SSeCKS/Gravin/AKAP12 attenuates expression of proliferative and angiogenic genes during suppression of v-Src-induced oncogenesis

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Abstract

Background: SSeCKS is a major protein kinase C substrate with kinase scaffolding and metastasis-suppressor activity whose expression is severely downregulated in Src- and Ras-transformed fibroblast and epithelial cells and in human prostate, breast, and gastric cancers. We previously used NIH3T3 cells with tetracycline-regulated SSeCKS expression plus a temperature-sensitive v-Src allele to show that SSeCKS re-expression inhibited parameters of v-Src-induced oncogenic growth without attenuating in vivo Src kinase activity.

Methods: We use cDNA microarrays and semi-quantitative RT-PCR analysis to identify changes in gene expression correlating with i) SSeCKS expression in the absence of v-Src activity, ii) activation of v-Src activity alone, and iii) SSeCKS re-expression in the presence of active v-Src.

Results: SSeCKS re-expression resulted in the attenuation of critical Src-induced proliferative and pro-angiogenic gene expression including Afp, Hif-1α, Cdc20a and Pdgfr-β, and conversely, SSeCKS induced several cell cycle regulatory genes such as Ptpn11, Gadd45a, Ptdlad1, Cdkn2d (p19), and Rbpb7.

Conclusion: Our data provide further evidence that SSeCKS can suppress Src-induced oncogenesis by modulating gene expression downstream of Src kinase activity.

Background

SSeCKS, originally identified as a transcriptionally-suppressed gene in v-src and ras-transformed rodent fibroblast cells [1], is the orthologue of human GRAVIN/AKAP12 gene that encodes a kinase-scaffolding protein [2] that is targeted as an autoantigen in some cases of myasthenia gravis [3]. SSeCKS/Gravin/AKAP12 expression is severely downregulated in human prostate, breast and gastric cancer, partially relating to the mapping of the human gene to 6q24-25.1 [4], a cancer deletion hotspot [5]. Re-expression of SSeCKS to physiologic levels in Src- or Ras-transformed fibroblasts or epithelial prostate cancer cells suppresses morphological transformation, anchorage- and growth factor-independent proliferation, and metastatic potential, while restoring normal actin-based cytoskeletal architecture and cell-cycle controls on cyclin D1 expression [4,6,7]. SSeCKS also seems to control the blood-brain barrier by suppressing astrocyte-expressed vascular endothelial growth factor (VEGF) during the switch to normoxic conditions after birth [8]. A recent study indicates that the ability of SSeCKS to suppress lung metastasis formation by MatLyLu prostate can-
cell lines. Our previous data indicated that SSeCKS overexpression in untransformed fibroblasts induces cell flattening as well as G1 phase growth arrest, the latter due to the suppression of growth factor-induced cyclin D1 expression [7]. The forced re-expression of SSeCKS could suppress v-Src-induced oncogenic growth in vivo without grossly affecting the ability of v-Src to phosphorylate cellular substrates [6]. Therefore, in order to identify genes differentially regulated by SSeCKS during G1 arrest or during suppression of Src-induced oncogenesis, we used NIH3T3-derived lines with tetracycline (Tet-Off)-inducible SSeCKS expression ("S24 cells") as well as S24 cells expressing a temperature-sensitive v-Src allele ("S24/ts72v-Src"). The ectopic expression of SSeCKS was strongly induced (>25-fold) in the absence of tetracycline (tet), yet in the presence of tet, ts72v-Src activation (35°C) resulted in a >10-fold decrease in endogenous SSeCKS levels (Fig. 1A), confirming our initial identification of SSeCKS as a Src-suppressed gene [1]. Moreover, SSeCKS overexpression did not affect Src autophosphorylation activity, as monitored by phospho-Y416 levels (Fig. 1A). The relationship between increased Src activity levels and

RT-PCR
1 µg of total RNA was primed with oligo-dT, reverse transcribed into first strand cDNA and then amplified in the linear range using the SuperScript® III RT SYBR One-Step RT-PCR Kit (Invitrogen), as described previously [1], using the primer sets described in Table 1. The primer sets for Grp56, Maff, Socs3, Afp, Gadd45a, Marcks, Hif1a, Afp, PDGFRB and HIF1A were purchased as QuantiTect Primer Assays (Qiagen). Optimization of RT-PCRs for semi-quantitative analysis was carried out after normalization using the β-actin mRNA as an internal control. PCR products were electrophoresed in 1.6% agarose gels, stained with ethidium bromide, and digitally imaged using a Chemi-Genius® Biolagher (Syngene). Relative intensities of PCR bands were quantified using GeneTools image software analysis (Syngene).

