Regulation of vascular smooth muscle cell proliferation, migration and death by heparan sulfate 6-*O*-endosulfatase1

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Abstract Migration, proliferation and death of vascular smooth muscle cells (VSMC) are important events in vascular pathology regulated by heparan sulfate proteoglycans and hence potentially by cell surface HS 6-*O*-endosulfatase1 (sulf1). Sulf1 mRNA expression was increased in cultured VSMC compared to rat aorta. Furthermore, adenovirus mediated overexpression of quail sulf1 decreased adhesion, and increased proliferation and apoptosis of VSMC. Overexpression of a dominant negative variant also decreased adhesion of VSMC and increased proliferation, apoptosis, migration and chemotaxis of VSMC. Our results imply that only normal levels of 6-*O*-sulfation maintained by sulf1 are optimal for several functions of VSMC.

Keywords: Heparan sulfate proteoglycan; 6-*O*-Endosulfatase; Smooth muscle cells

1. Introduction

Thickening of the inner intimal layer of arteries is an important component of atherosclerosis and the vascular response to injury, for example after balloon injury or in vein grafts. If the lumen is narrowed excessively it can lead to distal ischemia resulting in clinical symptoms such as angina pectoris and intermittent claudication. Intimal thickening involves accumulation of vascular smooth muscle cells (VSMC) and their associated extracellular matrix (ECM) [1]. VSMC are normally confined to the medial layer where they are maintained quiescent and contractile by interactions with their surrounding basement membrane, which contains predominately collagen IV, laminin and heparan sulfate proteoglycans (HSPGs) [2]. The relationship between HSPGs and VSMC behavior is complex. For example, HSPGs are an essential component of the low affinity receptors that in part mediate FGF-2-induced VSMC proliferation [3]. On the other hand, HSPGs have a number of inhibitory actions. They sequester growth factors, including FGF-2, which would otherwise activate VSMCs. They also interact directly with cell surface receptors, through which they inhibit migration and proliferation of VSMCs and promote differentiation towards a quiescent state [4–6]. Remodeling of HSPGs occurs during response to vascular injury and may promote neointima formation. For example, heparin lyase activity is increased 3–14 days after balloon catheter injury of rabbit carotid arteries [7]. Moreover, macrophage-derived lysosomal heparin lyases removed all of the ^{35}S labeled HS and induced a more proliferative VSMC phenotype in culture [8].

The highly heterogeneous HS glycosaminoglycan (HS) chains of HSPG determine ligand binding and hence function. They contain N-sulfated, acetylated and unsubstituted glucosamine and *O*-sulfate groups at characteristic positions [9]. Previous work suggests that the level of 6-*O*-sulfation of HS chains, in particular, can influence the function of HSPGs [10,11]. Consistent with this, a cell surface HS 6-*O*-endosulfatase with a preference towards trisulfated iduronic acid 2S-glucosamine-NS6S disaccharide units was recently cloned from quail embryos and shown to have a role in development of the skeletal muscle system [12]. Rat, human and mouse orthologs (sulf1, sulf2) were subsequently cloned and the quail enzyme relates closely to sulf1 [13–15]. These sulfatases were implicated in tumor invasion and metastasis [13,16], which has mechanistic similarities with invasion of VSMC into the intima. We therefore questioned whether Sulf1 could regulate the level of HS 6-*O*-sulfation and thereby affect the behavior of VSMC. As a first step, we prepared adenoviruses to overexpress wild-type and a dominant negative variant of sulf1 and investigated their effects on VSMC behavior in culture.

2. Materials and methods

2.1. Semi-quantitative RT-PCR analysis

Total RNA was extracted from isolated VSMCs and rat aortic segments using a fibrous tissue RNA extraction (Qiagen). First-strand cDNA was synthesized by random priming using the first-strand synthesis kit (ProStar, Stratagene). Semi-quantitative PCR was performed using primers for rat sulf1 [13] (forward, 5′-CAAGTGTTACCG-CCAAGGAG-3′, and reverse, 5′-TGTAGGCCTTGTGGTCCTTC-3′); 18S (forward 5′-CTCGATGCTCTTAGCTGAGT-3′ and reverse 5′-CTT CAA ACC TCC GAC TTT CG-3′) for various numbers of cycles to ensure that reactions did not reach saturation. Products were separated on agarose gels and analyzed by densitometry using AlphaImage analysis software (AlphaInnotech Corporation). Representative bands were excised, eluted (Qiagen) and sequenced (Lark technologies) to confirm their identity.

