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Review

Tudor tells about new twists in the story tale of SMURFs

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Abstract: Smad ubiquitin regulatory factors (SMURFS) belong to the HECT- family of E3 ubiquitin ligases. This family has two members, SMURF1 and SMURF2. SMURFs have emerged as well studied negative regulators of TGF induced intracellular signaling. However, increasingly it is being realized that SMURFs tactfully modulate an array of proteins in different cancers. This review sets spotlight on how SMURF1 and SMURF2 communicate with effectors of different signaling pathways during the multistep progression to cancer. We also summarize how microRNAs (miRNAs) effectively control SMURFs in different cancers. Role of SMURFs is context dependent in different cancers and better concepts related to miRNA regulation of SMURFs in different stages and steps of cancer will be helpful in efficient translation of laboratory findings to clinic.

Key words: Cancer, apoptosis, signaling, SMURF, therapy.

Introduction

Cancer is a multifaceted and genomically complex disease and research over the decades has substantially helped us in developing a better knowledge of wide ranging molecular mechanisms which underpin cancer development and metastasis. Overexpression of oncogenes, inactivation of tumor suppressor genes, deregulation of spatio-temporally controlled intracellular signaling cascade are some of the mechanisms which drive carcinogenesis. Transforming growth factor-β1 (TGF-β1) induced intracellular signaling is a deeply studied molecular mechanism reported to trigger wide ranging cellular activities. TGF-β1 undergoes activation before its release as an active cytokine (1,2). It is now clear that TGF-β1 transduces the signals intracellularly through TGF receptor. Proteomic studies revealed that interaction of ligand-receptor induced receptor autophosphorylation followed by an increase in phosphorylated levels of R-SMAD (5). It has been shown that binding of inhibitory SMADs and SMURF induced degradation of TGFR. SMURF, a C2-WW-HECT-domain E3 ubiquitin ligase is instrumental in tight control of TGF induced signaling cascade (3,4) (Figure 1). Phosphorylated R-SMADs heteromerically and/or homomerically complex with co-mediator SMAD (Co-SMAD) and accumulate in nucleus to interact with DNA-binding cofactors, corepressors and/or coactivators to transcriptionally modulate target gene network (1,2). The review is partitioned into different sections which include oncogenic and tumor suppression roles of SMURF1 and how SMURF1 interacts with different proteins in different cancers. Next we summarize role of SMURF2 in cancer regulation and how different microRNAs effectively control SMURF expression in different cancers. We start with SMURF1 in upcoming section and briefly review the findings which have provided us a near complete resolution of SMURF mediated signaling landscape.

Oncogenic role

It had previously been convincingly revealed that sphere cells generated from head and neck squamous cell carcinoma (HNSCC) cells had CSC-like characteristics. Protein levels of SMURF1 were considerably higher in sphere cells grown in cell culture (7 days) as compared to levels of SMURF1 in monolayered cells (5). Interestingly, increased expression of SMURF1 was noted in $CD44^{high}$ as compared to $CD44^{low}$ cell populations. SMURF1 suppressed of BMP induced intracellular signaling for maintenance of CSC-like population. The results indicated that SMURF1 knockdown partially reactivated BMP signaling cascade as evidenced by an increase in pSMAD1/5/8 levels (5). Mechanistically it has been shown that SMAD1 structurally interacted with intracellular domain of CD44 however SMURF1 inhibited the interaction via degradation of SMAD proteins. Moreover, SMURF1 knockdown sphere cells lost their ability for self-renewal and anchorage-independent