Results and discussion

Conditional expression of SSeCKS and v-Src activation in mouse fibroblast cell lines
Our previous data indicated that SSeCKS overexpression in untransformed fibroblasts induces cell flattening as well as G1 phase growth arrest, the latter due to the suppression of growth factor-induced cyclin D1 expression [7]. The forced re-expression of SSeCKS could suppress v-Src-induced oncogenic growth in vivo without grossly affecting the ability of v-Src to phosphorylate cellular substrates [6]. Therefore, in order to identify genes differentially regulated by SSeCKS during G1 arrest or during suppression of Src-induced oncogenesis, we used NIH3T3-derived lines with tetracycline (Tet-Off)-inducible SSeCKS expression ("S24 cells") as well as S24 cells expressing a temperature-sensitive v-Src allele ("S24/ts72v-Src"). The ectopic expression of SSeCKS was strongly induced (>25-fold) in the absence of tetracycline (tet), yet in the presence of tet, ts72v-Src activation (35°C) resulted in a >10-fold decrease in endogenous SSeCKS levels (Fig. 1A), confirming our initial identification of SSeCKS as a Src-suppressed gene [1]. Moreover, SSeCKS overexpression did not affect Src autophosphorylation activity, as monitored by phospho-Y416 levels (Fig. 1A). The relationship between increased Src activity levels and
decreased SSeCKS levels is also maintained when comparing the metastatic human colon cancer cell line, HT29 (high Src activity, low Gravin), with the low metastatic line, HCT116 (low Src activity, high Gravin) (Figure 1B).

We previously showed that growth of S24/ts72v-Src cells in 0.04 μg/ml tet resulted in re-expression of ectopic SSeCKS to the physiologic levels found in parental NIH3T3 cells [6]. Therefore, as described in Table 2, we compared differential gene expression for the following conditions: i) SSeCKS overexpression in untransformed cells (S24 grown in + vs. – tet at 37°C), ii) v-Src activation without SSeCKS re-expression ([S24/ts72v-Src grown in the presence of tet at 35°C vs. 39.5°C] minus [S24 grown in the presence of tet at 35°C]), or iii) SSeCKS re-expression in the presence of activated v-Src (S24/ts72v-Src at 35°C grown in + vs. – tet).

Profile of SSeCKS-regulated gene expression

We identified 45 genes whose expression was altered 2-fold or more by SSeCKS overexpression using at least three independent Affymetrix microarray analyses, as described in Materials and Methods, of RNA from S24 cells grown at 37°C in the presence or absence of tet. We then subtracted gene expression effects induced by the tTA transactivator alone in the absence of SSeCKS expression, that is, RNA derived from S2-6 parental cells [13] grown in the absence of tet. Given our prior results that SSeCKS overexpression inhibits G1→S transition in untransformed fibroblasts [7], it was not unexpected that ectopic SSeCKS could induce tumor suppressor/cell cycle negative control genes such as those encoding Rb, p19INK4a or Rassf5, while suppressing cell cycle promoting genes, such as cyclins D, A, B and F, Fos-like antigen 1, Afp [14] and the CDC20 homolog, or neoplasia-promoting genes such as Hmgb3 [15] or Cebpb [16] (Table 3). Similarly, SSeCKS induction of TGF-α and -β, and of Ngf-β, and the downregulation of Mtap7, is consistent with the pseudo-differentiated, non-polar phenotype displayed during SSeCKS-induced cell flattening [13]. The loss of PDGFR-β expression, which we have described elsewhere [17], likely relates to the ability of SSeCKS to suppress angiogenesis in the brain [8] and in tumor neovasculature. Of the signal transduction genes, the upregulation of Ptplad1, Traf6 [18] and Lmo4 [19] were consistent with the ability of SSeCKS to control cell cycle progression. Stam2 upregulation or Peg3 downregulation may facilitate SSeCKS-mediated anti-apoptosis based on reported roles for Stam2 role T cell survival signaling [20] and for Peg3 in neuronal death [21]. In contrast, SSeCKS unexpectedly upregulated several genes such as Gpr56, Prkce and Btbd2 which have been reported to be upregulated in human cancers [22,23] or involved in inducing cell cycle progression [24], whereas it downregulated several genes such as Nasp, known to antagonize cell cycle progression [25]. The role of other genes, such as Gng2, in SSeCKS regulation remain unclear although they are typically involved in specialized neuronal signaling responses [26]: upregulation might suggest that SSeCKS induces signaling pathways more associated with differentiated, especially neuronal, cells. Interestingly, we previously published that SSeCKS overexpression in S24 cells induces long axonal-like extensions which, when stained for SSeCKS, exhibit periodic, pearl-like enrichments of SSeCKS that are strikingly similar to SSeCKS-enriched structures in developing hippocampal axons [27]. The severe downregulation of Dusp9- which normally antagonizes insulin-mediated adipogenesis [28] may reflect an ability by SSeCKS to induce glucose uptake and/or metabolism, though this has not been studied. Mycbp is known to inhibit PKA activation at various cell sites by preventing recruitment of the PKA catalytic subunit to the scaffolding proteins, S-AKAP84 and AKAP95 [29]. Thus, Mycbp upregulation by SSeCKS- also a known AKAP [2]-may
reflect a higher level of temporal and/or spatial control of PKA activity by SSeCKS.