2.2. Construction of recombinant adenoviral vectors

Previously described plasmids containing cDNA coding for Qsulf1 wild-type and a dominant negative (CC89,90AA) form and a carboxy-terminal myc tag were obtained [12]. The coding sequences were excised and subcloned into a commercially available shuttle vector, pDC 515 (Microbix Biosystems Canada), downstream from the mouse cytomegalovirus promoter. Replication-deficient adenoviruses were generated by site-specific FLP-mediated recombination of the cotransfected

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shuttle and genomic plasmids in 293 cells. Viral stocks were amplified, CsCl banded, and titrated as previously described [17].

2.3. Culture and infection of SMC

Isolated VSMCs were prepared from the thoracic aorta of male Sprague–Dawley rats using a modification of the explant technique described previously [18]. VSMC of passages 2–7 were plated at a density of 4×10^4 cells/cm^2 and infected where indicated with 300 plaque-forming units (pfu) of adenovirus/cell. A previously described adenovirus expressing β-galactosidase [17] was used to control for the non-specific effects of viral infection.

2.4. Western blotting

Cells were lyzed in 50 mM Tris–HCl, pH 6.8, 10% glycerol, 1% SDS. Lysates were analyzed for protein content (Micro BCA assay kit, Pierce) and 20–60 μg of reduced protein were separated by polyacrylamide gel electrophoresis and transferred to PVDF membrane (Bio-Rad). Membranes were blocked with PBS, pH 7.4 containing 5% milk powder and incubated with antibodies to the myc tag (9E10, Santa Cruz), phospho p44 and p42 ERK1/2 and total ERK1/2 (Cell Signaling) or phospho FAK_{Y397} (Upstate) for 3 h at room temperature. Bound antibodies were detected using horseradish peroxidase-conjugated (HRP) secondary antibodies (Dako). Peroxidase activity was detected using enhanced chemoluminescence (Amersham Biosciences). Blots were stripped using Re-Blot™ (Chemicon).

2.5. Determination of cell surface 6-O-sulfate content

1×10^4 VSMC were plated in 96 well plates, infected at 300 pfu/cell of the adenovirus. After 48 h, the cells were washed twice with PBS, fixed with 4% formaldehyde/PBS for 10 min at room temperature, blocked in 1% BSA for 30 min and incubated with a 1:200 dilution of 10E4 antibody that recognizes the HS 6-*O*-sulfated glucosamine residues known to be hydrolyzed by sulf1 [11] for 1 h, mouse IgG at the same concentration was used as negative control. After 3 washes in PBS, a 1:200 dilution of goat anti-mouse HRP was added, incubated for 30 min, washed 3 times in PBS and incubated for up to 10 min with 2,2′-azino-di[3-ethylbenzthiazoline sulfonate] (Roche, Lewes, East Sussex, UK) and the absorbance read at 405 nm. The absorbance units/cell is expressed as a percentage of the β-gal control.

2.6. Immunofluorescence

VSMC cultured and infected on coverslips for 48 h were fixed with ice-cold 4% paraformaldehyde/PBS for 20 min at 4 °C. After incubation in 1% Triton X-100 at 4 °C for 5 min, VSMCs were incubated with 4% BSA/PBS at room temperature for 5 min followed by mouse 10E4 antibody at 10 μg/mL or mouse IgG (Sigma) in 1% BSA/PBS for 2 h at room temperature. VSMCs were then incubated for 45 min with biotinylated goat anti-mouse IgG (Dako) diluted 1:200 in 1% BSA/PBS and then for 30 min with extravidin-FITC diluted 1:200 in 1% BSA/PBS.

2.7. Proliferation

Cell numbers were counted 48 h after infection. VSMC proliferation was quantified by incorporation of 10 μM bromodeoxyuridine (BrdU) for 12 h at 36 h after infection. Incorporated BrdU was detected as previously described [19].

2.8. Apoptosis measurements

Apoptosis was analyzed at 48 h after infection by immuno-fluorescent detection of cleaved caspase-3 and by in situ end labeling (ISEL) as previously described [20].

2.9. Migration

VSMC migration was analyzed 48 h after infection using modified Boyden chambers as previously described [21]. Briefly, infected VSMCs were detached using 0.02% EDTA/PBS and placed at 5×10^4 cells per well in the chambers containing uncoated membranes or membranes coated with 0.75 mg/cm^2 Matrigel (Becton Dickinson Labware).

