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Carcinogen-induced squamous papillomas and oncogenic progression in the absence of the SSeCKS/AKAP12 metastasis suppressor correlates with FAK upregulation

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Abstract

The ability of SSeCKS/Gravin/AKAP12 (SSeCKS) to negatively regulate cell cycle progression is thought to relate to its spatiotemporal scaffolding activity for key signaling molecules such as protein kinase A and C, calmodulin, and cyclins. SSeCKS is downregulated upon progression to malignancy in many cancer types, including melanoma and non-melanoma skin cancer. The forced re-expression of SSeCKS is especially potent in suppressing metastasis through the inhibition of VEGF-mediated neovascularization. We have previously shown that SSeCKS-null (KO) mice exhibit hyperplasia and focal dysplasia in the prostate marked by activated Akt. To address whether KO-mice exhibit increased skin carcinogenesis, WT and KO C57BL/6 mice were treated topically with 12-O-tetradecanoylphorbol-13-acetate and 7,12-dimethylbenzanthracene. Compared to WT mice, KO mice developed squamous papillomas more rapidly and in greater numbers, and also exhibited significantly increased progression to squamous cell carcinoma. Untreated KO epidermal layers were thicker than those in age-matched WT mice, and exhibited significantly increased levels of FAK and phospho-ERK1/2, known mediators of carcinogen-induced squamous papilloma progression to carcinoma. Compared to protein levels in WT mouse embryo fibroblasts (MEF), SSeCKS levels were increased in FAK-null cells whereas FAK levels were increased in SSeCKS-null cells. RNAi studies in WT MEF cells suggest that SSeCKS and FAK attenuate each other's expression. Our study implicates a role for SSeCKS in preventing of skin cancer progression possibly through negatively regulating FAK expression.

Keywords

SSeCKS; TPA; DMBA; FAK; AKAP12; Gravin; papilloma; squamous cell carcinoma; skin cancer

Introduction

SSeCKS, src-suppressed C kinase substrate, encoded by the rodent orthologue of human *Gravin/AKAP12*, was originally identified as a suppressor of v-Src oncogenic signaling and progression [1–4]. Re-expression of SSeCKS inhibits Src-induced anchorage- and growth factor-independence and Matrigel-invasiveness, without affecting Src tyrosine kinase activity or the level of total phosphotyrosine-containing proteins [5]. SSeCKS exhibits metastasis-suppressing activity *in vivo* through the inhibition of VEGF-mediated neovascularization and through the downregulation of a PKC-Raf/MEK/ERK-mediated

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pathway controlling invasiveness [6][7]. *Gravin/AKAP12* maps to chromosome 6q24-25.2, a deletion hotspot in advanced human prostate, breast and ovarian cancer [1][7]. Downregulation of SSeCKS/Gravin/AKAP12 expression has also been documented in lung, ovarian, gastric, colon cancers, gliomas, sarcomas, melanomas and various leukemias, mostly involving hypermethylation of promoter CpG islands [2].

SSeCKS-null (KO) mice exhibit prostatic hyperplasia and focal dysplasia, correlating with high levels of SSeCKS in normal luminal epithelial cells in the prostate [8]. KO prostate tissues expressed significantly higher levels of Akt^{poS473}, suggesting that SSeCKS promotes pre-cancer initiation through the attenuation of PI-3K signaling. Based on its tumor suppressive activity, it is conceivable that the loss of SSeCKS results in a tumor-prone condition. SSeCKS is expressed in keratinocytes and other cells in the epidermal layer [9], and SSeCKS is downregulated in sarcomas cell lines compared to control keratinocytes [10]. In order to address whether SSeCKS-null mice are prone to carcinogen-induced skin carcinogenesis, age-matched WT or KO C57BL/6 mice were treated topically with DMBA and TPA, a treatment regimen known to induce squamous hyperplasia and in some cases, progression to squamous cell carcinoma [11][12]. The KO mice showed increased numbers and rates of growth of squamous papillomas, and they exhibited increased progression to squamous cell carcinoma (SCC) compared to WT mice. Untreated KO epidermal layers had increased levels of FAK and phospho-ERK1/2, known mediators of oncogenic progression in this model [13–16], and RNAi studies suggest that SSeCKS and FAK can attenuate each other's expression. Our findings suggest that SSeCKS attenuates carcinogen-induced oncogenesis, in part, by downregulating FAK.

