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Sulf-2, a heparan sulfate endosulfatase, promotes human lung carcinogenesis

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Abstract

Heparan sulfate proteoglycans (HSPGs) bind to multiple growth factors/morphogens and regulate their signaling. 6-*O*-sulfation (6S) of glucosamine within HS-chains is critical for many of these ligand interactions. Sulf-1 and Sulf-2, which are extracellular neutral-pH sulfatases, provide a novel post-synthetic mechanism for regulation of HSPG function by removing 6S from intact HS-chains. The Sulfs can thereby modulate several signaling pathways, including the promotion of Wnt signaling. We found induction of *SULF2* transcripts and Sulf-2 protein in human lung adenocarcinoma and squamous cell carcinoma, the two major classes of non-small cell lung cancers (NSCLC). We confirmed widespread Sulf-2 protein expression in tumor cells of 10/10 surgical specimens of human lung squamous carcinomas. We studied five Sulf-2⁺ NSCLC cell lines, including two which were derived by cigarette-smoke transformation of bronchial epithelial cells. shRNA-mediated Sulf-2 knockdown in these lines caused an increase in 6S on their cell surface and in parallel reversed their transformed phenotype in vitro, eliminated autocrine Wnt signaling, and strongly blunted xenograft tumor formation in nude mice. Conversely, forced Sulf-2 expression in non-malignant bronchial epithelial cells produced a partially transformed phenotype. Our findings support an essential role for Sulf-2 in lung cancer, the leading cancer killer.

Keywords

lung cancer; Sulf-2; sulfatase; heparan sulfate proteoglycans

INTRODUCTION

Lung cancer is the leading world-wide cause of cancer deaths with the majority of cases resulting from the exposure of airways to tobacco-smoke carcinogens (Sato *et al.*, 2007).

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Further understanding of the molecular basis of lung cancer is needed, since current treatment options are frequently inadequate (Osada and Takahashi, 2002; Sato *et al.*, 2007). Although heparan sulfate proteoglycans (HSPGs) are ubiquitous elements of the cell surface/ECM and perform critical signaling functions in development and normal physiology (Bernfield *et al.*, 1999; Bishop *et al.*, 2007; Habuchi *et al.*, 2004), they have received scant attention in lung cancer. HSPGs consist of heparan sulfate (HS) chains containing repeating uronic acid and glucosamine disaccharide units with the chains covalently linked to a restricted set of core proteins. The functions of HSPGs are mediated through their binding to a myriad number of ligands, including growth factors and morphogens. The pattern of four sulfation modifications on the sugars largely dictates binding specificity (Esko and Lindahl, 2001; Gallagher, 2001). The 6-*O*-sulfation (6S) of glucosamine is known to be critical in the binding of many protein ligands (Gallagher, 2001; Habuchi *et al.*, 2004). One mechanism for the spatiotemporal regulation of 6S in the embryo and adult is through expression of the heparan sulfate 6-*O*-sulfotransferases, which add sulfate esters to nascent HS chains (Brickman *et al.*, 1998; Habuchi *et al.*, 2004; Habuchi *et al.*, 2007).

A novel post-synthetic mechanism for regulating 6S on HSPGs emerged several years ago with the cloning and characterization of two novel extracellular sulfatases (Sulf-1 and Sulf-2) in human (Morimoto-Tomita *et al.*, 2002), following the original identification of Sulf-1 in the quail embryo (Dhoot *et al.*, 2001). These neutral-pH endosulfatases remove 6S in highly sulfated subdomains of intact heparin/HSPGs (Ai *et al.*, 2003; Lamanna *et al.*, 2008; Morimoto-Tomita *et al.*, 2005; Viviano *et al.*, 2004). Desulfation of HSPGs/heparin by the Sulfs reduces interactions with protein ligands, including Wnts, FGFs, and GDNF (Ai *et al.*, 2003; Ai *et al.*, 2007; Uchimura *et al.*, 2006), and modulates their signaling. The importance of these enzymes in development is established by the abnormal phenotypes observed in single and double *sulf* knockouts in mice (Ai *et al.*, 2007; Holst *et al.*, 2007; Lum *et al.*, 2006; Ratzka *et al.*, 2008). Consistent with many precedents for onco-developmental genes, Sulf-2 has been suggested to be relevant in several cancers (Lai *et al.*, 2008; Morimoto-Tomita *et al.*, 2005; Nawroth *et al.*, 2007). Here, we present evidence that Sulf-2 promotes human lung carcinogenesis.

RESULTS

Sulfs are expressed in lung tumors and lung cancer cell lines

Initially, we looked for dysregulated expression of *SULF* genes in human lung cancer by mining public microarray data. Relative to normal lung, *SULF1* was markedly increased by 3–6 fold in both adenocarcinoma ($p = 0.006$) and squamous cell carcinoma ($p = 0.004$) (Supplementary Fig. 1a), the two major classes of non-small cell lung carcinoma. Data from the Consortium for Functional Glycomics (<http://www.functionalglycomics.org/glycomics/publicdata/microarray.jsp>) provided information about both *SULFs* in lung cancer (Fig. 1a). Paired samples of lung squamous carcinoma and nonmalignant neighboring tissue were obtained from 10 patients undergoing surgical resection. *SULF1* increased in 10/10 pairs with a mean increase of 18 ± 2.4 -fold ($p=0.0005$). *SULF2* increased in 8/10 pairs with a mean increase of 3 ± 0.3 -fold ($p=0.003$). qPCR analysis of *SULF1* and *SULF2* in archived

cases of lung carcinoma verified these findings (Fig. 1b,c). (SULF1 and SULF2 increased 12 ± 1.5 fold ($p=0.008$) and 4 ± 0.3 fold ($p=0.05$), respectively in squamous carcinomas and 3 ± 0.4 fold for both ($p=0.003$ and $p=0.002$, respectively) in adenocarcinomas).

We next determined *SULF* expression in a series of NSCLC cell lines, all of which form tumors in immunocompromized mice (Supplementary Table 1). In addition, we evaluated two novel cell lines, B-ST and P-ST, which were obtained by exposing BEAS2B cells (a non-malignant human bronchial epithelial cell line, denoted “B-C” for “BEAS2B-control”) and primary human bronchial epithelial cells, (denoted “P-C” for “primary-control”) to an aqueous extract of cigarette smoke (Lemjabbar-Alaoui *et al.*, 2006). B-ST and P-ST (“ST” denotes “smoke-transformed”) are immortalized, exhibit a transformed phenotype in culture, and form tumors when injected into nude mice (Lemjabbar-Alaoui *et al.*, 2006). The tumors have features of squamous cell carcinoma (Supplementary Fig. 2), and thus B-ST and P-ST cells can be classified as NSCLC lines. By RT-PCR (Fig. 1d) and qPCR (Supplementary Fig. 1b), B-ST and P-ST cells strongly expressed *SULF2*, whereas the parental cells (B-C and P-C) had minimal levels. In addition, 3/16 of the conventional NSCLC cell lines were positive for *SULF1* and 5/16 were positive for *SULF2*. No line showed simultaneous expression of both *SULFs*. Among the *SULF*⁺ cell lines, four could be classified as squamous cell carcinomas, five as adenocarcinomas, and one as a large cell carcinoma (Supplementary Table 1). There was no statistically-significant correlation between *SULF* expression and whether or not the cell lines harbored oncogenic mutations in *KRAS* or *EGFR* (Supplementary Table 1).

