Dependence of proliferative vascular smooth muscle cells on CD98hc (4F2hc, *SLC3A2*)

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Activation of vascular smooth muscle cells (VSMCs) to migrate and proliferate is essential for the formation of intimal hyperplasia. Hence, selectively targeting activated VSMCs is a potential strategy against vaso-occlusive disorders such as in-stent restenosis, vein-graft stenosis, and transplant vasculopathy. We show that CD98 heavy chain (CD98hc) is markedly up-regulated in neointimal and cultured VSMCs, and that activated but not quiescent VSMCs require CD98hc for survival. CD98hc mediates integrin signaling and localizes amino acid transporters to the plasma membrane. SMC-specific deletion of CD98hc did not affect normal vessel morphology, indicating that CD98hc was not required for the maintenance of resident quiescent VSMCs; however, CD98hc deletion reduced intimal hyperplasia after arterial injury. Ex vivo and in vitro, loss of CD98hc suppressed proliferation and induced apoptosis in VSMCs. Furthermore, reconstitution with CD98hc mutants showed that CD98hc interaction with integrins was necessary for the survival of VSMCs. These studies establish the importance of CD98hc in VSMC proliferation and survival. Furthermore, loss of CD98hc was selectively deleterious to activated VSMCs while sparing resident quiescent VSMCs, suggesting that activated VSMCs are physiologically dependent on CD98hc, and hence, CD98hc is a potential therapeutic target in vaso-occlusive disorders.

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Abbreviations used: Adeno-Cre, adenovirus encoding Cre recombinase; Adeno-LacZ, adenovirus encoding β-galactosidase; CA, carotid artery; CD98hc, CD98 heavy chain; GMF, geometric mean fluorescence; PDGF, platelet-derived growth factor; PI, propidium iodide; TUNEL, Tdt-mediated dUTP-biotin nick-end labeling; VSMC, vascular smooth muscle cell.

Vaso-occlusive disorders such as in-stent restenosis, vein-graft stenosis, and transplant vasculopathy involve formation of intimal hyperplasia, i.e., accumulation of vascular smooth muscle cells (VSMCs) in the intimal vessel wall layer (Motwani and Topol, 1998; Schwartz and Henry, 2002; Mitchell and Libby, 2007). Intimal hyperplasia can significantly narrow the vessel lumen, and it also provides a substrate for lipoprotein retention leading to accelerated atherosclerosis (Schwartz et al., 1995; Williams and Tabas, 1995; Nakashima et al., 2008). Activation of quiescent VSMCs to respond to migratory and proliferative stimuli is an important step in the formation of intimal hyperplasia (Clowes et al., 1983; Newby and Zaltsman, 2000). Hence, selectively blocking expansion of activated VSMCs is a potential strategy to reduce intimal hyperplasia without affecting normal vasculature with resident quiescent VSMCs.

CD98 (4F2 antigen, FRP-1) is a transmembrane heterodimer composed of one CD98 heavy chain (CD98hc), the product of the *Slc3a2* gene (also known as 4F2hc), and one out of six different CD98-associated light chains (Wagner et al., 2001). CD98 is involved in cell proliferation and survival (Yagita and Hashimoto, 1986; Diaz et al., 1997; Liu et al., 2004; Féral et al., 2005) and is highly expressed in proliferating tissues (Hashimoto et al., 1983; Masuko et al., 1985). CD98 has two distinct functions, integrin signaling and amino acid transport, and these functions are dependent on different domains of CD98hc. The intracellular and transmembrane domains of CD98hc interact with β1 and β3 integrins and promote integrin outside-in signaling, (Zent et al., 2000; Fenczik et al., 2001; Féral et al., 2005; Prager et al., 2007), whereas the extracellular domain of CD98hc binds to CD98 light chains, targeting them to the plasma membrane

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where they mediate amino acid transport (Fenczik et al., 2001; Wagner et al., 2001).