Profile of v-Src-regulated gene expression
24 genes were identified whose expression varied 2-fold or more by v-Src activation. Specifically, we compared microarray data from RNAs derived from S24/ts72v-Src cells grown in the presence of tet (i.e.- no ectopic SSeCKS) at either 35°C (the PT for Src kinase activity) or 39.5°C (the NPT) (Table 4, column A). To identify possible gene changes induced by incubation at 39.5°C (and not by the loss of Src activity), we compared microarray data derived from S24/ts72v-Src cells versus S24 cells grown in the presence of tet at 35°C (Table 4, column B). However, gene expression changes that occur under the latter conditions only (e.g.- Sh3bp5) might also reflect bona fide v-Src-

**Table 1: Summary of primer sequences and positions**

| Target gene | Sequence of forward and reverse primers | position |
|-------------|----------------------------------------|----------|
| Mouse       |                                        |          |
| Pdgfr-β     | 5'-AGCTACATGGCCCTTTATGA-3' 2749–2768   |          |
|             | 5'-GGATCCCAAGACACCAGACA-3' 3069–3115   |          |
| Tgfβ-1      | 5'-CGGGGGCGACCTGGGCA 1597–1623         |          |
|             | 5'-CTGCTCCACCTGTCGAGCGACCAC-3' 1975–2001 |          |
| Cdc20       | 5'-GCTGCTGTCCGAGATCCT-3' 757–776       |          |
|             | 5'-TGTTCAACTGGAGCCTT-3' 939–958        |          |
| Ptpn11      | 5'-AGTCCAAATGACCCAGGCTC-3' 623–643     |          |
|             | 5'-AGCGTCTCTCAAATCCTTC-3' 874–893      |          |
| Id2         | 5'-GGACATACGATCCGCTTCT-3' 383–402      |          |
|             | 5'-AACGGTGACAGTCCCATGGG-3' 659–678     |          |
| Illr1       | 5'-CGCTCGACTATCTCTGTTGA-3' 299–318     |          |
|             | 5'-AGCTGGGCGGCTTTTATGT-3' 490–509      |          |
| Cdkn2d      | 5'-AGCTGGGCGGCTTTTATGT-3' 212–229      |          |
|             | 5'-CGCTCCACCTTGTCAC-3' 460–442         |          |
| β-actin     | 5'-GCTGGTTCGCTGGACATCCT-3' 757–776     |          |
|             | 5'-TGTTCAACTGGAGCCTT-3' 939–958        |          |