We concentrated on Sulf-2 for mechanistic studies because of its more frequent expression in the lines (7/18). We confirmed that all seven *SULF2*⁺ lines (including B-ST and P-ST) were strongly positive for Sulf-2 protein in both conditioned media (75 kDa) and detergent lysates (125 kDa) (Fig. 1e). The 125 and 75 kDa components correspond respectively to the unprocessed pro-protein and the amino-terminal subunit of Sulf-2 (Morimoto-Tomita *et al.*, 2002; Tang and Rosen, 2009). B-C and P-C cells showed no detectable signal. Thus, smoke-induced transformation is accompanied by a dramatic induction of Sulf-2.

We performed immunocytochemistry for Sulf-2 in NSCLC tumors (ten cases each of squamous cell carcinoma and adenocarcinoma) selected from archived surgical specimens (Supplementary Table 2). Normal lung tissue from the same specimens served as controls. Paraffin sections were stained with a newly developed Sulf-2 mAb (Supplementary Fig. 3). All ten cases of squamous cell carcinoma showed some degree of staining for Sulf-2 in the tumor cells. The staining was patchy and accentuated in the cells at the periphery of nests (Fig. 2). The percentage of positive cells was variable and the intensity of staining ranged from faint to intense (Supplementary Table 2). No correlation was found between tumor differentiation and the percentage of positive cells or intensity of staining. In striking contrast, there was no staining of adenocarcinoma cells in any of the cases. This was surprising since three of the NSCLC cell lines with *SULF2* expression (Calu-3, Calu-6, and A549) could be classified as adenocarcinomas (Supplementary Table 1). However, both adenocarcinomas and squamous cell carcinomas showed conspicuous staining of tumor stroma, including spindle-shaped cells and endothelial cells of blood vessels. In control

tissue distant from the tumors, endothelial cell staining was also seen (Fig. 2b), but normal airway epithelium, except for rare basal cells, was negative for Sulf-2.

Knockdown of Sulf-2 or expression of dominant-negative Sulf-2 reduces growth of lung cancer cells

We employed a previously developed lentiviral shRNA strategy (Nawroth *et al.*, 2007) to achieve knockdown of Sulf-2 in NSCLC lines. We transduced five of the Sulf-2⁺ lines (H460, H292, Calu-6, P-ST and B-ST cells) with either a specific shRNA (PLV-1413 or PLV-1143) (Nawroth *et al.*, 2007) or a control shRNA (PLV-Ctrl) containing an irrelevant sequence. Sulf-2 protein expression was strongly reduced (>90%) in all of the lines after transduction with PLV-1413 or PLV-1143, as compared to the control cells (Fig. 3a). To determine the effects of Sulf-2 knockdown on the sulfation status of HSPGs, we used a phage-display antibody (RB4CD12), whose binding to HSPGs depends on 6S (Dennissen *et al.*, 2002). We have verified that this antibody can be used as a cell surface-staining reagent to report the presence of the Sulfs within cells (M. Hossain, T. Hosono, R. Tang, T. H. van Kuppevelt, G. J. Jenniskens, S. D. Rosen, and K. Uchimura, manuscript submitted). Transduction of Sulf-2⁺ lines with PLV-1413 increased the RB4CD12 epitope on the cell surface relative to that of control-treated cells by 30–37% (Fig. 3b). The same treatment had no effect on the epitope in a Sulf-2⁻ line (H1975).

Whereas no difference in the growth was observed between the PLV-Ctrl transduced cells and the parental cells, both *SULF2*-specific shRNAs induced a marked reduction in the 5 Sulf-2⁺ lines (46–50 % reduction) (Fig. 3c). PLV-1413 transduction had no effect on H1975 cells compared to controls. Mutation of two cysteines in the catalytic domain of HSulf-2 (S2 CC) totally abrogates its sulfatase activity (Morimoto-Tomita *et al.*, 2002) and results in a dominant negative form (Nawroth *et al.*, 2007). Transfection of S2 CC into the five Sulf-2⁺ lines decreased cell growth but had no effect on H1975 cells (Supplementary Fig. 4).

Transduction of cells with either of the two Sulf-2 specific shRNAs inhibited cell proliferation, assayed by measuring BrdU incorporation, by 44 to 50% in all five Sulf2⁺ lines, when compared to controls (Fig. 3d). Sulf-2 knockdown increased apoptosis (annexin V staining) by 2–3 fold in the Sulf-2⁺ lines (Fig. 3e). In both cases, there were no effects on H1975 cells. These results indicate that Sulf-2 promotes cell growth in lung cancer cell lines by increasing both proliferation and cell survival.

Sulf-2 knockdown also induced 40–60% increased cell-substratum adhesion to fibronectin or collagen (Supplementary Fig. 5a), and 15–45% decreased serum-induced cell migration (Supplementary Fig. 5b). Correlated with this increased adhesion, the mobility of PLV-1413 transduced cells (H292 and P-ST), measured after “wounding” cell monolayers, was reduced by 37–50% (Fig. 3f). There was no difference in wound closure between PLV-1413-transduced and control H1975 cells (not shown).

Anchorage-independent cell growth is one of the hallmarks of transformed cells and is exhibited by the NSCLC lines and the smoke-transformed lines studied above. We therefore investigated the effect of Sulf-2 knockdown on the ability of the cells to grow in soft agar. Sulf-2 knockdown in the Sulf2⁺ lines reduced the number of colonies formed in soft agar by

70–82% (Fig. 3g), as compared to controls. In contrast, transduction of PLV-1413 had no effect on colony formation by H1975 cells.

Sulf-2 over-expression in non-malignant bronchial epithelial cells induces a transformed phenotype in culture

These loss-of-function findings suggested that Sulf-2 may have a transforming capability in lung epithelial cells. To examine the functional consequences of Sulf-2 expression in lung cells, we overexpressed either active or inactive Sulf-2 (S2^{CC}) in two non-malignant human bronchial epithelial cell lines (BEAS2B and 16HBE14o-), both of which are negative for the Sulfs. Transduction resulted in Sulf-2 expression levels comparable to those found in high-expressing lung cancer cell lines (Fig. 4a).

Sulf-2 expression in both BEAS2B and 16HBE14o- cell lines resulted in decreased staining by RB4CD12, indicating that the enzyme had the expected activity on cell surface HSPGs (Fig. 4b). However, S2^{CC} expression in these cells had no effect on this epitope, confirming the inactivity of this Sulf-2 mutant. Cells expressing S2^{CC} retained the flattened shape of the parental cells, whereas cells with active Sulf-2 (Supplementary Fig. 6a) were more rounded. In keeping with these morphological changes, the cells with active Sulf-2 exhibited decreased cell-substrate adhesion by 40–60% (Supplementary Fig. 6b). Furthermore, in both the wound repair assay (Fig. 4c) and chemotaxis assay (Supplementary Fig. 6c), the migration of active Sulf-2 expressing cells was markedly enhanced relative to that of cells with S2^{CC} or parental cells. With respect to cell growth, active Sulf-2, but not S2^{CC}, induced a modest (25–28%) but statistically significant increase (Fig. 4d). As for anchorage-independent growth, BEAS2B and 16HBE14o- cells, expressing Sulf-2, formed colonies in soft agar after 18 days, whereas neither parental cells nor S2^{CC}-transduced cells formed any colonies (Fig. 4e). We established 9 clones of Sulf-2-expressing BEAS2B cells from colonies in soft agar, all of which showed increased cell proliferation (not shown), cell survival (Fig. 4f), and anchorage-independent growth upon re-testing (not shown). Thus, over-expression of Sulf-2 in lung epithelial cells induced phenotypic characteristics of transformed cells.