Materials and Methods

Animals

SSeCKS-null mice were generated as described previously [8]. All mice used in this study were inbred to at least five generations with C57BL/6 mice. All experiments were approved by the Roswell Park Cancer Institute Animal Care and Use Committee.

Cell culture and reagents

FAK (–/–) and FAK (+/+) MEF were obtained from T. Yamamoto and S. Aizawa (National Cancer Institute, Tokyo), and maintained in 10% FBS/DMEM at 37°C in 5% CO₂. WT-MEF were isolated as described previously [8]. siRNA-FAK and siRNA-AKAP12 (SSeCKS) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All reagents were obtained from Sigma (St. Louis, MO) unless otherwise stated.

Chemical Carcinogenesis

Chemical carcinogenesis was performed on WT or KO 10-week-old female C57BL/6 mice (12/group). Squamous papillomas were induced on shaved dorsal portions of the mice, as previously described [11][12], with a minor modification. Briefly, mice were treated topically twice a week for 5 weeks with 150 µl of acetone containing 25 µg of 7,12-dimethylbenzanthracene (DMBA) and 6.25 µg of 12-O-tetradecanoylphorbol-13-acetate (TPA), then twice a week for 10 weeks with 6.25 µg of TPA, followed by 13 weeks of monitoring without further treatment.

Immunohistochemistry

An H&E section of each skin lesion was performed and imaged digitally as described below. Immunohistochemistry (IHC) was performed in the core facility of the Department of Pathology and Laboratory Medicine using FAK mouse monoclonal antibody (clone 4.47; Millipore, Billerica, MA) followed by anti-mouse IgG conjugated with horseradish

peroxidase (DAKO, Glostrup, Denmark) and 3,3-diaminobenzidine chromogen (Biogenex Laboratories, San Ramon, CA). After counterstaining with Mayer's hematoxylin, dehydration, and coverslipping, digital images were produced using an Olympus DP25 camera (Olympus, Center Valley, PA) mounted on an Olympus BX45 microscope, and the data were analyzed using DP2-BSW software (Olympus).

Immunoblotting

Immunoblot analysis was performed as described previously [8] using either antibodies specific for SSeCKS [4], FAK (A-17, Santa Cruz Biotechnology; Santa Cruz, CA) or GAPDH (Santa Cruz Biotechnology). For siRNA experiments, WT-MEF were transfected with 60 pM of siRNA in Oligofectamine (Invitrogen, Carlsbad, CA), incubated for 48 h, and then retransfected with siRNA. Cell lysates were collected after an additional 48h.

Imaging and Quantification of IHC

IHC-stained tissue slides were scanned in an Aperio ScanScope CS, viewed using ImageScope software, and quantified using Spectrum software (Aperio Technologies, Inc., Vista, CA). To obtain relative staining levels, five fields/sample containing roughly 100 cells each were analyzed for total cell staining levels over 10 intensity stacks, and then the averages of positive staining (1+ [stacks 2, 3, 4], 2+ [stacks 5, 6, 7] and 3+ [stacks 8, 9, 10]) versus no staining (stacks 0, 1) were compared between WT and KO tissues.

Statistical analysis

Statistical analysis was performed using Student's T-test with two-tailed distribution and two-sample equal variance (for two-group comparison). $P < 0.05$ was judged to be significantly different.