Because CD98 is involved in cell proliferation and survival, we hypothesized a role for VSMC CD98 in the formation of intimal hyperplasia after vascular injury. In this paper, we show that CD98 is markedly up-regulated in VSMCs activated by vascular injury or by growth factors, and that VSMCs with high CD98 expression are dependent on CD98hc for survival. Using a CD98hc conditional null mouse, we show that VSMC CD98hc is not needed for normal vascular morphology but is required for neointimal formation after arterial injury. Deletion of VSMC CD98hc both suppresses proliferation and induces apoptosis of VSMCs, thus providing a mechanism for its effect on intimal hyperplasia. Furthermore, using reconstitution with CD98hc mutants, we show that the region of CD98hc that interacts with integrins is essential for VSMC survival, whereas the region that mediates amino acid transport is dispensable. These findings show that loss of CD98hc is selectively deleterious to activated VSMCs with high CD98 expression while sparing resident quiescent VSMCs, suggesting CD98hc as a potential therapeutic target in vaso-occlusive disorders.

RESULTS

Expression of CD98 in VSMCs

To assess a potential role of CD98 in VSMC biology, we first analyzed CD98 expression in uninjured and injured arteries of wild-type mice. In uninjured carotid arteries (CAs) and aortas ($n = 3$), faint CD98 staining was seen among the quiescent VSMCs in the media (Fig. 1 A and not depicted). To examine VSMC CD98 expression in injured vessels, we modified a mouse model of carotid intimal hyperplasia (Fig. 1 B, illustrations; Simon et al., 2000). Medial injury was induced by pressure dilation, the endothelium was removed with a nylon fishing line, and flow was reduced by ligation of the external and internal CAs, thus leaving only the occipital artery open at the carotid bifurcation. In this model, a circumferential neointima was formed 3 wk after injury (Fig. 1 B, photomicrographs). Importantly, this model allowed a neointima to be formed in the presence of the remaining medial VSMCs as well as in the presence of a blood flow, making it suitable for SMC-specific gene knockout. In the injured CA ($n = 3$), there was a dramatic increase in CD98 expression in the neointima and among scattered cells in the underlying media (Fig. 1 C). We used flow cytometry to quantify CD98 expression in VSMCs and excluded a contribution from endothelial cells or leukocytes by eliminating CD31- and CD18-positive cells (Fig. S1). In uninjured vessels, we detected a single population of VSMCs that expressed modest levels of CD98. In sharp contrast, there was an additional CD98 bright population in the injured vessels (Fig. 1 D). Quantification of these data revealed an approximately sixfold increase in CD98 expression in the CD98 bright population (uninjured geometric mean fluorescence (GMF) = 40, injured CD98 dull GMF = 38, and CD98 bright GMF = 249). We also examined CD98 expression in cultured VSMCs plated on a mixed fibronectin/gelatin matrix in the presence of 0.2% fetal calf serum. These cultured VSMCs uniformly expressed CD98 at high levels (GMF = 325). Because the formation of intimal hyperplasia involves a change of extracellular matrix (e.g., a change from laminin to fibronectin) and secretion of growth factors, we examined how these factors regulated CD98 expression in cultured VSMCs. The VSMCs were first made quiescent on Matrigel for 2 d and then replated on laminin-1 or fibronectin with or without platelet-derived growth factor (PDGF)–BB for 24 h. Fibronectin significantly increased the CD98 expression compared with laminin-1, and PDGF-BB up-regulated the CD98 expression on both matrices (Fig. 1 E). In summary, quiescent VSMCs in uninjured vessels expressed CD98 at low levels, whereas vascular injury and tissue culture conditions dramatically increased VSMC CD98 expression. Furthermore, the VSMC CD98 expression was regulated by both extracellular matrix and growth factors.