Sulf-2 knockdown reduces growth of tumors arising from lung cancer cells

To examine the contribution of Sulf-2 to the tumorigenicity of the NSCLC cell lines, we injected control-transduced or Sulf-2 knockdown cells subcutaneously into nude mice and monitored tumor growth. Tumors derived from H460, H292, Calu-6, P-ST and B-ST cells with knockdown grew at markedly slower rates than those from the control cells (60–85% reduction) (Fig. 5). Furthermore, the harvested tumors were greatly reduced in weight (Supplementary Fig. 7), consistent with an observed decrease in cell proliferation and an increase in apoptosis within the tumors (Supplementary Figs. 8a and 8b). Transduction of H1975 cells with *SULF2* shRNA had no effect on tumorigenicity (Fig. 5).

To examine the effect of Sulf-2 overexpression on non-malignant bronchial epithelial cells, we injected BEAS2B or 16HBE14o- cells transduced with Sulf-2 into nude mice. Even after 2 months, no tumors formed from either cell type with or without Sulf-2 (not shown). Thus, although Sulf-2 overexpression in bronchial epithelial cells induced many features of

malignant transformation in culture, the introduction of Sulf-2 alone did not result in full transformation to a tumorigenic state.

Sulf-2 knockdown or expression of catalytically inactive Sulf-2 inhibits autocrine Wnt signaling in lung cancer cells

Previous work has implicated the Sulfs as positive regulators of Wnt signaling in development (Dhoot *et al.*, 2001; Freeman *et al.*, 2008) and in pancreatic cancer cell lines (Nawroth *et al.*, 2007). These enhancing effects were demonstrated for canonical Wnt signaling, which involves the translocation of β -catenin to the nucleus and its participation in a transcriptional complex (Clevers, 2006; Logan and Nusse, 2004). The analysis of Emerson and colleagues (Ai *et al.*, 2003) strongly supports a model whereby HSPGs sequester Wnt ligands and the Sulf acts to reduce the affinity of HSPG for Wnt, freeing or altering Wnt association with HSPGs, and thus allowing Wnt access to its signal-transduction receptors on the cell surface. To determine whether the five Sulf-2⁺ lines exhibited canonical Wnt signaling, we used the TOP/FOP flash assay (Clevers, 2006), which quantifies β -catenin-dependent transcriptional activity. All five cell lines exhibited TOP/FOP flash activity, with a range from 4 to 13 (Supplementary Fig. 9a). To check whether the signaling was autocrine in nature (i.e., triggered by extracellular Wnts), we employed sFRP and WIF-1, which are soluble extracellular inhibitors of Wnt signaling (Kawano and Kypta, 2003). When the five cell lines were transfected with sFRP or WIF cDNA, TOP/FOP flash activity was inhibited by 50–60% (Supplementary Fig. 9b). Consistent with these results, the addition of either sFRP1 or WIF protein to the culture medium of the cells reduced TOP/FOP flash activity to a comparable extent (Fig. 6a). Furthermore, the inhibitors markedly reduced cell growth by 30–50% (Fig. 6b). We next asked whether Wnt signaling was regulated by Sulf-2. Knockdown of Sulf-2 in the five cell lines resulted in a 45–50% inhibition of TOP/FOP flash activity (Fig. 6c). In addition, Sulf-2 knockdown cells demonstrated a favored plasma membrane localization for β -catenin, whereas a nuclear localization was favored in the control cells (Fig. 6d, Supplementary Fig. 9c). Also, Sulf-2 knockdown (H292 cells) led to a 3–4 fold reduction in the expression of several Wnt target genes (*Cyclin D1*, *Cyclin D2*, *Cyclin D3*, *JUN*, and *MYC*), which are involved in cell growth (<http://www.stanford.edu/~rnusse/wntwindow.html>) (Supplementary Table 3). Importantly, the addition sFRP1 or WIF-1 to the culture medium of knockdown cells produced no further inhibition of Wnt signaling (Supplementary Fig. 9d) or cell growth (Fig. 6e), consistent with the model in which Sulf-2 directly regulates the mobilization of Wnt ligands from HSPG sequestration (Ai *et al.*, 2003). It is conceivable that Sulf-2 knockdown could also have indirect effects on Wnt signaling as there was a \approx 2-fold reduction in the expression of two *WNT* genes among the six *WNTs* expressed in H292 cells (Supplementary Table 3).

Complementing the knockdown approach, we found that overexpression of the dominant negative S2 CC in the five cell lines inhibited Wnt activity by 40–60%, while overexpression of wild-type Sulf-2 produced no augmentation of Wnt signaling (Fig. 6f). In contrast, forced expression of Sulf-2 in cells that lacked it (BEAS2B and 16HBE14o-) increased TOP/FOP flash activity 4–5 fold from a baseline value of 1 (Fig. 6g).

DISCUSSION

The early view in the literature was that both Sulfs were tumor suppressors (Dai *et al.*, 2005; Lai *et al.*, 2003; Lai *et al.*, 2004; Li *et al.*, 2005). This belief originated from experiments in which forced expression of a Sulf in several tumor lines caused reduced growth-factor signaling by HB-EGF, FGF-2 or HGF, and diminished tumorigenicity. The negative effects of Sulfs on FGF-2 signaling are consistent with the requirement for 6S on HSPGs in the FGF-2 signaling complex (Gallagher, 2001). The ability of Sulf-1 to inhibit certain growth factor responses may explain why *SULF1* expression is reduced in subsets of tumors (ovarian, hepatocellular) (Lai *et al.*, 2003; Lai *et al.*, 2004) and in certain developmental contexts (Freeman *et al.*, 2008; Langsdorf *et al.*, 2007; Wang *et al.*, 2004). However, consistent with a potential oncogenic role, one or both *SULF* genes are over-expressed in subsets of multiple tumors (breast, pancreatic, hepatocellular carcinoma, head and neck, and lung) (Castro *et al.*, 2008; Grigoriadis *et al.*, 2006; Kudo *et al.*, 2006; Lai *et al.*, 2004; Lai *et al.*, 2008; Morimoto-Tomita *et al.*, 2005; Nawroth *et al.*, 2007). A strong case for a positive contribution has emerged for Sulf-2 in particular. Not only is *SULF2* overexpressed in hepatocellular carcinoma, but high expression is associated with a worse prognosis (Lai *et al.*, 2008). Unbiased screening studies have identified *SULF2/sulf2* as a candidate cancer-causing gene in human breast cancer and mouse brain cancer (Johansson *et al.*, 2004; Sjoblom *et al.*, 2006). Finally, knockdown of Sulf-2 in pancreatic adenocarcinoma cell lines (Nawroth *et al.*, 2007) and hepatocellular cell lines (Lai *et al.*, 2008) reverts their transformed phenotypes in culture and impedes their tumorigenicity in nude mice. The present study is the first to investigate Sulf-2 in NSCLC. The expression of Sulf-2 in NSCLC tumors, taken together with the loss-of-function experiments in NSCLC cell lines and gain-of-function experiments in non-malignant bronchial epithelial cells, constitute the strongest evidence to date for a positive contribution in malignancy. Notably, two of the NSCLC lines studied represent novel models of tobacco smoke-induced carcinogenesis, which is responsible for 85% of lung cancer (Sato *et al.*, 2007). Our findings provide impetus for the further investigation of Sulf-2 in NSCLC and other cancers in which it is over-expressed.