Results

Increased squamous papilloma formation in the absence of SSeCKS

In order to address whether SSeCKS-null mice are prone to skin cancer-inducing carcinogens, we employed a well-accepted protocol wherein topical treatment with TPA induces squamous papilloma formation, and the additional use of DMBA causes progression of these hyperplastic lesions to SCC [11][12]. C57BL/6 background mice were chosen because they are relatively resistant to squamous papillomas and progression to carcinoma, compared to more susceptible strains such as FVB [17–19]. Indeed, after 18 weeks of treatment, none of the WT mice developed squamous papillomas whereas 50% of the KO had lesions by 13 weeks and 100% had lesions by 18 weeks of treatment (Fig. 1A). After 24 weeks, treated KO mice had roughly twice the number of independent skin lesions compared to WT mice (Fig. 1B). Finally, the average lesion size in KO mice after 28 weeks of treatment was roughly 4-fold larger than in WT mice (Figs. 1C and D).

The loss of SSeCKS promotes epidermal neoplasia

Our data indicate that progression to carcinogen-induced skin neoplasia increases in the absence of SSeCKS. Specifically, after 28 weeks, most of the lesions in the treated KO mice showed invasive moderately differentiated SCC, with a significant amount of parakeratosis and atypia at the skin surface (Fig. 2). In contrast, fewer than one squamous papilloma lesion per WT mouse showed any evidence of invasive SCC (Fig. 2), whereas the remaining lesions exhibited benign squamous papillomatous hyperplasia with no parakeratosis or atypia at the skin surface. It is noteworthy that one KO mouse exhibited a 750 mm³ lesion below its ear (i.e., not in the area painted with carcinogens) that contained spindle cell

carcinoma (data not shown), suggestive of a limited transition to a more aggressive metastatic phenotype in the absence of SSeCKS.

FAK is upregulated in the absence of SSeCKS

FAK plays a key role in squamous papilloma formation and progression to SCC based on decreased skin carcinogenesis after treatment of either FAK^{+/-} or keratinocyte-specific FAK-null mice with DMBA and TPA [13][14]. We noted that the epidermal layers in KO mice were 2.133-fold thicker than those in WT mice, averaging roughly two cell layers compared to the normal single cell layer in WT mice (Fig. 3A). The thicker epidermal layers in KO mice displayed significantly increased levels of FAK (Fig. 3B) compared to age-matched WT controls. As a staining control, parallel tissue slices stained with pre-immune IgG showed little or no background staining in the squamous area. Compared to untreated skin (Fig. 3A), the carcinogen-induced SCC lesions in WT and KO mice showed considerably less FAK staining, although there was a clear shift from homogeneous cytoplasmic staining to cell edge staining in the SCC (Fig. 3C). Whereas the SCC lesions in WT mice showed patches of low-level staining, the KO-SCC lesions had 2.4-fold higher FAK staining levels throughout the lesion (Fig. 3C).

In order to determine whether FAK is upregulated in other cells following the loss of SSeCKS, KO and WT MEF were analyzed by immunoblotting and showed at least a 3-fold increase in FAK protein levels when normalized to GAPDH loading controls (Fig. 3D). Lysates were also probed with SSeCKS-specific Ab to show the absence SSeCKS protein in the KO MEF. Interestingly, a similar immunoblot analysis of FAK^{+/+} versus FAK^{-/-} cells [20] showed a roughly 3-fold increase in SSeCKS protein levels in FAK-null cells (Fig. 3E), indicating a reciprocal inverse relationship between FAK and SSeCKS. Moreover, the siRNA-mediated knockdown of SSeCKS in WT MEF resulted in a 2.7-fold increase in FAK protein levels, and conversely, the knockdown of FAK resulted in a 3.7-fold increase in SSeCKS (Fig. 3F). Taken with the data above, these findings strongly suggest that SSeCKS attenuates FAK levels in the squamous skin layer and in fibroblasts, and that in the absence of SSeCKS, higher levels of FAK correlate with increased transition of papillomas to SCC.