**Human**

| Target gene | Sequence of forward and reverse primers | position |
|-------------|----------------------------------------|----------|
| CDC20A      | 5'-GGGGTCCTCCTCGAGACATTCC-3' 1034–1053 |          |
|             | 5'-TTCTTGTACGCTTCTGCTTGCCG-3' 33–56   |          |
| PSCDDBP     | 5'-GCTGGTTCGCTGGACATCCT-3' 757–776     |          |
|             | 5'-TGTTCAACTGGAGCCTT-3' 939–958        |          |
| FOSL1       | 5'-CGGGTCGTACGCCCAAG-3' 473–492        |          |
|             | 5'-CTCCTCGCTCATCTGAGC-3' 874–893       |          |
| HMGA2       | 5'-GGGGTCCTCCTCGAGACATTCC-3' 1034–1053 |          |
|             | 5'-TTCTTGTACGCTTCTGCTTGCCG-3' 33–56   |          |
| HMG1B       | 5'-ACAACCGAGACAAACCATGG-3' 1107–1126   |          |
|             | 5'-CCCCTTGTCCACAGCTTAAG-3' 1290–1310   |          |
| β-ACTIN     | 5'-GCTCCTCTTCGACACAGCGCTC-3' 93–113    |          |
|             | 5'-TTCTTGTACGCTTCTGCTTGCCG-3' 33–56   |          |
| AFP         | 5'-ACAACCGAGACAAACCATGG-3' 1107–1126   |          |
|             | 5'-CCCCTTGTCCACAGCTTAAG-3' 1290–1310   |          |
| APF         | 5'-GCTCCTCTTCGACACAGCGCTC-3' 93–113    |          |
|            | 5'-TTCTTGTACGCTTCTGCTTGCCG-3' 33–56   |          |
| PDGFRB      | Qiagen QuantiTect Assay, cat. #QT0008183 |          |
|             | Qiagen QuantiTect Assay, cat. #QT0008237 |          |
| HIF1A       | Qiagen QuantiTect Assay, cat. #QT00083644 |          |
|             | Qiagen QuantiTect Assay, cat. #QT00174020 |          |
induced changes that are negated at the NPT, and thus, we included both column A and B data in our overall analysis.

Six of the 24 genes (25%) were identified in Table 3 as being inversely regulated by SSeCKS, i.e.- if they were induced by SSeCKS, they were suppressed by v-Src, or vice-versa. These include Peg3, Fosl1, Hmgb3, Gfra1, I1r1r1 and Afp. In contrast, Igfbp4 is downregulated both by SSeCKS overexpression and v-Src activation, lessening confidence that this gene plays a significant role in oncogenesis.

Rgs16, Id2 and Socs3 have paradoxical roles in that they play both positive and negative functions in cancer. For example, Rgs16- whose expression is induced by v-Src in our system- is a GTPase activating protein for Ga, [30], and Gαi is known to cooperate with Src in oncogenic transformation [31]. However, Rgs16 activity is required for retinoid-induced growth inhibition [32], suggesting that it could antagonize cancer progression. Similarly, Socs3- whose expression is also induced by v-Src, is known to inhibit insulin-mediated proliferation signaling [33] and to be downregulated in head and neck squamous cell carcinomas [34], yet it is upregulated in breast cancer [35]. Id2 plays positive roles in cancer, possibly by inhibiting parameters of differentiation [36], yet decreased Id2 levels correlate with increased invasive potential in human breast cancer cases [37].

The increased levels of the transcription factors Maff, Runx1 and Etv4, and the decreased levels of Cemtd3, Id4, Spon2, and Cbr2 by v-Src seem to directly relate to cancer progression. Specifically, Maff is overexpressed in some forms of cancer [38] and Maf proteins play direct roles in controlling v-Src-regulated gene expression [39]. Runx1 induces cell cycle progression [40] and increased Runx1 levels are found in chronic myelogenous leukemias [41]. Etv4 levels are increased in gastric cancers [42]. Cemtd2 is a GAP for RhoA whose activity is blocked by phosphorylation by Src-family kinases [43]. Id4 is downregulated in colon [44] and gastric cancers [45]. Spon2 and Cbr2 levels are decreased significantly in lung cancers [46,47]. In contrast, v-Src induction of Prkg2 does not make apparent sense given that higher Prkg2 levels correlate with increased patient survival rates for lung cancer [48].

Finally, it is unclear how the differential regulation of Sh3bp, Atoh6, Tcfec and Pscdbp would affect v-Src-induced oncogenesis.