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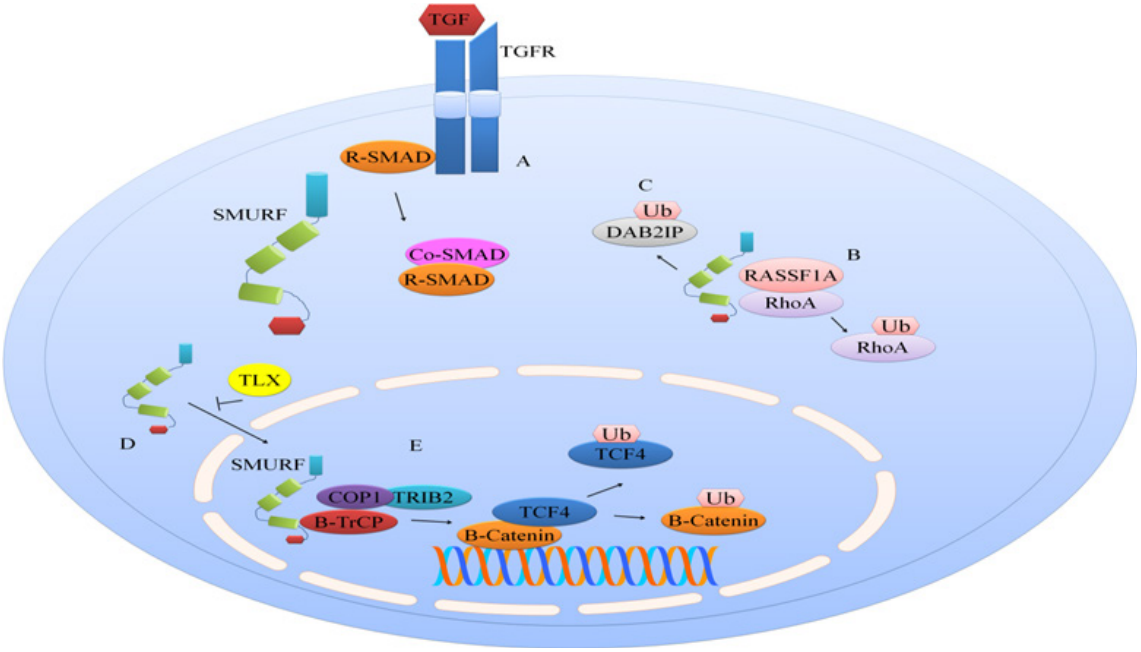

Figure 1. shows (A) TGF mediated intracellular signaling that involved receptor phosphorylation and consequent activation of R-SMADs. These SMADs interacted with co-SMADs and migrated into the nucleus to stimulate expression of target genes. (B) SMURF1 interacted with RASSF1A to ubiquitinate RhoA. (C) DAB2IP is also ubiquitinated by SMURF1. (D) Nuclear accumulation of SMUF1 is also reported. However, TLX inhibited translocation of SMURF1 into nucleus. (E) SMURF1 interacted with TRIB2 for shuttling into the nucleus to ubiquitinate its substrates.

survival (5).

mTOR, a Ser/Thr kinase translationally controlled SMURF1. EGF/PI3K/mTOR signaling axis modulated expression of SMURF1 (6). Endogenous levels of SMURF1 had been noted to increase considerably after treatment with EGF, but these levels declined significantly in cells treated combinatorially with inhibitors LY294002 or rapamycin and EGF (6). Casein kinase-2 interacting protein-1 (CKIP-1) enhanced SMURF1 auto-degradation. CKIP-1 inhibited activity of Akt in colon SW480 and HCT116 cancer cells and reduced SMURF1 levels (6).

10 ng/ml of EGF transcriptionally increased expression levels of SMURF1 in MDA-MB-231 cells (7). Interaction of growth factor with EGFR induced homo-dimerization or hetero-dimerization with members of the ErbB family to transduce signals to downstream effectors, including phospholipase Cγ1 (PLCγ1)/PKC and Ras/MAPK pathways (7). EGF-induced rise in expression levels of SMURF1 was notably reduced in cancer cells treated combinatorially with EGF and ERK1/2 inhibitor or PKC inhibitor (7).

Expression levels of SMURF1 were significantly higher in cancer cell lines with 7q22.1 gain/amplification (8). SMURF1 amplification efficiently promoted anchorage independent growth and cellular invasion. Contact inhibition was significantly lost in SMURF1 overexpressing pancreatic cancer cells (8).