Previous data indicated that SSeCKS suppresses metastasis formation by attenuating tumor- and stromal-encoded VEGF and its subsequent induction of neovascularization [6]. We addressed whether the increased SCC formation in KO versus WT correlated with differences in lesion-specific VEGF levels. However, the carcinogen-induced SCC lesions showed relatively low angiogenic beds, and moreover, no difference in VEGF staining was identified between WT and KO SCC lesions (data not shown).

Increased ERK activation in absence of SSeCKS

MEK-ERK signaling is known to facilitate TPA/DMBA-induced transition of papillomas to SCC [16]. Therefore, normal WT and KO skin samples were stained for either phospho-ERK1/2 levels- the downstream marker of MEK activation. Phospho-ERK levels were significantly higher in KO vs. WT skin (3.2-fold $\pm$ 0.6) (Fig. 3G). As a negative control, these tissues showed no significant staining levels of phospho-JNK/SAPK, a signaling mediator not known to contribute to TPA/DMBA-induced SCC.

Loss of SSeCKS/Gravin/AKAP12 in human SCC

Although it is unlikely that human SCC of the skin arises solely through the pathway induced by TPA/DMBA carcinogens in the mouse skin model, we addressed whether human SCC lesions showed changes in human SSeCKS/Gravin/AKAP12 levels compared to local normal skin. 10 independent SCC lesions were stained with human Gravin/AKAP12-specific Ab [7], and the SCC lesions in all samples showed >3-fold lower staining than in

local normal skin, as typified by the levels shown in Fig. 3H. Moreover, human AKAP12 staining levels were quite variable within parts of a given SCC lesion (data not shown).

Discussion

The current study strengthens the notion that SSeCKS/Gravin/AKAP12 functions as a tumor suppressor based on the finding that SSeCKS-null mice exhibit epidermal hyperplasia and increased susceptibility to carcinogen-induced skin cancer. SSeCKS seems to attenuate several parameters of tumor progression in this DMBA/TPA model: increased frequency and growth rate of squamous papilloma, and increased oncogenic progression to SCC. Although this carcinogen model is usually performed in susceptible genetic backgrounds such as FVB [18], we and others used the more resistant C57BL/6 background [17][19] in order to maximize the cancer-prone effect from the loss of SSeCKS. One example of a spindle cell carcinoma was found in a KO mouse distal to the carcinogen-painted skin area, suggesting a possible increase in progression to metastasis in the absence of SSeCKS. Indeed, this phenomenon may show statistical significance if the mice were in a FVB background.

The increased spontaneous hyperplasia in KO epidermal layers is consistent with the ability of SSeCKS to suppress growth factor and adhesion-mediated Raf/MEK/ERK activation [21] [22] by scaffolding PKC isozymes and inhibiting their ability to activate Raf, and by disengaging activated FAK-Src from activating Raf/MEK/ERK [22]. Indeed, although SSeCKS-null prostates show hypercellularity, there is increased proliferation (Ki67 staining) and apoptosis (TUNEL staining) [8], the former correlating with increased MEK activity levels [22]. MEK is known to drive carcinogen-induced papilloma development, and MEK1 is required genetically for squamous papilloma formation [16][23]. Therefore, it is conceivable that the loss of SSeCKS activates Raf/MEK/ERK-pathway leading to enhanced squamous papilloma formation through enhanced MEK/ERK signaling.

The loss of SSeCKS correlated with increased FAK protein levels in squamous layer and in embryo fibroblasts. Given that FAK promotes malignant conversion in this carcinogen-induced skin cancer model [13–15], our results strongly suggest that the increased oncogenic progression in the absence of SSeCKS is facilitated by increased FAK levels. Indeed, FAK is known to promote proliferation through the upregulation of cyclin D expression [24], although it also possible that its ability to promote Src-induced proliferative and oncogenic growth signaling may play a parallel role. The reciprocal inverse relationship between SSeCKS and FAK protein levels strongly suggests that SSeCKS antagonizes specific FAK-mediated pathways involved in proliferation and/or survival. We also noted that *Cdc2l* (cyclin-dependent kinase 11), which suppresses DMBA/TPA-induced skin carcinogenesis [25], is downregulated in KO compared to WT MEF in gene expression microarrays (data not shown). Interestingly, our finding that SCC lesions exhibit lower FAK IHC staining levels compared to untreated skin suggests that FAK regulates oncogenic progression but not necessarily tumor maintenance in this context.