**Profile of gene expression in cells expressing activated Src plus physiologic SSeCKS levels**

46 genes were identified whose expression was altered under conditions of activated v-Src and SSeCKS to physiologic levels (Table 5). 16 of these genes (36%) were identified in screens described in Tables 3 or 4. Of these, 12 genes (Gpr56, Gng2, Ptplad1, Marcks, Cdkn2d, Cdc2a, Hmgb3, Ngfb, Gfra1, I1r1r1, Pdgfrb and Afp) exhibited the same expression control (i.e.- up-or downregulation) as detected after SSeCKS overexpression alone (Table 3). This strongly suggests that these genes are markers for SSeCKS-mediated growth arrest and/or tumor suppression. The remaining 4 pre-identified genes (Socs3, Maff, Id2 and Atoh6) are regulated in the same manner as in v-Src-transformed cells (Table 4) and thus, it is likely that these genes remain controlled by v-Src yet are not sufficient for the oncogenic phenotype.

SSeCKS reverses the expression of several genes that might either antagonize v-Src-induced oncogenesis or function as markers for non-transformed cells. Specifically, SSeCKS induces the expression of Gadd45a- a gene known to inhibit progression at either S or G2/M phases [49] or to induce density-dependent G1 phase arrest [50]. Cdkn2d, Rbbp7- which inhibits mitogen- and oncogene-induced c-Fos activation [51], and Dub1- whose overexpression induces G1 arrest [52] in the same context, SSeCKS suppresses the expression of Map2kb- whose activity is increased in Ras-mediated invasiveness and metastatic potential [53], Rgs2- whose increased expression in mantle cell lymphomas correlates with increased metastatic potential [54], Tcfap4- a regulator of caspase-9 mediated apoptosis [55], Cdc2a, Hif1a- a mediator of tumor angiogenesis induced by v-Src [56,57] and activated c-Src [58,59], Ddit4- an Hif1a-inducible gene [60], and Pdgfrb. Interestingly, SSeCKS re-expression may facilitate increased immune surveillance of tumor cells by the induction of Hsp4a, a tumor antigen carrier that increases the immunogenicity of colon cancer cells in a murine model [61]. Although the decrease in Caspase-7 (Casp7) expression correlates with the decreased apoptotic index of v-Src cells re-expressing SSeCKS [6], it is unclear how

| Cells/Growth condition | Comparison |
|------------------------|------------|
| NIH3T3/S24 cells, + vs. – tet, 37°C | SSeCKS overexpression |
| ([S24/ts72v-Src cells, 35°C vs. 39.5°C] minus S24 cells, 35°C, + tet (1 µg/ml)) | v-Src activation |
| S24/ts72v-Src cells, 35°C, 1.0 vs.0.04 µg/ml tet | SSeCKS re-expression plus v-Src activation |
the concomitant increase in Stk4, a pro-apoptotic serine/threonine kinase [62], affects cell survival after SSeCKS re-expression.

A set of genes seems to remain regulated by v-Src during SSeCKS re-expression, suggesting that their expression levels are not sufficient to induce or maintain oncogenic transformation in the presence of physiologic levels of SSeCKS. These include Ptpn11, which can activate Src-family kinases [63] and which is required for v-Src-induced morphological transformation [64]. Pip5k1b, whose product is activated by Rho-family GTPases in cancer cells [65], Spk2, a gene upregulated after retinoid-induced differentiation of HL60 leukemia cells [66], Dusp10, a negative regulator of MAP kinases and antagonist of prostate cancer cell proliferation [67], Marcks, Timp3, a gene downregulated in squamous cell carcinomas and prostate adenocarcinomas [68,69], Gdf5, a growth arrest inducer in mouse B cells [70], and Gas1, a gene downregulated in EGF-induced hepatocellular carcinoma [71] and in Ras-transformed cells [72]. The involvement of S100A8 is unclear because although its expression is induced in prostate cancer [73], its expression is downregulated in the metastatic breast cancer cell line, MDA-MB-231, compared to the non-metastatic MCF-7 [74].