Grossly normal vasculature in the absence of VSMC CD98hc

To assess the importance of CD98 for VSMC function in vivo, we specifically deleted CD98hc in SMCs by crossing mice bearing a conditional null *Slc3a2* allele (*Slc3a2*$^{fl/fl}$; Fig. 2 A; Féral et al., 2007) with *SM22α-Cre* transgenic mice that express Cre recombinase under an SMC-specific promoter (Lepore et al., 2005). In the *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice, the *Slc3a2*$^{fl/fl}$ gene was deleted in >97% of aortic SMCs and CD98 protein was undetectable (Fig. 2, B and C). Despite almost complete loss of VSMC CD98, vascular morphology was apparently normal as judged by histological examination of the CA, the aorta, the femoral artery, the internal jugular vein, and the inferior vena cava (Fig. S2). The functional integrity of the vasculature was confirmed by the normal morphology of highly vascularized tissues such as lung and liver (Fig. S2), and the normal viability, fertility, and weight gain of *Slc3a2*$^{fl/fl}$ *SM22α-Cre* male and female mice (not depicted). We also examined the dorsal aorta at embryonic day 13.5 in *Slc3a2*$^{fl/fl}$ *SM22α-Cre* embryos ($n = 7$) and *Slc3a2*$^{fl/fl}$ controls ($n = 2$). In control embryos, the vascular cells expressed CD98 at high levels. In the *Slc3a2*$^{fl/fl}$*SM22a-Cre* embryos, the CD98 expression was significantly lower, but most vascular cells still stained positive for CD98 (Fig. S3). However, there was no obvious morphological difference between the two groups. Collectively, VSMC CD98hc is not necessary for normal vessel morphology under conditions where VSMCs manifest the quiescent phenotype.

VSMC CD98hc is required for neointima formation in injured CAs

Because cultured and neointimal VSMCs expressed CD98 at high levels, we assessed whether CD98hc is necessary for neointima formation after arterial injury. *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice ($n = 8$) and littermate controls (*Slc3a2*$^{fl/fl}$; $n = 10$) were subjected to the carotid injury described in Fig. 1 B. 3 wk after surgery, there was a dramatic decrease (>70%) in neointimal area in the *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice compared with littermate controls (Fig. 3, A and B). Furthermore, in

Slc3a2$^{fl/fl}$*SM22α-Cre* mice, the injured vessels (intima + media) contained some CD98-positive VSMCs (Fig. 3 C). In these flow cytometry studies, endothelial cells (CD31 positive) and leukocytes (CD18 positive) were excluded in the analyses. The presence of these CD98 bright cells suggests that there was a strong selection for CD98-positive cells during the neointima formation. Collectively, VSMC CD98hc is required for intimal hyperplasia, and the neointima that forms in the *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice is likely caused by selection of cells in which the *Slc3a2*$^{fl/fl}$ gene is retained intact.

CD98hc is important for VSMC explant outgrowth, proliferation, and survival

To study VSMCs ex vivo, we isolated the medial vessel wall layer of thoracic aortas and incubated these VSMC explants in DMEM with 10% serum (n = 6 aortas in each group). In a preliminary experiment, the number of VSMCs that grew out

