoma Mouse Prostate” model, TRAMP (Fig. 3B) (Shannon, Kinney, Barbara Foster, Irwin Gelman and Adam Karpf, unpublished data).

Recent studies have elucidated several mechanisms by which AKAP12 is transcriptional regulated. In rodents and humans, *AKAP12* transcription is driven by three independent promoters, α , β and γ , the latter expressed only in the testes [26]. The α and β promoters, which are equally active in most cells and which are co-downregulated in cancer cells [20], splice to a common long exon, resulting in α - and β -AKAP12 proteins that only differ in their N-terminal 7%. The Src-induced downregulation of both α and β AKAP12 promoters involves *cis*-acting sequences proximal to the transcription start site that bind USF1, Sp1, Sp3, HDAC1 [27], plus a form of TFII-I that converts to a transcriptional repressor once tyrosine phosphorylated by activated Src [28]. Once downregulated, the AKAP12 promoter undergoes hypermethylation at promoter CpG islands in many cancer types such as colon, gastric, prostate, lung, pancreatic and skin [29–37], although a recent case of AKAP12 silencing in multiple myeloma by changes in histone acetylation has been reported [38].

Given that AKAP12 downregulation might, in itself, represent only a bystander effect, it is important to note that AKAP12 loss is associated with increased cancer susceptibility. For example, AKAP12 silencing via promoter hypermethylation [39, 40] has been shown to function as a neoplastic biomarker in the progression of Barrett’s esophagus to esophageal adenocarcinoma [41]. AKAP12 loss correlates with increased cancer incidence in breast cancer [42, 43], hepatocellular carcinoma [44] and chronic myeloid leukemia [45], and increased lung metastasis in cases of pancreatic cancer [37].

Upregulation of AKAP12 by antagonists of oncogenic transformation

Additional evidence that strengthens a role for AKAP12 as an oncogenic suppressor stems from its induction by genes or treatments that antagonize oncogenic transformation. In this regard, AKAP12 expression is upregulated in cancer cells by the re-expression of the p53 [46] or p21 [47] tumor suppressors, or by antagonizing STAT3 β -dependent oncogenic signaling [48]. Similarly, AKAP12 is upregulated during growth arrest induced by differentiating agents such as retinoids [49, 50] or vitamin D3 [51, 52], or following Smad-4-dependent, TGF- β -induced cell cycle arrest [53], but not by generic growth arrest induced by serum removal [54]. Multiple studies identify AKAP12 as androgen-inducible in primary human prostate epithelial cells or in androgen-dependent cancer lines such as LNCaP [55–57], in contrast to androgen-independent LNCaP lines, which exhibit AKAP12 downregulation [58].

Tumor- and metastasis suppression by AKAP12

An important method in the validation of an oncogenic suppressor is to show that its re-expression decreases specific parameters of primary- and/or metastatic oncogenic growth *in vitro* and *in vivo*, and in the case of AKAP12, it can have tumor or metastasis suppressing activity depending on the assay used. For example, AKAP12 re-expression significantly decreased v-Src-, Jun- or Ras-induced *in vitro* oncogenic growth parameters such as anchorage- and growth factor-independence, focus formation, Matrigel invasiveness, and invadosome formation, concomitant with the induction of cell flattening and the reestablishment of normalized cytoskeletal architecture including F-actin stress fibers flanked with focal adhesion plaques [21, 24, 29, 59–62]. The ability of AKAP12 to inhibit oncogenic motility *in vitro*, such as invasiveness and chemotaxis, correlates in part with the suppression of MMP-2/9 expression and secretion [60, 63, 62]. This involves disengaging Src from activating downstream RhoGTPase-family- and PKC-Raf/MEK/ERK-mediated pathways controlling MMP expression, cytoskeletal remodeling and invadosome formation. Interestingly, AKAP12 re-expression does not inhibit Src kinase activity in cells, in as much as Src autophosphorylation, total phosphotyrosine substrate levels, and the Src-induced tyrosine phosphorylation of specific downstream signaling or invasion mediators such as Shc or Tks5, respectively, is unaffected by AKAP12 re-expression [60, 63, 21]. In contrast, the loss of invadosome structures and Matrigel invasiveness correlates with an AKAP12-mediated suppression of RhoA and Cdc42 activation levels [60] and serum-induced PKC activation levels [63]. The continued activity of Src and the continued ability for serum-enhanced Src phosphorylation of Shc after AKAP12 re-expression [63] suggests that AKAP12 physically disengages active Src from downstream oncogenic signaling rather than inhibiting its intrinsic kinase activity.