By monitoring the RB4CD12 epitope, we confirmed that the transforming activity of Sulf-2 depends on its activity as an HSPG endosulfatase. Previous studies have shown that HSPGs facilitate the progression of tumors by promoting their growth through direct effects on tumor cells (Aikawa *et al.*, 2008; Alexander *et al.*, 2000; Capurro *et al.*, 2005) or indirect effects on the tumor microenvironment (Aikawa *et al.*, 2008; (Fuster and Esko, 2005). The upregulated Sulf-2 expression in the stroma of NSCLC tumors (in particular adenocarcinomas) may be involved in carcinogenesis mechanisms of the latter type, for example in promoting angiogenesis or metastasis (Aikawa *et al.*, 2008; Fuster and Esko, 2005). The extensive heparanase literature further highlights the importance of HSPGs in cancer (Parish *et al.*, 2001; Vlodavsky *et al.*, 2007). This endoglycosidase cleaves HS chains at a few sites and produces large fragments that retain growth factor-binding activity. Heparanase is upregulated in many tumors and promotes tumor growth and metastasis (Vlodavsky *et al.*, 2007). Like heparanase, the Sulfs provide a postsynthetic mechanism to edit HSPGs, but do so without cleaving its chains.

The Sulfs positively modulate several signaling pathways including Wnt, BMP, Hedgehog, and GDNF (Ai *et al.*, 2003; Ai *et al.*, 2007; Danesin *et al.*, 2006; Dhoot *et al.*, 2001; Nawroth *et al.*, 2007; Viviano *et al.*, 2004). Of these, promotion of canonical Wnt signaling is the best validated (Dhoot *et al.*, 2001; Freeman *et al.*, 2008; Nawroth *et al.*, 2007; Tang and Rosen, 2009). Aberrant activation of Wnt signaling is an important mechanism underlying the pathogenesis of many tumors (Clevers, 2006; Klaus and Birchmeier, 2008; Logan and Nusse, 2004; Miller *et al.*, 1999). In some cases (e.g., colorectal cancers), the pathway is activated by mutations in intracellular signaling intermediates such as APC, axin or β -catenin (Clevers, 2006). Wnt signaling can also be activated by altered receptor-ligand interactions at the level of the cell surface, involving changes in expression of Wnts, soluble extracellular Wnt antagonists, or Wnt receptors/co-receptors (Hoang *et al.*, 2004; Huguet *et al.*, 1994; Janssens *et al.*, 2004; Suzuki *et al.*, 2004). Tumors and cell lines driven by this type of mechanism (“autocrine Wnt signaling”) are subject to inhibition by extracellular administration of Wnt antagonists (Bafico *et al.*, 2004; DeAlmeida *et al.*, 2007; Derksen *et al.*, 2004; Groen *et al.*, 2008; Kim *et al.*, 2007; Mikami *et al.*, 2005; Schlange *et al.*, 2007; You *et al.*, 2004).

In NSCLC, there is emerging evidence for aberrant Wnt signaling, which is based on altered receptor/ligand interactions or membrane proximal events rather than mutations in downstream components (Furuuchi *et al.*, 2000; Ohgaki *et al.*, 2004; Sunaga *et al.*, 2001; Winn *et al.*, 2006). Thus, promoters of autocrine Wnt signaling (Wnt1, Wnt2, disheveled) are upregulated (Huang *et al.*, 2008; Nakashima *et al.*, 2008; Uematsu *et al.*, 2003; You *et al.*, 2004) and conversely, WIF-1 is down-regulated in NSCLC tumors (Mazieres *et al.*, 2004). Studies with NSCLC cell lines corroborate the importance of these dysregulated Wnt elements in promoting cell growth (Kim *et al.*, 2007). Strikingly, all five Sulf-2⁺ NSCLC lines studied exhibited autocrine Wnt signaling, which is compatible with the involvement of Sulf-2 in regulating the bioavailability of extracellular Wnt ligands through its endosulfatase action on HSPGs. Consistent with this role for Sulf-2, knockdown of Sulf-2 or the introduction of a dominant negative form of Sulf-2 in these lines caused pronounced inhibition of canonical Wnt signaling, while conversely the forced expression of Sulf-2 in normal bronchial epithelial lines induced Wnt signaling. Furthermore, inhibition of autocrine Wnt signaling or knockdown of Sulf-2 in the NSCLC lines produced equivalent effects on cell growth, which were not augmented by combining both treatments. These findings not only corroborate the importance of autocrine Wnt signaling in the growth of NSCLC lines (Kim *et al.*, 2007) but also establish that Sulf-2 is required for this autocrine pathway.

Lung cancer is a heterogeneous disease involving dysregulation of a number of signaling pathways, most frequently involving *EGFR* and *KRAS* (Osada and Takahashi, 2002; Sato *et al.*, 2007). The relationship of Sulf-2 to these pathways remains to be determined. Intriguingly, Hynes and colleagues have demonstrated that autocrine Wnt signaling can transactivate EGFR in breast cancer cell lines (Schlange *et al.*, 2007). Although further validation is required, the results presented above raise the possibility that Sulf-2 could be a therapeutic target in NSCLC. Sulf-2 is an extracellular enzyme and thus is potentially amenable to inhibition by either antibody-based or small molecule drugs. Since Sulf-2 is

secreted and may enter body fluids, it also merits consideration as a potential biomarker of lung cancer.

MATERIALS AND METHODS

Constructs

Descriptions are provided in Supplementary Materials

RT-PCR and data mining

For analyzing *SULF* expression in published gene microarray studies, we used the oncomine database (www.oncomine.org) and the Consortium for Functional Glycomics data of Dr. Rittenhouse-Olson (<http://www.functionalglycomics.org/glycomics/publicdata/microarray.jsp>, #887). For analyzing *SULF* expression by PCR and RT-PCR, methodology is provided in Supplementary Materials.

Sulf-2 antibodies

The peptide-based polyclonal antibody against HSulf-2 was produced as described (Morimoto-Tomita *et al.*, 2005). Monoclonal antibodies were produced by ProSci Inc. A *sulf2* null mouse (Lum *et al.*, 2006) (provided by Drs. Joanna Phillips and Zena Werb) was immunized with recombinant HSulf-2 protein (15 micrograms over three injections). 2B4, one of the resulting mAbs, was used. Its characterization is presented in Supplementary Fig. 3.

Immunohistochemistry

Under UCSF CHR approval (H1060-27616-01), archived paraffin-embedded tissue blocks were obtained for ten cases each of squamous lung carcinoma and adenocarcinoma. 4 μm sections were subjected to heat-induced epitope retrieval with Trilogy (Cell Marque) and stained with 2 $\mu\text{g}/\text{ml}$ of 2B4 for 2 hours. A mAb directed to Herpes Simplex virus I/II served as a control. Antibody binding was visualized by the Dako Envision (+) system (cat #K4011).