In order to verify the gene expression changes identified in the microarray studies, several genes were assayed by semi-quantitative RT-PCR using primer sets shown in Table 1. Thus, RNAs derived from S24 or S24/ts72v-Src cells grown at the PT in the presence or absence of tet were amplified by RT-PCR as described in Materials and Methods and then the electrophoresed products were quantified by digital densitometry. Fig. 2A and Table 6 show a strong concordance between the regulation of Cdc2a, Il1r1, Cdkn2d, Pdgfrb, Ptpn11, Tgfb1, Id2, Gpr56, Maff, Soc3, Afp, Ngfb, Gadd45a, Marcks and Hif1a by either SSeCKS alone or SSeCKS re-expression in the presence of v-Src as gauged by both the microarray and RT-PCR assays. This strengthens the notion that the genes identified by our microarray comparisons reflect bona fide examples of differential expression due to SSeCKS overexpression or SSeCKS re-expression in the presence of active v-Src. Finally, we performed semi-quantitative RT-PCR on several genes shown to be induced by active v-Src (Table 3) using RNAs from HT29 and HCT116, representing cells with high and low levels of c-Src activity, respectively (Fig. 1B). Fig. 2B shows significantly higher levels of HMGA2, FOSL1, CDC20A, PSCDBP, AFP, PDGFRB and HIF1A expression in HT29 cells, which correlates with the v-Src-induced levels found for the mouse orthologues in Table 4 (for both cell comparisons A and B). In contrast, HMGB3 transcript levels were similar in HT29 and HCT116 cells, correlating with the finding that its mouse orthologue was likely not induced by v-Src (Table 4, column B). Taken together, these data strongly suggest that activated Src and SSeCKS/Gravin control similar sets of
genes in both human and mouse fibroblasts and epithelial cells.

Conclusion
We have identified sets of genes whose expression is controlled either by SSeCKS or v-Src alone, or only after re-expression of SSeCKS in the presence of activated v-Src. Our data suggest that some or all of the SSeCKS-regulated genes may either directly antagonize v-Src-induced oncogenesis or may serve as markers for non-oncogenic cells. Moreover, our analysis has identified a set of genes previously thought to contribute to the oncogenic phenotype.
yet which are insufficient to induce oncogenesis in the presence of physiologic levels of SSeCKS. In sum, our data indicate that SSeCKS may antagonize Src-induced oncogenesis through the normalization of functions controlling mitogenic signaling pathways, cell cycle progression, transcriptional regulation and apoptosis.

**Abbreviations**

SSeCKS, Src Suppressed C Kinase Substrate; PKC, protein kinase C; RT-PCR, reverse transcription-polymerase chain reaction; VEGF, vascular endothelial growth factor; PT, permissive temperature; NPT, non-permissive temperature; PAb, polyclonal antibody; MAb, monoclonal antibody; Ig, immunoglobulin; tet, tetracycline.

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors' contributions**

YL performed the work as a postdoctoral fellow in the lab of IHG and thus, both contributed to the conceptual framework of this study. Both YL and IHG produced and approved the final written manuscript. LG performed many of the semi-quantitative RT-PCR assays.

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Table 5: Genes regulated by SSsCKS in v-Src transformed cells. A value ≥ 2 represents induction; a value ≤ 0.5 represents repression.