2.10. Cell adhesion assay

VSCM infected for 48 h were detached using 0.02% PBS/EDTA, resuspended in DMEM supplemented with 10% FCS and allowed to adhere to uncoated wells at 37 °C for 45 min and 3 h. The wells were washed with PBS to remove unattached cells, fixed in formalin, stained with 1% aqueous toluidine blue and quantified spectrophotometrically [22]. Results were expressed as percentage of cells adherent at 3 h.

2.11. Statistics

Experiments were performed at least 3 times with different VSMCs (n indicates the times the experiment was repeated). Data were analyzed using a two-tailed paired t test; significant differences were taken if $P < 0.05$.

3. Results

3.1. Expression of endogenous sulf1 and overexpression Qsulf1 and DNQsulf1 in rat VSMC

We used semi-quantitative RT-PCR to measure endogenous sulf1 mRNA in cultured rat VSMCs and aorta. Although rat sulf1 mRNA was present in rat aortas, significantly higher levels were detected in cultured aortic VSMC (Fig. 1A). This indicates that the expression of sulf1 is increased in the highly proliferative cultured VSMCs compared to the relatively quiescent VSMCs in the rat aorta.

To examine the function of sulf1 in VSMCs, we constructed recombinant adenoviruses that contained the entire coding sequence of the quail wild-type enzyme downstream of a mouse CMV promoter (Qsulf1). We also constructed viruses with the (CC89,90AA) variant, which has been shown previously [12] to be inactive and possess dominant negative properties (DNQsulf1). Both transgenes were tagged with myc. Western blots using an anti-myc antibody demonstrated that infection with adenoviruses resulted in the overexpression of Qsulf1 and DNQsulf1, respectively (Fig 1B). The majority of the recombinant sulfatases were retained within the cell, with less than 1% secreted into the medium (Fig. 1B).

Measurement of catalytic activity of sulf1 towards extracellular substrates is complicated by the presence of much larger amounts of lysosomal sulfatases in cell homogenates. We therefore designed a whole cell ELISA assay based on immunoreactivity to antibody 10E4, the epitope of which includes the HS 6-*O*-sulfated glucosamine residues known to be hydrolyzed by sulf1 [11]. Immunocytochemistry with 10E4 showed activity particularly at the margins between cells. Immunoreactivity appeared decreased in Qsulf1 overexpressing cells and increased in cells expressing DNQsulf1 compared to control VSMC overexpressing β-galactosidase (Fig. 2A). Consistent with these observations, overexpression of Qsulf1 significantly decreased immunoreactivity to antibody 10E4 detected using a whole-cell ELISA assay (Fig. 2B). The overall reduction was only approximately 10%, which suggests either that overexpressed Qsulf1 was inadequate to hydrolyze all 6-*O*-sulfated glucosamines or, more likely, that the antibody also recognized other sugars. Indeed 10E4 is known from other studies to also bind strongly to disaccharides containing terminal non-sulfated hexuronoic acid and unsubstituted *N*-glucosamine. [23]. DNQsulf1 significantly increased 10E4 immunoreactivity by 35% compared to control cells, consistent with it behaving as a dominant negative inhibitor of sulf1.

3.2. Adhesion, proliferation and apoptosis of VSMC

Cell adhesion is known to influence proliferation, migration and death of VSMC. Overexpression of either Qsulf1 or DNQsulf1 impaired adhesion of rat VSMC to tissue culture wells (Fig. 3A). This implies that sulf1 contributes to maintaining a level of HS 6-*O*-sulfation that appears optimal for adhesion.