Tumor suppressing role

SMURF1 structurally interacted with the N-terminal of the TRIB2 protein to post-translationally regulate TRIB2 (9). Shown in figure 1. There is evidence of greater accumulation of COP1, β-TrCP and SMURF1 in nucleus in TRIB2-FLAG-expressing Bel-7402 cells. TRIB2 contained 3 E3 ligase binding sites to interact with COP1, β-TrCP and SMURF1 as evidenced by corresponding antibodies in the immunoprecipitates pull down assay by using anti-TRIB2 antibodies which suggested that TRIB2 and these ligases assembled into multi-protein machinery (9). TRIB2 inhibited Wnt induced intracellular signaling by facilitating the transportation of SMURF1 into the nucleus. TRIB2 overexpression significantly reduced colony formation, cell proliferation and tumor growth in xenografted mice (9). Certain hints have emerged suggesting that p70 S6 kinase (p70S6K) phosphorylated TRIB2 to promote SMURF1 mediated degradation (10). There is a rapidly increasing list of proteins reported to interact with SMURFs and recently emerging molecular studies have greatly improved our understanding of interaction between regulatory proteins and SMURF1 in different cancers.

Interaction of SMURF1 with different proteins

RASSF1A

RASSF1A modulated SMURF1-mediated ubiquitination of RhoA by directly binding to SMURF1 (11). Shown in figure 1. Detailed mechanistic insights revealed that RASSF1A interacted with SMURF1 and formed a protein complex with SMURF1 and RhoA. HECT domain of SMURF1 interacted with N-terminal amino acids 69-82 of RASSF1A. Mutant RASSF1A lacking either – RhoA or SMURF1-binding motif failed to degrade RhoA (11) Mutant RASSF1A expressing cells had remarkably higher tumor cell proliferation, epithelial to mesenchymal transition (EMT), drug resistance and metastasis (11).

TLX

TLX (NR2E1), an orphan nuclear receptor is frequently overexpressed in glioblastoma. TGF-β mediated intracellular signaling in TLX silenced glioma cells as evidenced by increased Smad2 phosphorylation (12). Furthermore, there was a noteworthy rise in

levels of type II and type I TGF-β receptors. Immunofluorescence assays indicated a rise in nuclear levels of SMURF1 and decrease in cytosolic levels in TLX silenced cells. The results revealed that TLX interacted with and retained SMURF1 in the cytoplasm. Shown in figure 1. Surprisingly, SMURF1 itself is regulated by both auto-ubiquitination and SMURF2-dependent ubiquitination (12). Levels of SMURF1 were markedly reduced in 293T cells exogenously expressing SMURF2 and SMURF1. However there was a rise in SMURF1 levels in cells coexpressing TLX, SMURF2 and SMURF1 (12).

DAB2IP

Deletion of ovarian carcinoma 2/disabled homolog 2 (DOC-2/DAB2) interacting protein (DAB2IP), a tumor suppressor is frequently downregulated in cancer. SMURF1 interacted with and triggered DAB2IP degradation (13). Shown in figure 1. Half-life of SMURF1-T145E mutant was higher as compared to wild-type SMURF1, and a non-phosphorylatable T145A had a shorter half-life. Data suggested that SMURF1 is controlled by Akt-mediated phosphorylation to stabilize SMURF1 protein. Akt/SMURF1 oncogenic signaling cascade promoted proliferation and migration of the cells largely through degradation of the DAB2IP (13).

MCAM and CD166

Both Melanoma Cell Adhesion Molecule (MCAM) and Cluster of Differentiation 166 (CD166) have been shown to contribute in maintenance of transformative phenotype of Hepatocellular Carcinoma (HCC) cells (14). CD166 tactfully regulated MCAM by protecting it from ubiquitin-mediated degradation. CD166 has been shown to down-regulate β-TrCP and SMURF1 which degrade MCAM protein (14). Moreover, reduction in transformative phenotype of SMURF1 and β-TrCP overexpressing cells was reversed partially by MCAM thus providing evidence of MCAM targeting by β-TrCP and SMURF1 (14). c-Raf/MEK/ERK signal transduction axis acted as a downstream effector of CD166/PI3K/AKT axis to trigger ubiquitination and degradation of SMURF1 and β-TrCP (14).