Immunoblotting assays

Methodology is provided in Supplementary Materials

Cell lines and cell culture

H460, H292, A549, Calu-6, Calu-3, and BEAS2B cells were from American Type Culture Collection. B-ST and P-ST are described in **Results** 16HBE14o- cells were from Dr. Keith Mostov (UCSF). All cells were grown in RPMI-1640 with 10% FBS and penicillin/streptomycin. HEK 293 cells were maintained in DMEM with 10% FBS and penicillin/streptomycin.

HSPG sulfation analysis

To determine the effects of Sulf-2 knockdown on HSPGs, we used a phage-display antibody (RB4CD12) (Dennissen *et al.*, 2002) with an irrelevant antibody as a control. A Cy3-

conjugated mouse anti-VSV secondary antibody (Sigma) was used and cells were analyzed by flow cytometry.

Cell growth, proliferation and survival assays

Methodology is provided in Supplementary Materials.

In vitro cell migration and cell adhesion assays

Methodology is provided in Supplementary Materials.

Monolayer wound healing assays

Cells were grown to confluency on 6-well plates. Scratches were made with a pipette tip. Cell migration was quantified by measuring the width of the wounds at 0, 24, 48 and 72 hours.

Colony formation in soft agar

Methodology is provided in Supplementary Materials.

Wnt luciferase reporter assays

Methodology is provided in Supplementary Materials.

Tumorigenicity assays and histological evaluation of xenografts

5×10^6 cells, transduced with either control or Sulf-2 shRNA were injected into the flanks of 5-week-old athymic male BALB/c nu/nu mice (5 mice/group). Tumor growth was monitored every 2–3 days. Groups of animals were sacrificed when the largest tumor nodules attained 0.8 cm (after 25 – 40 days). Tumors were harvested, weighed and processed for histology. All procedures had UCSF IACUC approval. Some of the mice were injected intraperitoneally with BrdU 2 hrs prior to sacrifice. Tissue sections were immunostained with a BrdU antibody (BD Biosciences) or with the active caspase 3 antibody. Tissue sections were incubated with the corresponding peroxidase-conjugated secondary antibody with visualization with Nova Red and counterstained with hematoxylin. The numbers of BrdU+ or active caspase3+ cells were counted in four high power field (HPF) for each tissue section with 3 sections per tumor.

Statistical analysis

All numerical data were calculated as means \pm standard deviations or \pm SEMs. Differences between groups were compared with a Student's *t*-test. Paired Tumor/Control samples were compared with a paired two-tailed *t*-test. A *p* value ≤ 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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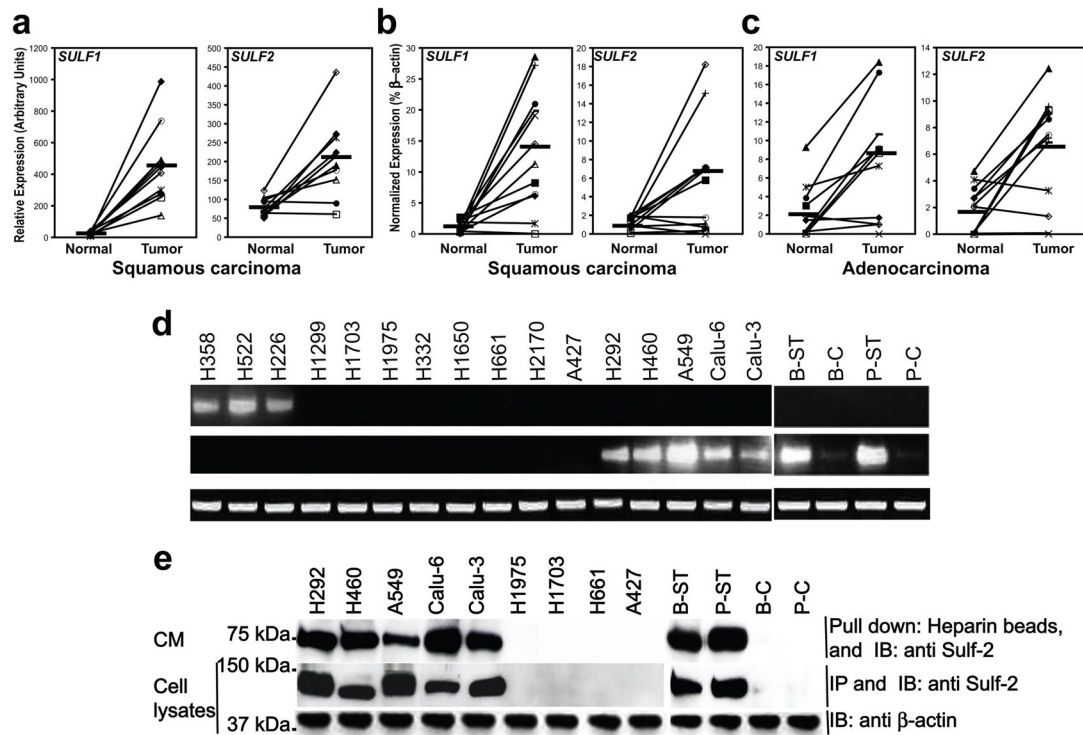


Figure 1. SULF transcript and protein expression in NSCLC tumors and lung cancer cell lines: (a) DNA microarray analysis of SULF1 and SULF2 expression in squamous cell lung carcinomas and adjacent normal lung. Results were mined from (www.functionalglycomics.org, core E, #887). SULF1 (Left) and SULF2 (Right) transcripts in normal lung vs. lung squamous cell carcinomas (10 cases, lines connect individual patient values). Mean values (horizontal black bars) increased 18-fold for SULF1 ($p=0.0005$) and 3-fold for SULF2 ($p=0.003$). qPCR determinations of SULFs (normalized to % β -actin) in (b) lung squamous cell carcinomas (12 cases) and (c) lung adenocarcinomas (12 cases) vs. adjacent normal lung samples. Relative to normals, SULF1 and SULF2 increased 12-fold ($p=0.008$) and 4-fold ($p=0.05$) in squamous carcinomas and 3-fold for both ($p=0.003$ and $p=0.002$, respectively) in adenocarcinomas. (d) RT-PCR analysis of SULFs in NSCLC cell lines (H358; H522; H266; H1299; H1703; H1975) and smoke transformed cells (B-ST; P-ST) and their respective parentals (B-C; P-C) with β actin as control. (e) Immunoblotting of Sulf-2 in conditioned medium (Upper) and lysates (Middle) of indicated cells with β -actin as loading control (Lower).