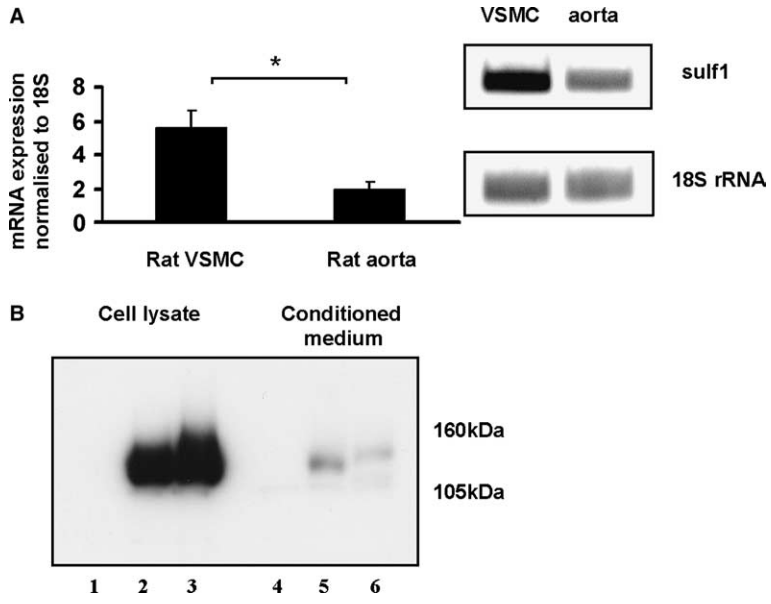

Fig. 1. Expression of endogenous and recombinant sulf1 in VSMC. (A) Semiquantitative RT-PCR analysis for sulf1 was performed on total RNA from rat aorta or cultured VSMC as shown and expression was normalized to18S rRNA. A representative gel is shown, PCR was performed for 19 cycles for 18S rRNA and 29 for sulf1. $^{*}P < 0.05$, $n = 3$. (B) VSMCs were infected with either control adenovirus (β-Gal, lanes 1 and 4), wild-type Qsulf1 expressing adenovirus (lanes 2 and 5) or DNQsulf1 (lanes 3 and 6). Cell lysate and conditioned medium were analyzed for myc-tag expression by Western blotting 48 h after infection. Blots were overexposed so as to detect the proteins in the conditioned medium.

Overexpressing either Qsulf1 or DNQsulf1 significantly increased VSMC proliferation measured by BrdU incorporation 94% and 72%, respectively, compared to control cells overexpressing β-galactosidase (Fig. 3B). Interestingly, increased BrdU incorporation was not accompanied by increased cell counts in VSMC overexpressing Qsulf1 compared to β-galactosidase, while cultures overexpressing DNQsulf1 had significantly decreased cell numbers relative to both other treatments (Fig. 3C). To explain this discrepancy, we investigated the levels of apoptotic cell death. DNA fragmentation measured by ISEL revealed that VSMC overexpressing Qsulf1 had 2.8-fold higher levels of apoptotic cell death than β-galactosidase overexpressing VSMC (Fig. 4A), while cells overexpressing DNQsulf1 showed 11.5-fold higher levels. Immunostaining of VSMC for cleaved caspase3, a downstream executioner of the apoptosis cascade, confirmed that overexpression of Qsulf1 significantly increased apoptosis and that this was further increased by DNQsulf1 (Fig. 4B). Clearly increased cell death caused by Qsulf1 overexpression nullified the effects on cell proliferation while death caused by DNQsulf1 overexpression more than overcame the increase in proliferation.

3.3. Migration of VSMC

VSMC migration also plays a fundamental role in intimal thickening and we therefore investigated the effect of altering HS 6-*O*-sulfation on chemotaxis and invasion. Overexpressing Qsulf1 did not affect chemotaxis of VSMC through uncoated wells or invasion through reconstituted basement membranes (Matrigel) relative to β-galactosidase controls (Fig. 5A and B). On the other hand increasing 6-*O*-sulfation by overexpressing DNQsulf1 significantly increased invasion through (Matrigel) and chemotaxis compared to control cells (Fig. 5A and B).

3.4. Effects on ERK1/2 and Focal adhesion kinase (FAK) phosphorylation

To gain a first insight into the mechanisms underlying the effects of sulf1, we measured ERK1/2 phosphorylation and total ERK levels by Western blotting under conditions similar to those used for the proliferation measurements (Fig. 6A). Both Qsulf1 and DNQsulf1 increased ERK phosphorylation 2.8–7.4-fold in quiescent VSMC and 1.5–3.2-fold after 2 h and 1.2–3.1-fold after 4 h of serum stimulation (either treatment $P < 0.05$ vs. β-gal, Kruskal–Wallis test). By contrast when we measured FAK phosphorylation under conditions similar to those used for the adhesion measurements there was no effect of either treatment (Fig. 6B).