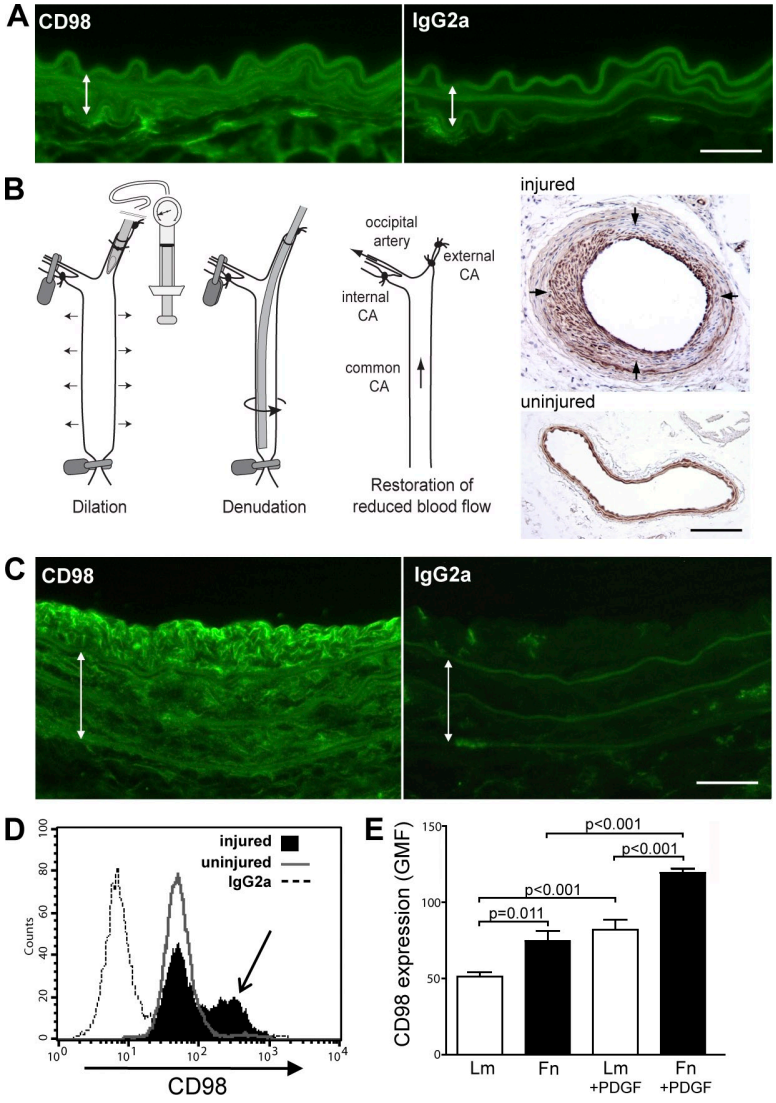

Figure 1. CD98 expression in VSMCs. (A) Uninjured mouse CA stained for CD98 (green; left) or isotype control (right). The lumen is facing up, and double arrows indicate the vessel media. Note the faint green CD98 staining of VSMCs in between the autofluorescent elastic laminae. Data in this panel and in C are representative of one out of three mice so studied. Bar, 25 µm. (B) Mouse CA injury model. (illustrations) Saline dilates the common CA (91 Pa for 20 s), followed by endothelial denudation with a nylon wire and restoration of a reduced blood flow. (photomicrographs) Injured (day 21) and uninjured CA. Arrows indicate the internal elastic lamina. Brown staining indicates SM22α immunostaining. Bar, 100 µm. (C) Expression of CD98 14 d after carotid injury. Note the bright CD98 staining in the neointima. (D) Cellular expression of VSMC CD98 in CAs (intima + media) analyzed by flow cytometry. Endothelial cells (CD31 positive) and leukocytes (CD18 positive) were excluded during the data analyses. Note the CD98 bright VSMC population after vascular injury (arrow). The experiment was performed twice. Three injured CAs and three uninjured CAs were pooled in each experiment. (E) Extracellular matrix and PDGF-BB regulate CD98 expression in cultured mouse VSMCs. VSMCs were differentiated on Matrigel and replated on either laminin-1 (Lm) or fibronectin (Fn) with or without 20 ng/ml PDGF-BB. After 24 h, the replated cells were analyzed for CD98 by flow cytometry. Data are presented as means ± SEM (n = 6 in each group). Data are from two independent experiments.

from *Slc3a2*$^{fl/fl}$*SM22α-Cre* explants after 4 d was markedly reduced (>70%) compared with *Slc3a2*$^{fl/fl}$ controls (Fig. 4 A). Within the *Slc3a2*$^{fl/fl}$*SM22α-Cre* explant tissues, all VSMCs were negative for CD98 protein (Fig. 4 B, left). However, a selection for CD98-positive cells was observed in the outgrown cells; specifically, 78% of VSMCs that grew out from *Slc3a2*$^{fl/fl}$*SM22α-Cre* explants were CD98 positive (Fig. 4 B, right). The strong selection against CD98-null VSMCs in injured vessels in vivo and in explant outgrowths ex vivo could have been caused by increased cell death or a lack of proliferation. To examine these possibilities, explant tissues at day 4 were processed for histological analyses of proliferation (BrdU incorporation over 2 h; $n = 6$ in each group) and apoptosis (Tdt-mediated dUTP-biotin nick-end labeling [TUNEL] assay; $n = 6$ in each group). In *Slc3a2*$^{fl/fl}$*SM22α-Cre* explants, there were very few proliferating cells (0.3 ± 0.1%), in contrast to *Slc3a2*$^{fl/fl}$ control explants that exhibited 2.9 ± 0.4% proliferating cells (Fig. 4 C, left). The *Slc3a2*$^{fl/fl}$*SM22α-Cre* explants also exhibited a 1.8-fold increase in apoptosis relative to control explants (Fig. 4 C, right). Thus, CD98hc is important for VSMC proliferation and survival.