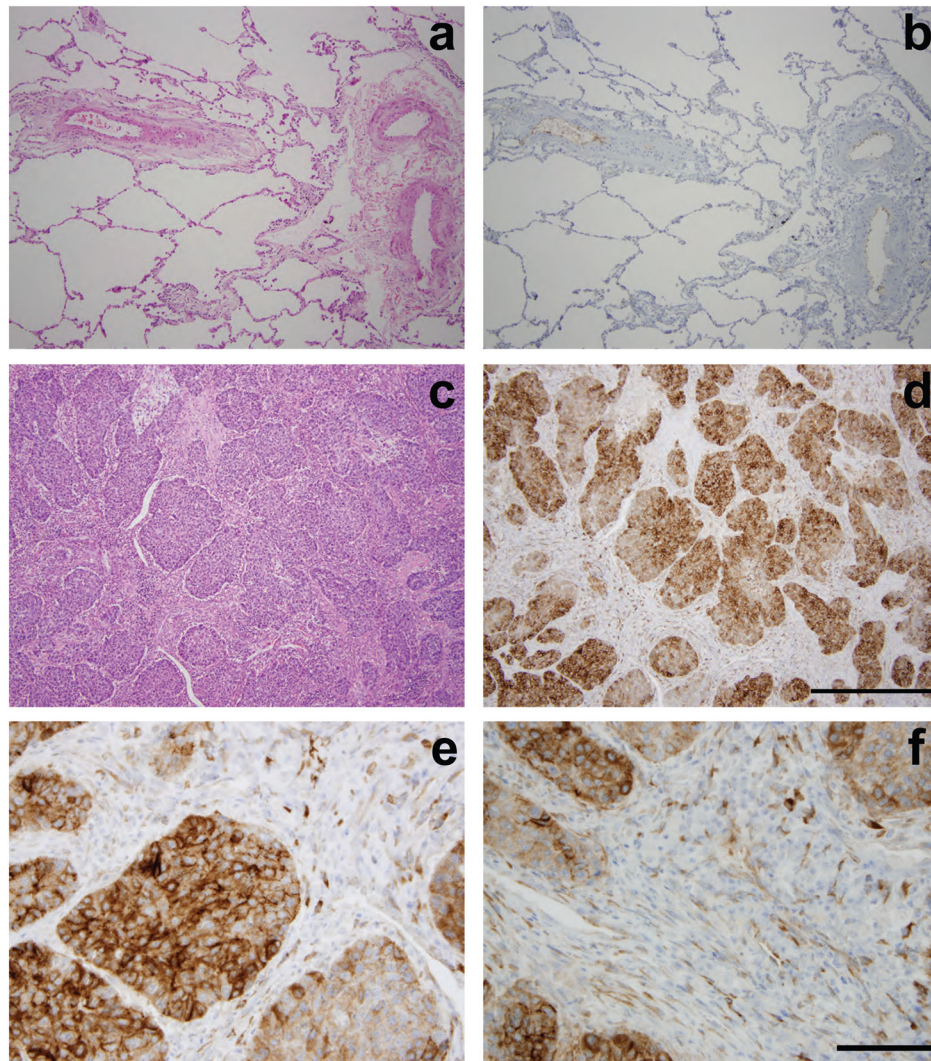
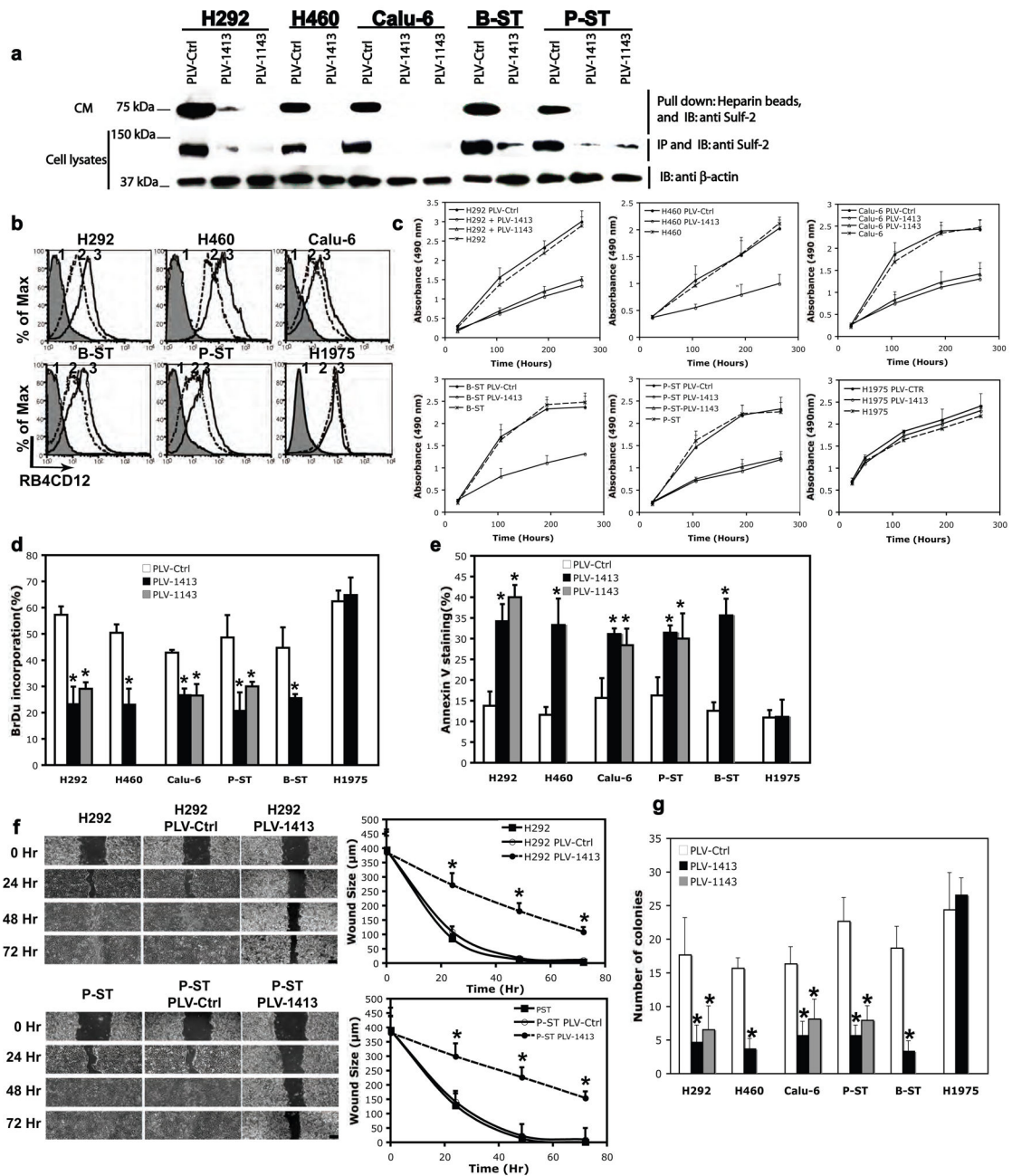
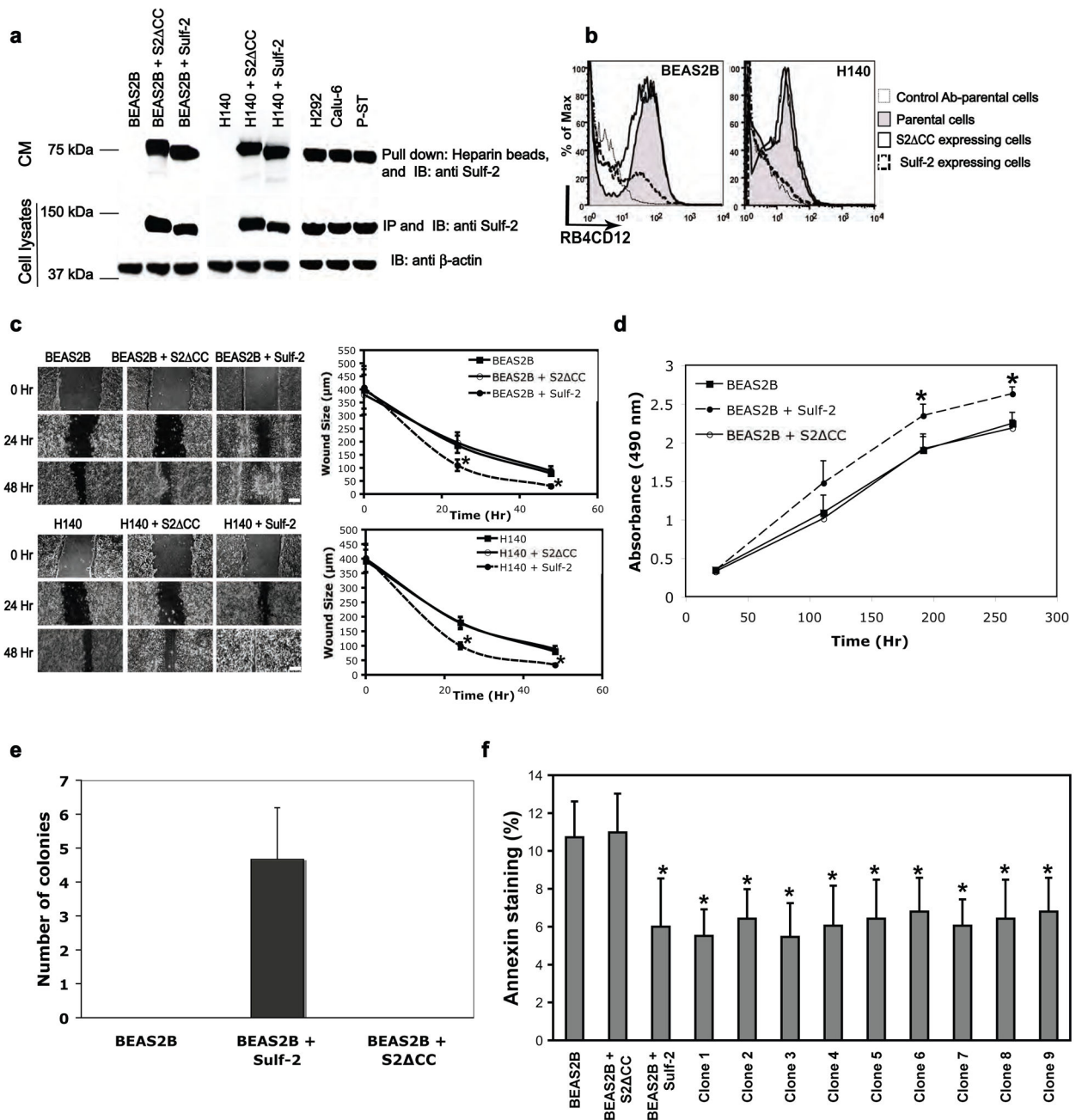