SND1 and RhoA

SND1, an AEG-1/MTDH/LYRIC-binding protein is frequently over-expressed in different cancers. Tumor growth was significantly lower in nude mice orthotopically implanted with SND1 silenced breast cancer MDA-MB-231 cells (15). It is intriguing to note that TGF transcriptionally upregulates SND1 in MCF-7 cells. SMURF1 promoted degradation of the small GTP protein RhoA by increasing its ubiquitination (15). Considerably enhanced SMURF1 levels and markedly reduced levels of RhoA were noted in breast cancer cells that ectopically overexpressed SND1 (15). SND1 remarkably enhanced metastasizing capability of breast cancer cells by SMURF1-mediated degradation of RhoA (15).

Nedd8

Detailed studies have shown that SMURF1 interacted with Nedd8 E2 (Ubc12) and Nedd8 and utilized a unique activation site within HECT N-lobe to facilitate transfer of Nedd8 to conjugate covalently (16). SMURF1 has been shown to be conjugated with Nedd8 as evidenced by migration of SMURF1 as high molecular weight (HMW) bands in presence of NAE, Nedd8 and Ubc12 (16). C699 ubiquitylation site was identified in the C-lobe, whereas C426 neddylation site was identified in N-lobe larger sub-domain of HECT. These sites have structural similarities. SMURF1 auto-neddylation stimulated its ubiquitin E3 ligase activation and consequent substrate degradation. SMURF1 is frequently over-expressed in colorectal cancer tissues (16). C426 was noted to be critically important for auto-ubiquitylation of SMURF1 however C699 did not play any role in auto-neddylation of SMURF1 that provided a clue that SMURF1 neddylation preceded SMURF1 ubiquitylation (Xie et al, 2014). Expectedly, C426A mutation dramatically reduced auto-neddylation of SMURF1 comparable to the results obtained after treatment with MLN4924 or Ubc12 and Uba3 depletion. C426A mutation severely impaired formation of SMURF1 thioester bond with Nedd8. Auto-ubiquitylation of SMURF1(wild-type) was markedly enhanced in cells that ectopically expressed Nedd8 however no such findings were noted in cells that expressed ligase-defective C699A mutant (16). Auto-ubiquitylation of SMURF1 was also significantly compromised in Nedd8 depleted cells. Both tumor weight and volume were drastically enhanced in mice implanted with cancer cells reconstituted with SMURF1-WT or C530A (16). Contrarily, cells reconstructed with either SMURF1 C699A or C426A did not trigger tumor growth and invasion (16).

TRAF4

Tumor necrosis factor receptor-associated factor 4 (TRAF4), a member of TRAF family of adaptor proteins is frequently upregulated in breast cancer. K190 was noted as the site for SMURF1-induced mono-ubiquitination that promoted cellular migration (17).

IQGAP1

IQ motif containing GTPase activating protein 1 (IQGAP1) has been shown to efficiently inhibit TβRII-transduced signals intracellularly in pericytes to suppress differentiation of myofibroblasts in tumor microenvironment (18). TGF-β1 temporally increased binding of IQGAP1 to TβRII, thus showing that TGF-β1 treatment enhanced binding of IQGAP1 to TβRII-containing signaling complexes. Ubiquitination of TβRII was markedly inhibited in IQGAP1 silenced HSCs (18). Another important finding of the study was that both distribution of SMURF1 at plasma membrane and TβRII/ SMURF1 co-presence at plasma membrane were dramatically reduced in IQGAP1 silenced HSCs (18).

USP9X/FAM

Ubiquitin-specific peptidase 9, X-linked (USP9X/FAM) has previously been reported to effectively deubiquitinate SMURF1 (19). Carboxyl terminal of USP9X and 2nd WW domain of SMURF1 interacted to stabilize SMURF1. SMURF1 (wild type) degradation was notably higher in USP9X-depleted cells as compared to ligase activity deficient SMURF1 (19). In line with the findings that SMURF1 (wild type) can be auto-ubi-

quitinated through its own E3 ligase, it was suggested that USP9X stabilized SMURF1 by exerting inhibitory effects on auto-ubiquitination activity of SMURF1. SMURF1-mediated cellular migration was notably reduced in USP9X depleted MDA-MB-231 cells (19).