CD98hc is required for VSMC survival in vitro

To precisely define the mechanism by which CD98hc mediates VSMC expansion, we acutely deleted the CD98hc gene in cultured VSMCs. *Slc3a2*$^{fl/fl}$ VSMCs were isolated from the thoracic aorta and the CD98hc gene was deleted using adenovirus encoding Cre recombinase (Adeno-Cre); control *Slc3a2*$^{fl/fl}$ VSMCs were infected with adenovirus encoding β-galactosidase (Adeno-LacZ). The efficiency of CD98hc gene deletion was judged by flow cytometry for CD98. At day 4 after

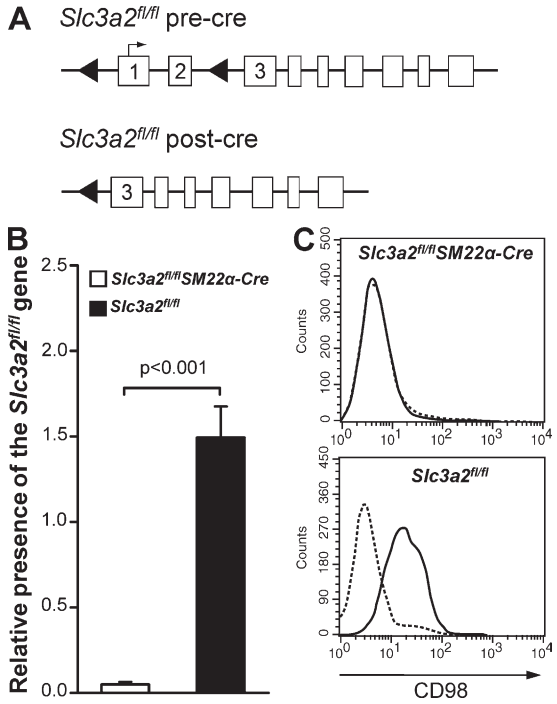

Figure 2. Deletion of CD98hc in *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice. (A) Exons 1 and 2 in the *Slc3a2*$^{fl/fl}$ gene are flanked by loxP sites (closed triangles; top). After exposure to Cre recombinase, these exons, including the start codon (arrow on exon 1), will be excised (bottom) and CD98hc will not be expressed. (B) Efficiency of *Slc3a2*$^{fl/fl}$ gene deletion. The media from the thoracic aorta was isolated from *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice ($n = 3$) and *Slc3a2*$^{fl/fl}$ littermate controls ($n = 3$). The relative presence of the *Slc3a2*$^{fl/fl}$ gene (normalized to the α4 integrin gene) was quantified with real-time PCR. Data are presented as means ± SEM and are from one experiment. (C) Loss of VSMC CD98 protein expression. Single-cell suspensions were prepared from the media of thoracic aortas and analyzed by flow cytometry for CD98 (continuous lines; the dotted lines indicate isotype control). (top) VSMCs from *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice. (bottom) VSMCs from *Slc3a2*$^{fl/fl}$ mice. Two aortas were pooled in each group. The experiment was performed twice.