Figure 2. Sulf-2 protein expression in NSCLC tumors: representative sections of benign lung and squamous cell carcinoma were stained with hematoxylin and eosin (H&E) and adjacent serial sections were stained with anti-Sulf-2 antibody (2B4). (a) Normal lung, H&E. (b) Normal lung stained with 2B4. (c) Squamous cell carcinoma, H&E. (d) Squamous cell carcinoma stained with 2B4 antibody demonstrates islands of tumor cells strongly positive for Sulf-2 surrounded by weakly staining desmoplastic stroma. Panels a, b, c and d are low-power micrographs (100X, scale bar = 500 μ m). (e and f) High-power micrographs of squamous cell carcinoma stained with 2B4 antibody. Panel e shows staining of tumor-associated stromal cells with 2B4 antibody (400X, scale bar = 100 μ m).

**Figure 3.**

Effects of Sulf-2 knockdown in lung cancer cell lines: (a) Cells were transduced with Sulf-2-specific (PLV-1413, PLV-1143) or control (PLV-Ctrl) shRNAs. Immunoblotting for Sulf-2 in CM (Upper) and cell lysates (Middle) with a loading control of β -actin (Lower). (b) Effects of Sulf-2 knockdown on the cell surface expression of the RB4CD12 epitope in 6 cell lines, one of which is negative for Sulf-2 (H1975). Mock-knockdown (PLV-Ctrl) (2) or Sulf-2-knockdown (PLV-1413) (3) cells were stained with RB4CD12 or with an irrelevant antibody as control (1). (c) Cell growth was monitored for 6 days using CellTiter-Blue. (d) Cell proliferation in mock- or Sulf-2 knockdown H292 and P-ST cells, as measured by BrdU incorporation. (e) Effect of Sulf-2 knockdown on cell apoptosis as determined by staining

with APC conjugated-Annexin V. (f) Wound-healing migration assay for mock-knockdown, Sulf-2 knockdown or non-transduced cells. Healing of scratch wounds was measured at the indicated times. Scale bar = 120 μ m. (g) The formation of colonies in soft agar by Sulf-2 knockdown cells and control cells after 21 days of culture. The values shown in graphs are means \pm SDs. (* indicates $p < 0.05$ relative to controls).

**Figure 4.**

Effects of Sulf-2 overexpression in non-malignant bronchial epithelial cells: (a) BEAS2B and H140 were transduced with either Sulf2 or inactive Sulf-2 (S2 CC). Immunoblotting of Sulf-2 protein in CM (Upper) and cell lysates (Middle) of transduced cells as compared to Sulf-2+ cells (H292, Calu-6 and P-ST) with b-actin as loading control (Lower). (b) Staining of parental and transduced cells with RB4CD12 or control antibody, BEAS2B (left), H140 (right). (c) Healing of scratch wounds by parental and transduced cells. Scale bar = 76μm. (d) Growth of parental and transduced cells by Cell Titer Blue assay. (e) Soft agar colony formation by parental and transduced cells after 21 days of culture. (f) 9 clones of Sulf-2

expressing BEAS2B cells were generated from colonies in soft agar. Apoptosis was determined for the clones, parentals, and transduced populations by measuring staining with APC conjugated-Annexin V. The values shown in graphs are means \pm SDs for 3 independent determinations (* indicates $p < 0.05$).