| Gene                                      | Symbol | Modulation |
|-------------------------------------------|--------|------------|
| **Signal transduction**                   |        |            |
| Cytokine inducible kinase                 | Plk3   | 52.35      |
| G protein-coupled receptor 56             | Gpr56  | 49.00*     |
| Guanine nucleotide binding protein (G protein), gamma 2 subunit | Gng2 | 7.16* |
| Protein tyrosine phosphatase, non-receptor type 1 | Ptpn1 | 5.86 |
| Serine/threonine kinase 4                 | Stk4   | 3.97       |
| RAB20, member RAS oncogene family         | Rab20  | 3.92       |
| Phosphatidylinositol-4-phosphate S-kinase, type 1 beta | Pip5k1b | 3.50 |
| Growth arrest and DNA-damage-inducible 45 alpha | Gad4d5a | 3.43 |
| Butyrate-induced transcript 1             | Ptplad1| 3.29*      |
| Suppressor of cytokine signaling 3        | Socs3  | 2.07***    |
| Serine/arginine-rich protein specific kinase 2 | Srpk2 | 0.45 |
| Mitogen activated protein kinase kinase 6 | Map2k6 | 0.44 |
| Regulator of G-protein signaling 2        | Rgs2   | 0.38       |
| Ras and Rab interactor 2                  | Rin2   | 0.33       |
| Dual specificity phosphatase 10           | Dusp10 | 0.17       |
| Myristoylated alanine rich protein kinase C substrate | Marcks | 0.12* |
| **Cell cycle and transcriptional regulator** |        |            |
| Histone 1, H2bp                           | Hist1h2bp | 3.61     |
| Cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4) | Cdkn2d | 2.46* |
| Retinoblastoma binding protein 7          | Rbbp7  | 2.39       |
| Histone 2, H3c2                           | Hist2h3c2 | 2.28 |
| v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F | Maff | 2.28*** |
| Inhibitor of DNA binding 2                | Id2    | 0.44***    |
| Transcription factor AP-4 (activating enhancer-binding protein 4) | Tclap4 | 0.26 |
| Cell division cycle 20 homolog (S. cerevisiae) | Cdc2a | 0.25* |
| Basic helix-loop-helix transcription factor 6 | Atoh6 | 0.24*** |
| High mobility group box 3                 | Hmgb3  | 0.24***    |
| Hypoxia inducible factor 1, alpha subunit | Hif1a | 0.22 |
| Kruppel-like factor 7 (ubiquitous)        | Klf7   | 0.10       |
| **Growth factor and receptor**            |        |            |
| Chemokine (C-C motif) ligand 2            | Ccl2   | 4.26       |
| Nerve growth factor, beta                 | Nfgb   | 2.38*      |
| Glial cell line derived neurotrophic factor family receptor alpha 1 | Gfra1 | 0.33*** |
| Interleukin 1 receptor-like 1             | II1r1  | 0.23***    |
| Platelet derived growth factor receptor, beta polypeptide | Pdgfrb | 0.18* |
| Transforming growth factor, beta 2        | Tgb2   | 0.14       |
| **Others**                                |        |            |
| S100 calcium binding protein A8 (calgranulin A) | S100a8 | 16.22 |
| Fatty acid desaturase 3                   | Fads3  | 8.00       |
| Heat shock protein 4                      | Hspa4  | 4.29       |
| Deubiquitinating enzyme I                 | Dub1   | 3.23       |
| alpha fetoprotein                         | Afp    | 0.50***    |
| Tissue inhibitor of metalloproteinase 3   | Timp3  | 0.33       |
| HIF-1 responsive RTP801                   | Ddit4  | 0.26       |
| S-azacytidine induced gene 1              | Az1    | 0.24       |
| Growth differentiation factor 5            | Gdf5   | 0.18       |
| Growth arrest specific 1                  | Gas1   | 0.18       |
| Caspase 7                                 | Casp7  | 0.18       |
| retinitis pigmentosa 1 homolog (human)    | Rph1   | 0.12       |
| protocadherin 18                          | Pcdh18 | 0.07       |

* S24/327v-Src MEF, 35 °C, + vs – tet.
* Similar effects when compared to SSsCKS overexpression alone (Table 3).
** Opposite effects when compared to v-Src activation (Table 4).
*** Similar effects when compared to v-Src activation (Table 4).
### Table 6: Comparison of microarray and RT-PCR data

| Gene       | SSeCKS Induction | SSeCKS induction plus v-Src activation | SSeCKS Induction | SSeCKS induction plus v-Src activation |
|------------|------------------|----------------------------------------|------------------|----------------------------------------|
| Cdc2a      | 0.46             | 0.25                                   | 0.27             | 0.25                                   |
| Il1r1      | 0.29             | 0.23                                   | 0.31             | 0.36                                   |
| Cdkn2d     | 2.73             | 2.46                                   | 3.10             | 3.50                                   |
| Pdgfrb     | 0.30             | 0.18                                   | 0.34             | 0.30                                   |
| Ptpn1      | NC⁰              | 5.86                                   | NC              | 3.04                                   |
| Tgfbi      | 2.33             | NC                                    | 2.50             | NC                                    |
| Id2        | NC              | 0.44                                   | NC              | 0.33                                   |
| Gpr56      | 126.24           | 49.00                                  | 45.0             | 28.0                                   |
| Maff       | NC              | 2.28                                   | NC              | 3.5                                    |
| Socs3      | NC              | 2.07                                   | NC              | 2.4                                    |
| Afp        | 0.32             | 0.50                                   | 0.4              | 0.2                                    |
| Ngb        | 2.28             | 2.38                                   | 8.0              | 2.5                                    |
| Gadd45a    | NC              | 3.43                                   | 4.0              | 3.0                                    |
| Marcks     | 0.48             | 0.12                                   | NC              | 0.2                                    |
| Hif1a      | NC              | 0.22                                   | NC              | 0.3                                    |

⁰, based on two independent experiments. SE < 0.005.
², from Table 3.
³, from Table 5.
NC, no change.

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