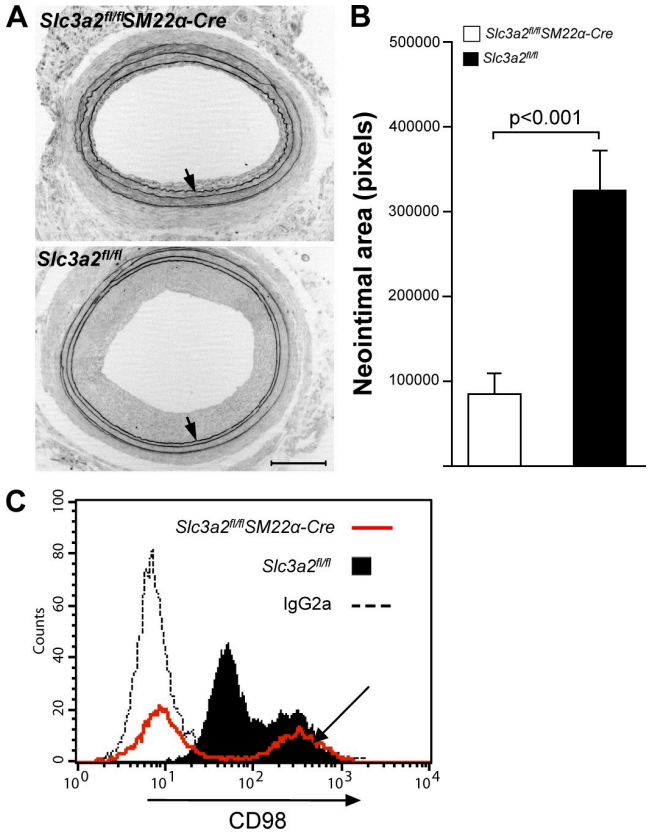

Figure 3. VSMC CD98hc deletion inhibits neointimal hyperplasia. (A) Representative images of neointima formation 3 wk after carotid injury in *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice (top) and *Slc3a2*$^{fl/fl}$ littermate controls (bottom). Arrows indicate the internal elastic lamina, which demarcates the intima from the media. Bar, 100 µm. (B) Quantification of neointimal area (pixels) in *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice ($n = 8$) and *Slc3a2*$^{fl/fl}$ littermates ($n = 10$). Data are presented as means ± SEM. P = 0.0006. (C) Cellular expression of VSMC CD98 in *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice ($n = 3$) and *Slc3a2*$^{fl/fl}$ mice ($n = 3$) 14 d after carotid injury. The adventitias were removed, and the remaining tissues (intima + media) in each group were pooled and processed for flow cytometry for CD98. Endothelial cells (CD31 positive) and leukocytes (CD18 positive) were excluded in the data analyses. The dotted line indicates IgG2a isotype control. Note the bright CD98-positive population in the injured CAs from *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice (arrow). The figure is representative of two independent experiments.

Adeno-Cre infection, >95% of the cells had reduced CD98 protein expression to <10% of that seen in Adeno-LacZ–infected cells (unpublished data). To assess the importance of CD98hc for VSMC survival, we enumerated the number of live cells (propidium iodide [PI]–negative cells) at various times after adenovirus infection. The cells were cultured in limiting serum (0.2% serum). Beginning 5 d after Adeno-Cre infection, there was a reduction of 85 ± 3.1% in viable VSMCs, and by day 6, 98 ± 0.5% of the VSMCs were lost (compared with Adeno-Cre–infected VSMCs at day 3). Among Adeno-LacZ–infected VSMCs, there was <10% loss of live cells at days 5 (9.2 ± 2.4%) and 6 (8.1 ± 7.5%) compared with day 3 (Fig. 5 A). The massive cell death was most likely caused by apoptosis, because the Adeno-Cre–treated cells stained positive for TUNEL assay (Fig. 5 B) and exhibited activated caspase-1/3, as assessed by staining with FITC-zVAD-FMK (CaspACE; Fig. 5 C). Because PDGF-BB up-regulated CD98 expression in cultured VSMCs (Fig. 1 E), we also examined the effect of this growth factor on VSMC survival after CD98hc gene deletion. Stimulation with PDGF-BB or 10% serum caused Adeno-Cre–treated VSMCs to undergo accelerated cell death compared with cells in low serum conditions (Fig. 5 D). In conclusion, CD98hc is necessary for the survival of cultured VSMCs; i.e., they are physiologically dependent on their CD98hc expression and the CD98hc dependence is exacerbated by growth factor stimulation.