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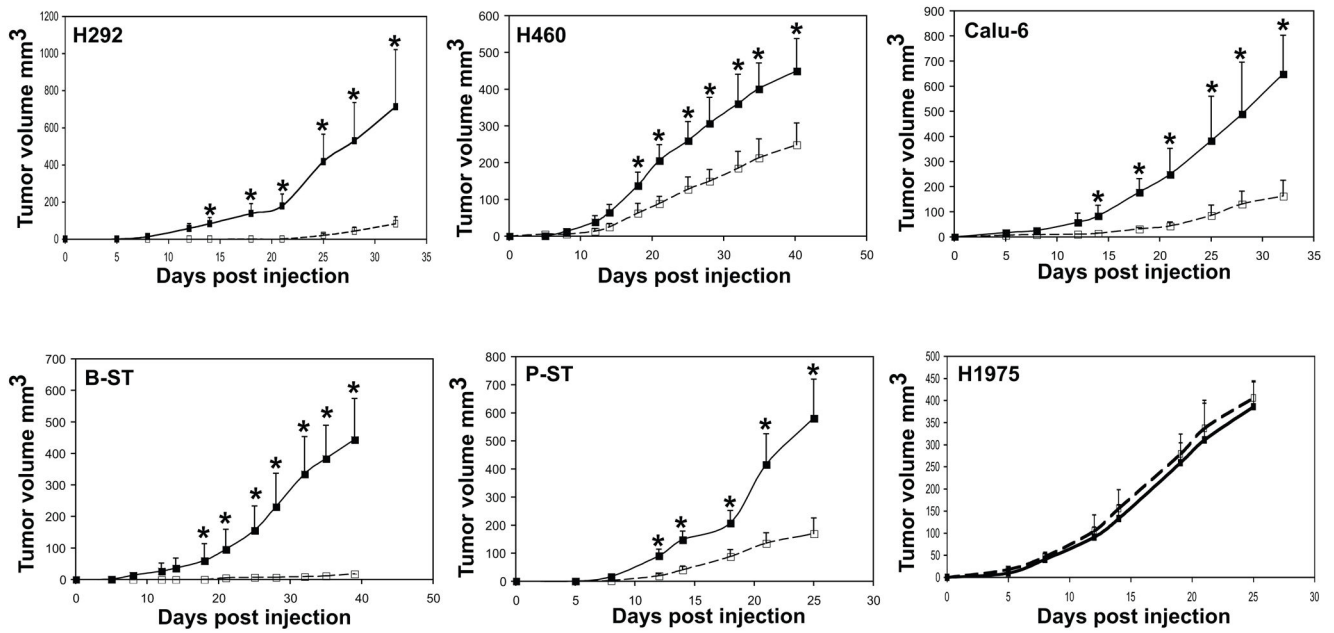
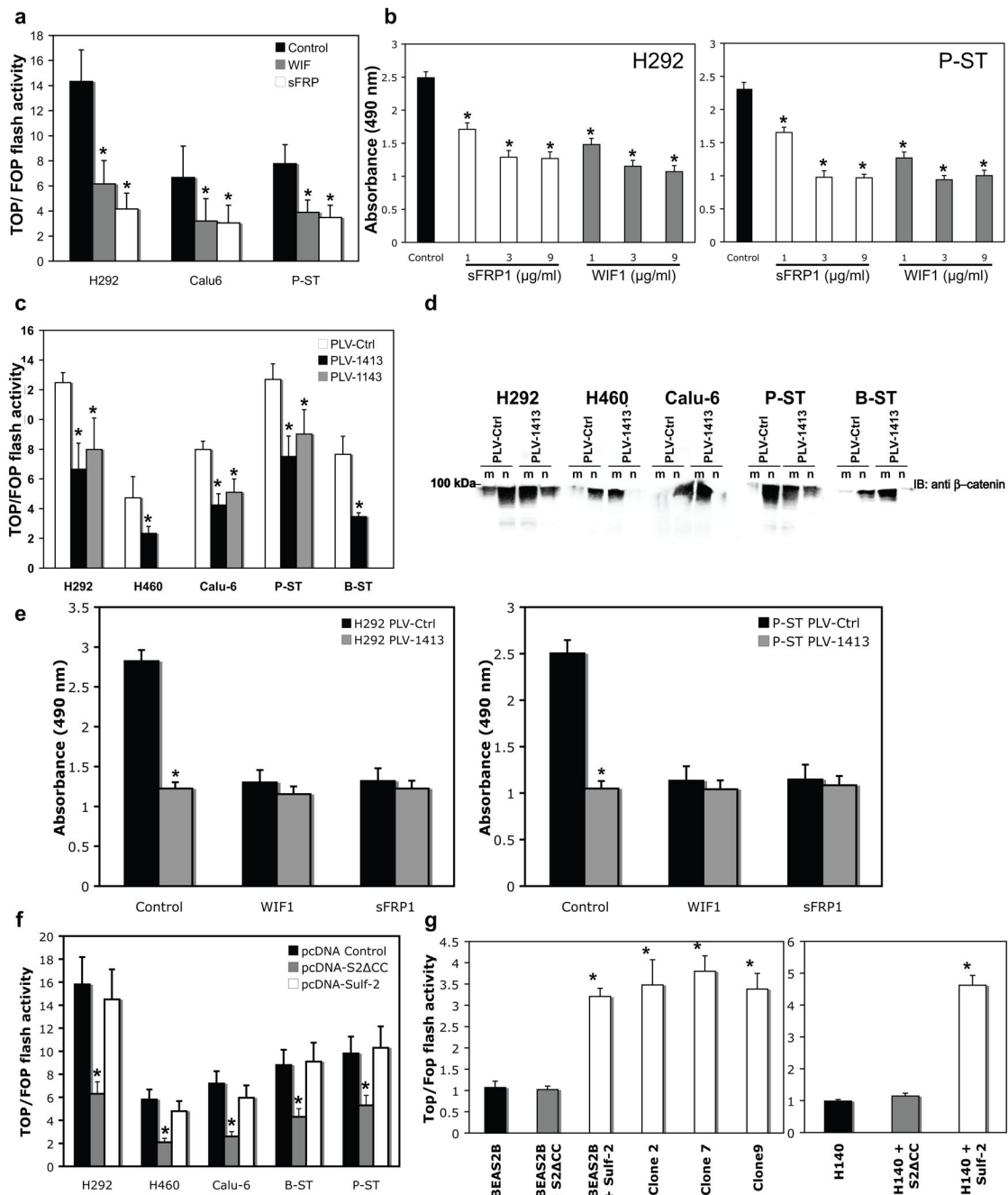


Figure 5.

Effects of Sulf-2 knockdown on the tumorigenicity of lung cancer cell lines: The indicated lines with mock knockdown (PLV-Ctrl, black lines) or Sulf-2 knockdown (PLV-1413, dashed lines) were injected subcutaneously into nude mice and tumor volume was monitored over time. The values shown are means (+SEM) of 4–5 mice. * indicates $p < 0.05$.

**Figure 6.**

Sulf-2 knockdown effect on autocrine Wnt signaling (a) H292, Calu6, and P-ST cells were transfected with TOP/FOP flash reporter system, then cultured in presence of 3 μ g/ml sFRP1 or WIF-1 or medium alone. Means \pm SD's for 3 determinations of TOP/FOP activity are shown. (b) Growth of H292 and P-ST cells in the presence of sFRP1 or WIF-1 or medium alone (Control). (c) Effect of Sulf-2 knockdown on TOP/FOP flash activity. (d) Effect of Sulf-2 knockdown on localization of b-catenin in nuclear (n) and plasma membrane (m) fractions, as determined by immunoblotting. (e) Growth of Sulf-2 knockdown or control-

treated cells in the presence of sFRP1 (3 µg/ml), WIF-1 (3 µg/ml) or medium alone (Control). (f) TOP/FOP flash was measured in the indicated lines after transfection with empty vector pcDNA (Control), Sulf-2 cDNA or S2 CC cDNA. (g) TOP/FOP flash was measured in Sulf-2 or S2 CC-expressing BEAS2B and H140 cells and their non transduced counterparts, as well as 3 clones (2, 7 and 9, see Fig. 5). Data shown are means \pm SD's. In all panels, * denotes $p < 0.05$.

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