4. Discussion

There is a wealth of evidence that HSPGs regulate the behavior of VSMC. For example addition of HSPGs, glycosaminoglycans derived from them or indeed heparin profoundly inhibit VSMC proliferation [4–6]. Conversely, transgenic mice with HS-deficient perlecan display increased VSMC proliferation and intima formation [24]. As detailed in Section 1, the status of 6-*O*-sulfation influences function of HSPGs. However, endogenous 6-*O*-sulfatases have only recently been cloned and hence our study is the first to demonstrate sulf1 expression in VSMC. Moreover, we demonstrated upregulation of sulf1 mRNA levels between intact aortas and VSMC in culture. It will be valuable to confirm this regulation at the level of protein should suitable antibodies become available. Many previous studies have compared gene expression in aortas as a model for quiescent cells and in cultured cells as a model for the cells involved in neointima formation [25]. This is valid because cultured VSMC share phenotypic charac-

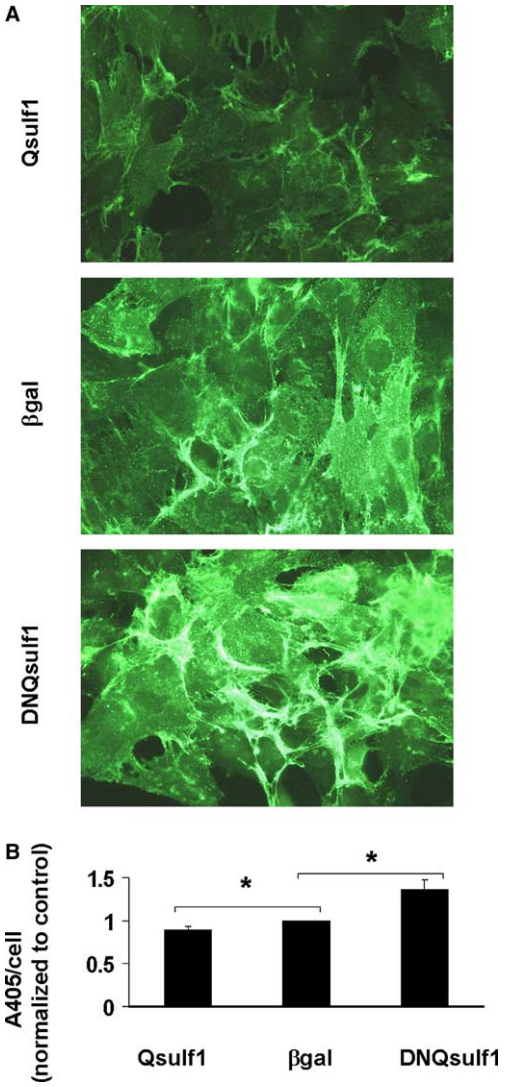

Fig. 2. Analysis of 6-*O*-sulfate glucosamine using antibody 10E4. VSMCs were infected with either control adenovirus (Ad:βGal), or adenovirus expressing wild-type Qsulf1 or DNQsulf1 as indicated. (A) By immmunofluorescence. (B) By ELISA, $n = 4$, $^{*}P < 0.05$.

teristics of intimal VSMC, they are free to migrate and they proliferate in response to serum many times faster than VSMC in intact aortas [18]. Interestingly previous studies demonstrated upregulation HSPG core protein genes in proliferating rat VSMC. For example, syndecan mRNAs increase beginning at day 2 after rat carotid balloon injury and perlecan transcripts increase after a week's lag to a peak after 2–4 weeks [26]. By contrast to sulf1, *N*-deacetylase/*N*-sulfotransferase, the enzyme responsible for the initiation of 6-*O*-sulfation of the disaccharide units, is downregulated in proliferating VSMC compared to uninjured arteries [27]. Increased sulfation [27] and decreased sulfatase (our present data) both imply that higher levels of 6-*O*-sulfation correlate with quiescence of VSMC.

Using overexpression of Qsulf1 to reduce and DNQsulf1 to increase 6-*O*-sulfation in cultured VSMC, we are also the first to demonstrate that sulf1 regulates the major properties of VSMC, namely adhesion, proliferation, apoptosis and migration. Interestingly, both Qsulf1 and DNQsulf1 overexpression