The region of CD98hc that interacts with integrins but not amino acid transporters supports VSMC survival

CD98hc can mediate both integrin signaling and amino acid transport. The integrin function is dependent on both the cytoplasmic and transmembrane domain, whereas the extracellular domain is necessary for amino acid transport. To assess which domains of CD98hc were important for VSMC survival, CD98hc-deficient VSMCs (Adeno-Cre treated) were reconstituted with full-length CD98hc, full-length CD69, and three CD98hc-CD69 chimeras in which the cytoplasmic (C69T98E98), the transmembrane (C98T69E98), or the extracellular (C98T98E69) domain had been switched to CD69 (Fig. 6 A; Fenczik et al., 2001; Féral et al., 2005). CD69 is a type II transmembrane protein, but it is not known to bind integrins or CD98 light chains. Full-length CD98hc and the chimera that supports integrin function (C98T98E69) rescued CD98hc-deficient VSMCs from apoptosis. In sharp contrast, full-length CD69 and the chimeras that support amino acid transport function (C69T98E98 and C98T69E98) did not rescue VSMCs from apoptosis (Fig. 6 B). Hence, the domains of CD98hc that associate with integrins are essential for VSMC survival.

DISCUSSION

Vaso-occlusive disorders, such as in-stent restenosis, vein-graft stenosis, and transplant vasculopathy, involve intimal hyperplasia, which is caused by an activation of VSMCs to migrate and proliferate (Motwani and Topol, 1998; Schwartz and Henry, 2002; Mitchell and Libby, 2007). In this paper, we show that CD98hc is markedly up-regulated in VSMCs stimulated by vascular injury and by in vitro cell-culturing conditions. Furthermore, using SMC-specific deletion of