Although it remains unclear how AKAP12 controls Rho GTPase family activity, control of PKC is likely facilitated by a direct scaffolding activity by AKAP12, such that saturated binding inhibits PKC kinase activity [9, 16]. This correlates with increased PKC isozyme activity in AKAP12-null MEF [16]. Interestingly, AKAP12-null MEF fail to exhibit phorbol ester-induced cell shape change, i.e.- actin cytoskeletal remodeling, and the ability of re-expressed AKAP12 to rescue this requires two separate, yet homologous PKC-binding sites in AKAP12 with the motif, EG^{I/V}T/SxWxSFK^{K/R}M/LVTP^{K/R}K^{K/R}x^{K/R}xxxExxxE^{E/D} [16]. This strongly suggests that AKAP12 physically transduces signals to downstream PKC substrates that control the actin cytoskeleton. Consistent with this notion, the ability of AKAP12 to suppress cancer cell invasiveness, chemotaxis and cell survival requires its PKC binding domains [63].

A mechanism by which AKAP12 might disengage active Src from downstream oncogenic signaling is based on the identification of a Src scaffolding domain in AKAP12, homologous to the Src-binding domain in caveolin1, which seems to facilitate increased cell adhesion by AKAP12 by inducing Src translocation from FAK-enriched focal adhesion complexes to lipid rafts. The notion that AKAP12 facilitates Src association with caveolae structures is based on the increased level of AKAP12-induced Y14 phosphorylation of Caveolin-1 (Su et al., manuscript under review), a known Src substrate site [64].

In addition to the correlation between AKAP12 loss and metastasis (above), AKAP12 fulfills the functional definition of an *in vivo* metastasis suppressor [65]: its re-expression does not significantly affect primary tumor growth yet it suppresses metastasis formation. As will be described below, there is mounting evidence that mice lacking AKAP12 are also metastasis-prone. We initially demonstrated metastasis-suppressing activity in rat MAT-LyLu (MLL) prostate cancer cells: the tetracycline-regulated AKAP12 re-expression had a marginal effect on the growth of primary-site tumors, yet there was a severe reduction in the spontaneous formation of macroscopic pulmonary metastases [24, 66]. AKAP12 neither altered generic cell motility (e.g. in monolayer wound healing assays) nor the rates at which cells colonized distal sites. Rather the micrometastases produced by the AKAP12-re-expressor MLL cells were avascular and reminiscent of experimental pulmonary micrometastases of Lewis lung carcinoma cells growing in Src-null hosts [67], a defect attributed to a failure to transduce VEGF-mediated recruitment of endothelial cells to the metastasis neovasculature [68, 69]. AKAP12 suppresses VEGF expression in MLL and many other cell types [66, 70–74], and indeed, the finding that the forced re-expression of VEGF in MLL/AKAP12-expressing cells rescues macrometastasis formation strongly argues that AKAP12 suppresses a late stage of metastasis, namely VEGF-induced neovascularization at distal sites. A recent study by Liu et al. [75] confirms the ability of AKAP12 to suppress the *in vivo* metastatic potential of colon cancer cells.

Loss of AKAP12 leads to a tumor- or metastasis-prone condition

Although AKAP12-null mice have normal longevity, they display hyperplasia in several tissues normally enriched for AKAP12 expression, including the prostate, lung and skin [18, 76]. Moreover, the increase AKAP12-null prostatic cellularity results from higher apoptosis levels and even higher proliferation levels- characteristics of increased senescence, a process thought to represent a protective response to the loss of an oncogenic suppressor function [77]. Indeed, AKAP12-null prostates and MEF display increased markers of senescence, such as senescence-associated β -galactosidase, p16^{Ink4a} and γ -H2AX (Fig. 4A–C), that correlate with increased polyploidy (Fig. 4D) and binucleation [22]. This senescence is Rb-dependent, resulting from hyperactive PKC α and γ due to the loss of AKAP12 scaffolding activity. Importantly, AKAP12 deficiency renders these MEF prone to oncogenic transformation by only one oncogene, such as Src or Ras, in contrast to WT-MEF, which require at least two oncogenes [22]. The notion that AKAP12 deficiency increases oncogenic susceptibility *in vivo* is borne out by two recent studies. In the first, Akakura et al. [76] demonstrate increased carcinogen-induced papilloma formation and conversion to squamous cell carcinoma in AKAP12-null mice, and although the frequency was low, several treated AKAP12-null mice developed spontaneous spindle cell carcinoma metastases. Interestingly, the increased carcinogenesis in the absence of AKAP12 correlates with induced levels of FAK, a known mediator of skin cancer progression [78]. In the second, AKAP12-null mice exhibited severely increased numbers of spontaneous peritoneal metastases compared to WT mice following the orthotopic injection of syngeneic B16F10 melanoma cells [79]. Importantly, AKAP12 deficiency had no effect on primary tumor growth. This indicates that AKAP12 can control metastatic progression through both tumor- and microenvironment-specific mechanisms.