Role of SMURF2

SMURF2 induced SMURF1 degradation, however SMURF1 did not trigger degradation of SMURF2. SMURF2 induced ubiquitination of catalytically inactive SMURF1 mutant in breast cancer cells. Frequency of bone metastasis in tibiae as well as whole body of mice inoculated with SMURF2 depleted breast cancer cells was higher as compared to controls (20). Interestingly, the area of bone metastasis in the tibiae of mice xenografted with SMURF2 depleted breast cancer cells was larger as compared to mice inoculated with control transfected or SMURF1 depleted breast cancer cells (20).

Bortezomib dose-dependently inhibited SMURF2 and SMURF1 mRNA levels and most effectively at 50 μmol/l after 72 h of treatment. Prostate cancer cell proliferation was notably reduced by Bortezomib at a dosage of 50 μmol/l (21).

Cancer promoting role of SMURF2

CNKSR2 (Connector enhancer of kinase suppressor of ras2), an evolutionarily conserved scaffold protein has been reported to be involved in different signaling cascades (22). Surprisingly, SMURF2 stabilized CNKSR2 levels in cancer cells. Depletion of SMURF2 notably reduced CNKSR2 levels in breast cancer and colon cancer cells. Phosphorylated AKT levels were also substantially reduced in SMURF2-depleted cancer cells. FoxO proteins are transcription factors which transcriptionally control expression of different genes in nucleus (22). AKT mediated phosphorylation induced nuclear export of FoxO proteins. However, cytosolic accumulation of FoxO proteins was notably reduced in SMURF2-depleted cancer cells. Resultantly, nuclear accumulation of FoxO proteins increased in SMURF2-depleted cancer cells that resulted in transcriptional upregulation of p27/Kip1 and p21/waf1 (22).

SMURF2 stabilized mutant KRAS levels in cancer cells. There were markedly reduced mutant KRAS levels in SMURF2 depleted mutant KRAS-driven lung adenocarcinoma cells H441 ($KRAS^{G12V}$) and H358 ($KRAS^{G12C}$) (23). β-TrCP1 has been studied to degrade mutant KRAS. Moreover, β-TrCP1 levels were notably reduced in SMURF2 (wild-type) overexpressing cancer cells. SMURF2-mediated mono-ubiquitination of UBCH5 has been observed to be significant for poly-ubiquitination of β-TrCP1 and stability of KRAS levels (23).

miRNA regulation of SMURF1 and SMURF2 in cancers

MicroRNAs (miRNAs) belong to a small non-coding RNA family reportedly involved in modulation of a wide array of biological processes including carcinogenesis (24). The rapidly increasing intricacy of this network has opened new avenues in cancer research as miRNAs, produced from what was once considered "genomic trash," are essentially important for cancer initiation, metastasis and resistance acquired by cancer cells against different therapeutic strategies (24). The following section deals mainly with regulation of SMURFs by miRNAs.

miR-497 reconstitution considerably reduced migration and invasion of ovarian cancer cells. SMURF1 is negatively regulated by miR-497 in different cancers (25, 26). Shown in figure 2. miR424–503 mediated inhibition of Smad7 and SMURF2 effectively promoted metastasis (27). Shown in figure 2. There was notable development of lung metastasis in mice injected with miR424–503 expressing breast cancer cells however, there was no detectable metastatic development in mice injected with control MCF7 cells (27). Similar results were obtained upon inoculation of miR424–503 expressing MDA-MB-231 breast cancer cells in mice. However transduction of cells either with miR424–503/SMURF2 ORF or miR424–503/Smad7 ORF inhibited metastasis-promoting features of miR424–503 (27). The results were indicative of the fact that Smad7 and SMURF2 inhibited miR424–503-induced breast cancer