In sum, our work has determined that the loss of SSeCKS causes increased susceptibility to squamous papilloma initiation and oncogenic progression in a two-step carcinogen-induced skin cancer model. Our data strongly suggest that SSeCKS suppresses carcinogen-induced skin cancer through the downregulation of FAK. Furthermore, they suggest that the decreased carcinogen-induced skin carcinogenesis in the absence of FAK [14] relates to SSeCKS upregulation. Further work is required to address whether SSeCKS-null mice generated in FVB-background exhibit even more advanced parameters of oncogenic progression such as metastasis.

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Abbreviation

SSeCKS	Src Suppressed C Kinase Substrate
DMBA	7,12-dimethylbenzanthracene
TPA	12-O-tetradecanoylphorbol-13-acetate
FAK	Focal Adhesion Kinase

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Fig. 1. SSeCKS deficiency facilitates squamous papilloma formation

(A) Squamous papilloma incidence per mouse (percentage over time). (B) Papilloma multiplicity (average papilloma numbers/mouse). Variation between mice in either WT or KO groups was less than 15%. (C) Papilloma size in mm². Horizontal line, mean of 16 randomly selected lesions. *, $P < 0.001$. (D) Representative images of skin papillomas (red arrows) in WT or KO mice after 28 weeks of treatment.

Fig. 2. SSeCKS deficiency promotes epidermal neoplasia

Pathologic analysis (H&E stained) of typical DMBA/TPA-induced (28 weeks) skin lesions from WT or KO mice. Note the limited SCC development in the WT lesion, which mostly contains benign hyperplastic epidermis. In contrast, most of the KO lesion contains SCC, including areas of more advanced, endophytic growth, containing moderately undifferentiated carcinoma. p, papilloma; t, SCC. Size bar, 50 μ m.

Fig. 3. The expression of FAK is increased in SSeCKS-null mice

(A; *upper panels*) H&E-stained sections of 3-month-old WT and KO epidermal layers (typical thickness shown by double-head arrows). Size bar, 10 μm . Double-head arrow, representative epidermis. *Lower panel*, average thickness (in μm) of epidermal layers between WT and KO mice (10/group; 3 measurements/mouse). *, P<0.005. (B) Immunohistochemical staining for FAK (*upper panels*) or IgG control (*bottom panel*) of epidermal layers from 3-month-old WT or KO mice. (C) FAK IHC staining in SCC lesions in WT vs. KO mice. KO SCC lesions exhibit 2.44-fold (± 0.62) higher FAK levels than WT SCC (mean staining levels in 5 independent fields for each mouse strain, as described in Materials and Methods. P<0.02). Immunoblot analysis for SSeCKS, FAK or GAPDH protein in lysates of SSeCKS^{+/+} (WT) or SSeCKS^{-/-} MEF (D), or FAK^{+/+} or FAK^{-/-} MEF (E), or in WT MEF treated with control (scrambled)-, mAKAP12 (SSeCKS)-, or FAK-siRNA (F). The blots in panels D–F are typical of three independent experiments. (G) IHC analysis of untreated skin of WT vs. KO mice stained for phospho-ERK1/2, phospho-JNK/SAPK or control IgG. (H) IHC staining of normal human skin vs. human SCC lesions for SSeCKS/Gravin/AKAP12. The 3-fold lower SSeCKS staining in SCC vs. normal skin was found in 10 independent tissue sets (not shown).