Future directions

Experiments are underway to more directly address the metastasis suppressor role of AKAP12, especially to characterize how this role is manifested at the molecular mechanism. These include crossing the *Akap12*^{-/-} background into mice with a prostate-specific deletion of *Rb* (Pb-Cre;*Rb*^{fl/fl}). The *Akap12*^{-/-};Pb-Cre;*Rb*^{fl/fl} mice exhibit significantly increased frequencies of lymph node metastases that display transitional basal-luminal phenotypes (Poster 56, AACR Advances in Cancer Research Meeting, Orlando, FL, Feb. 6–9, 2012). Additionally, we are determining the role of AKAP12 expressed by microenvironmental cells, such as fibroblasts and CD11b myeloid cells, in controlling metastatic potential to the peritoneum using the B16F10 melanoma model (above). It is still unclear why AKAP12 might manifest more suppressive roles in metastasis rather than in cancer initiation or maintenance, although this may translate to AKAP12's ability to attenuate Src-directed metastasis-specific pathways as described above. Nonetheless, it will be important to characterize treatments such as retinoids or inhibitors of histone deacetylation or DNA methylation as means to suppress metastatic progression via the re-expression of AKAP12.

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Figure 1. AKAP12 scaffolding domains

Binding domains on AKAP12 are shown for Src, cyclins (2 sites), calmodulin (4 sites), GalTase (2 sites), phosphatidylserine, PKC (2 sites), F-actin, β_2 -adrenergic receptor (β_2 -AR), and R_{II}-PKA (AKAP). Additionally, AKAP12 encodes three polybasic domains that facilitate binding to phosphoinositols, four SV40-type nuclear localization signals (NLS), and a domain that facilitates nuclear exclusion. Four major PKC phosphorylation sites as well as one major tyrosine phosphorylation site are shown. The α AKAP12 isoform is post-translationally myristoylated (Myr) at its N-terminus.

Figure 2. Deletion of AKAP12 chromosomal locus associated with lymph node metastasis in prostate cancer

CNV analysis of primary prostate cancers vs. lymph node metastases (“LN mets”) by Lapointe et al. [25] identifies deletions in the 6q13-22 region in 30–90% of cases, depending on CNV subtype. In contrast, roughly 30% of LN mets exhibit deletions of the AKAP12 locus at 6q24-25.2 [24] (Reprinted by permission from the American Association for Cancer Research).

Figure 3. Transcriptional downregulation of AKAP12 in metastases of human and mouse cancers

(A) Oncomine studies showing AKAP12 downregulation in metastases of sarcoma, melanoma and breast cancer compared to primary-site tumors [80, 81, 82] (cancergenome.nih.gov). (B) AKAP12 downregulation as determined by qRT-PCR in liver and kidney metastases from the TRAMP autochthonous model of prostate cancer.

Figure 4. Increased markers of senescence in AKAP12-null prostates and MEF

The hyperplastic prostates in *Akap12*^{-/-} (KO) vs. WT mice display increased staining for senescence-associated β -galactosidase (“SA β gal”) (**Panel A**)(inset: percent of SA β gal-positive cells), p16^{Ink4a} (**Panel B**)(insets: percent of p16-positive cells; AP, anterior prostate; VP, ventral prostate; LP, lateral prostate), and γ H2AX (**Panel C**) as described in Akakura et al. [22]. Karyotype analysis of AKAP12-null MEF shows near tetraploid chromosome numbers (78 in this cell, **Panel D**).