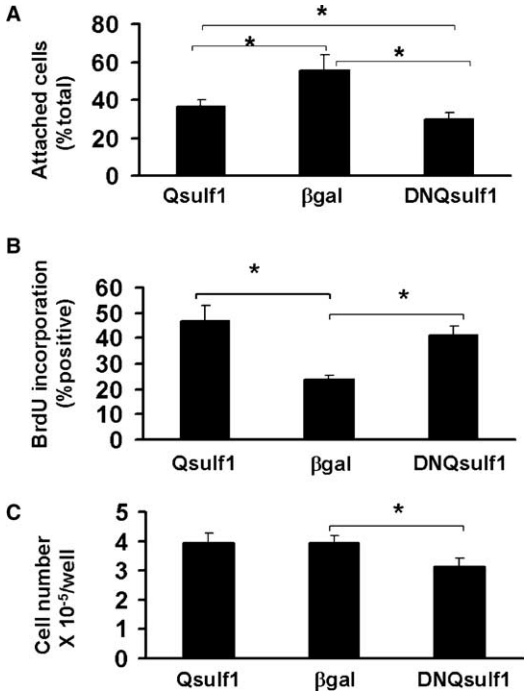

Fig. 3. Measurement cell adhesion and proliferation and apoptosis. VSMC were infected with adenovirus expressing β-galactosidase, Qsulf1 or DNQsulf1 for 48 h. (A) Infected VSMC were placed in 96-well plates (1.5×10^4/well) and allowed to attach for 45 m and 3 h. Following fixation and toluidine staining, absorbance at 595 nm was measured and percentage of cells adherent at 45 m vs. 3 h was calculated. (B) VSMC were labeled with BrdU for the last 12 h and then immunostained. (C) VSMC numbers by cell counting. $^{*}P < 0.05$, $n = 3$.

decreased adhesion and increased proliferation and apoptosis of VSMC. Our results imply that only the normal 'physiological' levels of 6-*O*-sulfation maintained by sulf1 mediate optimal levels of adhesion. Such adhesion appears to partially inhibit proliferation but has a major benefit in terms of avoiding apoptosis of VSMC. The relationship between cell adhesion and migration is also a complex one. Too great adhesion can physically retard migration but insufficient adhesion can result in failure to gain traction and therefore also inhibit migration. In our experiments, reducing adhesion by overexpressing sulf1 had little effect on migration but reducing adhesion further with DNQsulf1 significantly increased migration. These results imply that the normal 'physiological' levels of 6-*O*-sulfation maintained by sulf1 partly restrict migration.

The mechanisms responsible for the effects of sulf1 on VSMC are unknown. Qsulf1-mediated remodeling of cell surface HS was previously shown to promote proliferation through Wnt signaling [11,12]. However, we could detect no difference in β-catenin nuclear localization in VSMC infected with Qsulf1, DNQsulf1 or β-galactosidase (results not shown). We did however detect an equivalent stimulatory effect of Qsulf1 and DNQsulf1 on ERK phosphorylation that most likely contributed to their stimulatory effects on VSMC proliferation. By contrast we found no effect on FAK phosphorylation, which shows some specificity in the effects of Qsulf manipulations. Previous work suggests that Sulf1 could be active either in the Golgi apparatus as well as the cell surface. Moreover shedding of surface sulf1 occurs in CHO [15] and could therefore lead to a bystander effect. However, this was

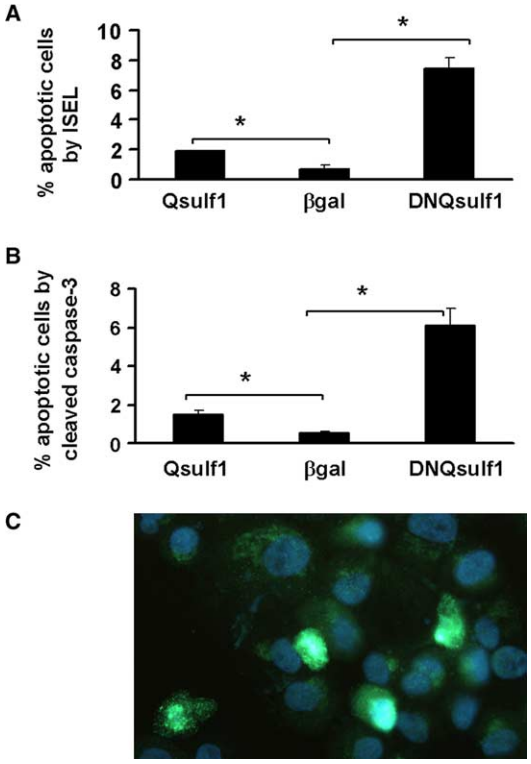

Fig. 4. Measurement of apoptosis. VSMC were infected with adenovirus expressing β-galactosidase, Qsulf1 or DNQsulf1 as indicated for 48 h. (A) Apoptotic cell death analyzed by ISEL. (B) Apoptotic cell death analyzed by immunofluorescence of cleaved caspase-3. (C) A representative micrograph showing caspase-3 green and nuclei blue. $^{*}P < 0.05$, $n = 3$.