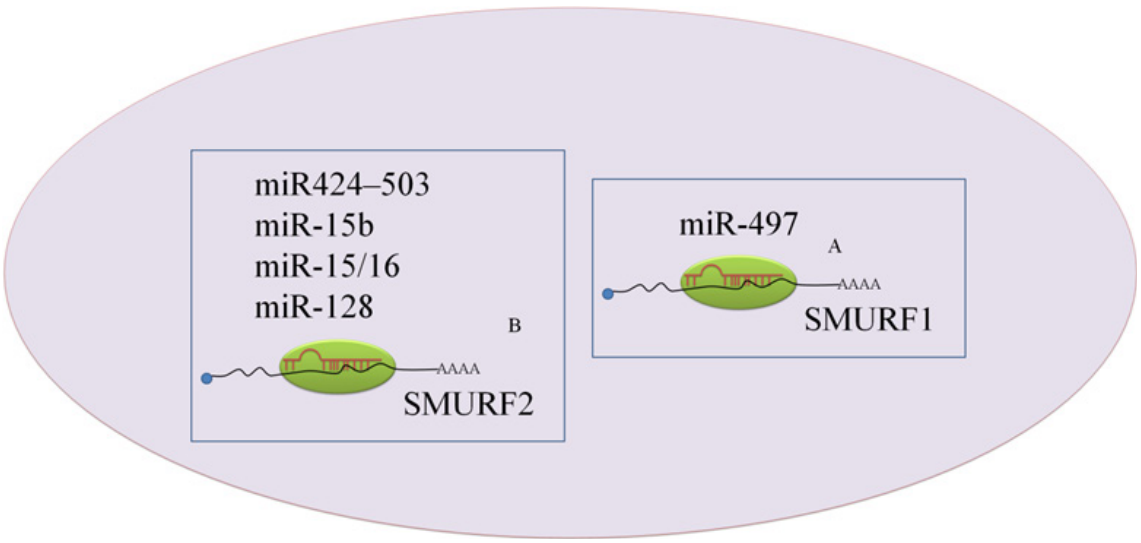

Figure 2. shows miRNA regulation of SMURF1 and SMURF2.

metastasis (27).

miR-15b had been noted to negatively regulate SMURF2 and promoted EMT in pancreatic cancer (28). miR-15b directly targeted 3'-UTR of SMURF2 in pancreatic cancer BxPC-3 cells as evidenced by luciferase reporter assay (28). Luciferase reporter assay provided evidence that luciferase activities of SMURF2-WT-luc plasmids were considerably repressed in pre-miR-15b transfected cancer cells (28). SMURF2 mRNA is a novel target of miR-322/503 and miR-322/503 are involved in translational repression of SMURF2 by interacting with SMURF2 3′-UTR (29).

miR-15/16 and miR-128 modulated downregulation of SMURF2. Shown in figure 2. SMURF2 levels were notably enhanced in triple negative breast cancer cells transfected with antagomirs of miR-16, miR-15a, miR-15b and miR-128 (30).

Conclusion

Wealth of information has helped us in developing a better comprehension of how cells read TGF-β induced signals and much progress has been made in demystification of underlying mechanisms. Targeting of structural association of SMURFs with their adaptor proteins, substrates or the regulators of these proteins will be helpful in identification of effective drug targets. Natural products mediated regulation of SMURFs in different cancers is insufficiently explored. Although attempts have been made to understand how natural products modulate SMURF mediated control of protein network in cancer cells. For example, Fucoidan, a polysaccharide isolated from brown seaweeds effectively increased conjugation of Smad7 and SMURF2 to TGFRs (31). Fucoidan treatment increased binding of SMURF2 and Smad7 to TGFRII and TGFRI in cancer cells. Lewis lung carcinoma (LLC1) cells were inoculated into the hypodermic dorsum of mice and tumor volume reduced dose-dependently in mice orally administered with Fucoidan (31).

In addition to functional studies, such as transgenic and gene knockout approaches, it will be interesting to further expand the proteins they target for ubiquitination. Systematic identification of substrates and substrate specificity for these SMURFs using protein array and global proteomics approaches is rquired.

Undoubtedly, deeper and sharper understanding related to SMURFs–adaptor and inhibitor–substrate interactions will be helpful in designing of the drugs with maximum efficacy and minimum off target effects.

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