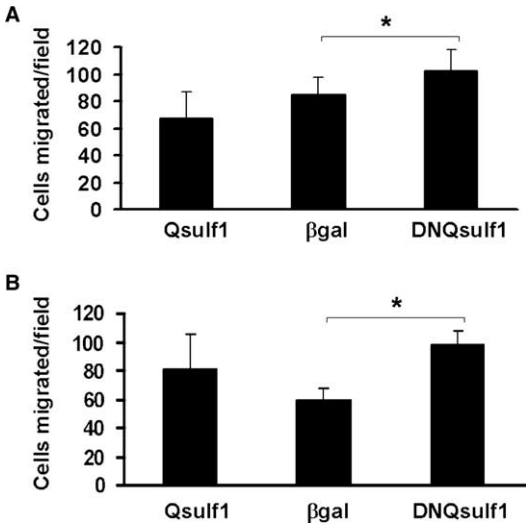

Fig. 5. Measurement of VSMC migration applying onto membranes. (A) Chemotaxis was analyzed using uncoated membranes. (B) Invasion was analyzed using Matrigel coated membranes. $^{*}P < 0.05$, $n = 3$.

not observed in COS, HeLa, CHO or 293 cells [12,13] or according to our studies in VSMC.

Taken together, our results provide the first evidence for a role of sulf1 in regulating the behavior of VSMC. Future studies beyond the scope of this paper using transgenic and knockout animals are now justified to investigate the role of sulf1 in neointima formation in vivo.

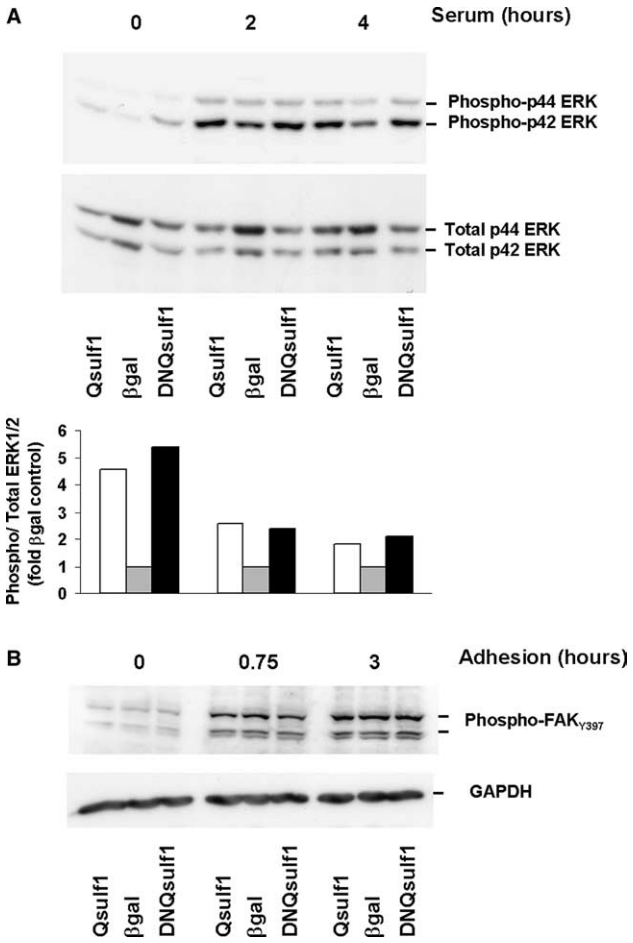

Fig. 6. Phosphorylation of ERK1/2 and FAK. (A) VSMC were infected with adenovirus for 30 h, quiesed in serum-free DMEM with 0.1% lactalbumin for 18 h, then 10% serum was added back for the times shown. 60 μg of total protein per lane was probed for phospho ERK, stripped and probed for total ERK. (B) VSMC were infected with adenovirus for 48 h, detached, rested at 2.5×10^5/ml in DMEM containing 10% serum plus 10 mM HEPES, pH 7.4, at 25 °C for 3 h and then adhered for the times shown. Total cell lysates were probed for phospho FAK, stripped and probed for GAPDH. Representative results of 2 separate experiments are shown.

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