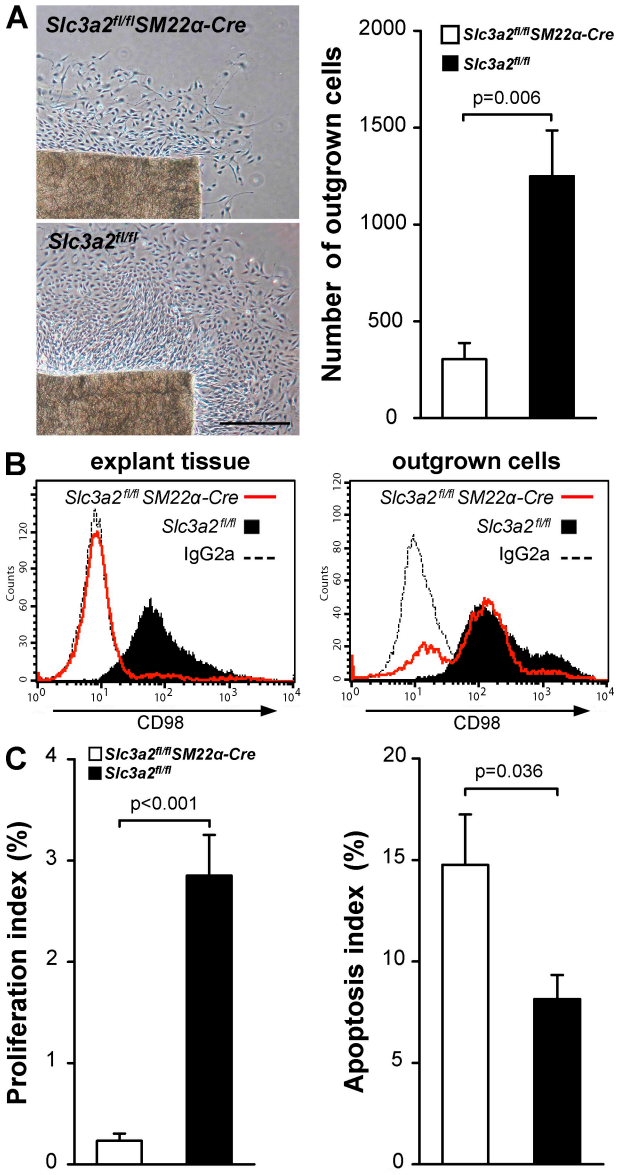

Figure 4. CD98hc is required for VSMC outgrowth, proliferation, and survival ex vivo. (A) Outgrown cells from media explants cultured in 10% serum were enumerated at day 4. (top) *Slc3a2*$^{fl/fl}$*SM22α-Cre* explant. (bottom) *Slc3a2*$^{fl/fl}$ littermate explant. The bar graph quantifies outgrown cells from *Slc3a2*$^{fl/fl}$*SM22α-Cre* explants and *Slc3a2*$^{fl/fl}$ controls (n = 6 explants from six mice in each group for a total of 72 explants). Data are presented as means ± SEM and are from one experiment. Bar, 100 µm. (B) CD98 expression in digested explant tissues (left) and in outgrown cells (right) analyzed by flow cytometry. The dotted line indicates IgG2a antibody control. Explants from six mice were pooled in each group. The experiment was performed twice with identical results. (C) Proliferation and apoptosis within explant tissues at day 4. (left) Proliferation index (percentage of BrdU-positive cells). (right) Apoptosis index (percentage of TUNEL-positive cells). Data are presented as means ± SEM (n = 6 explants from six mice in each group).

CD98hc, we show that loss of VSMC CD98hc is compatible with normal vessel morphology; however, CD98hc is required for formation of a neointima after arterial injury. The mechanism of this effect of VSMC CD98hc deletion was both suppression of proliferation and induction of apoptosis. CD98hc has two distinct functions, amino acid transport and interaction with integrins to enhance integrin signaling. Using reconstitution with CD98hc mutants, we show that the moiety of CD98hc that interacts with integrins and mediates integrin signaling is necessary and sufficient for the survival of VSMCs. These studies are the first to establish the role of CD98hc in VSMC proliferation and survival, and show that the ability of CD98hc to interact with integrins is essential for these functions. Furthermore, loss of CD98hc is selectively deleterious to activated VSMCs with high CD98 expression while sparing resident quiescent VSMCs, explaining its capacity to block neoinitima formation and indicating that CD98hc is a potential therapeutic target in vaso-occlusive disorders.

The present study shows that CD98hc expression is up-regulated when VSMCs are stimulated by vascular injury and growth factors. CD98hc expression was low in quiescent resting VSMCs in the aorta and CA but was markedly increased after vessel injury. Furthermore, CD98hc expression was high in VSMCs that grew out from tissue explants and in cultured VSMCs, and the CD98hc expression was up-regulated by fibronectin and PDGF-BB. Thus, in three different experimental systems (in vivo, ex vivo, and in vitro) CD98hc was up-regulated in activated VSMCs and can therefore be considered an activation marker. The first intron of the CD98hc gene contains a transcriptional enhancer element that is tightly regulated coordinately with cell proliferation, suggesting that this element is responsible for the increased CD98hc in activated VSMCs (Karpinski et al., 1989).

Strikingly, deletion of CD98hc in VSMCs did not seem to affect quiescent VSMCs in the normal vasculature. In the *Slc3a2*$^{fl/fl}$*SM22α-Cre* embryo, a slow turnover of the CD98 protein after gene deletion might have enabled the vasculature to develop. Indeed, we found residual CD98 expression at embryonic day 13.5 (Fig. S3), which is 4 d after the *SM22α-Cre* transgene is reported to be expressed (Lepore et al., 2005). The finding that a mature vasculature can form and function without VSMC CD98hc shows that CD98hc can be inhibited without lethal effects on resting quiescent VSMCs. In contrast to quiescent VSMCs, CD98hc was required for activated VSMCs during the formation of intimal hyperplasia and for cultured VSMCs. The selective effect of CD98hc deletion on activated VSMCs suggests that VSMC activation leads to a dependence on CD98hc for their survival. This dependence

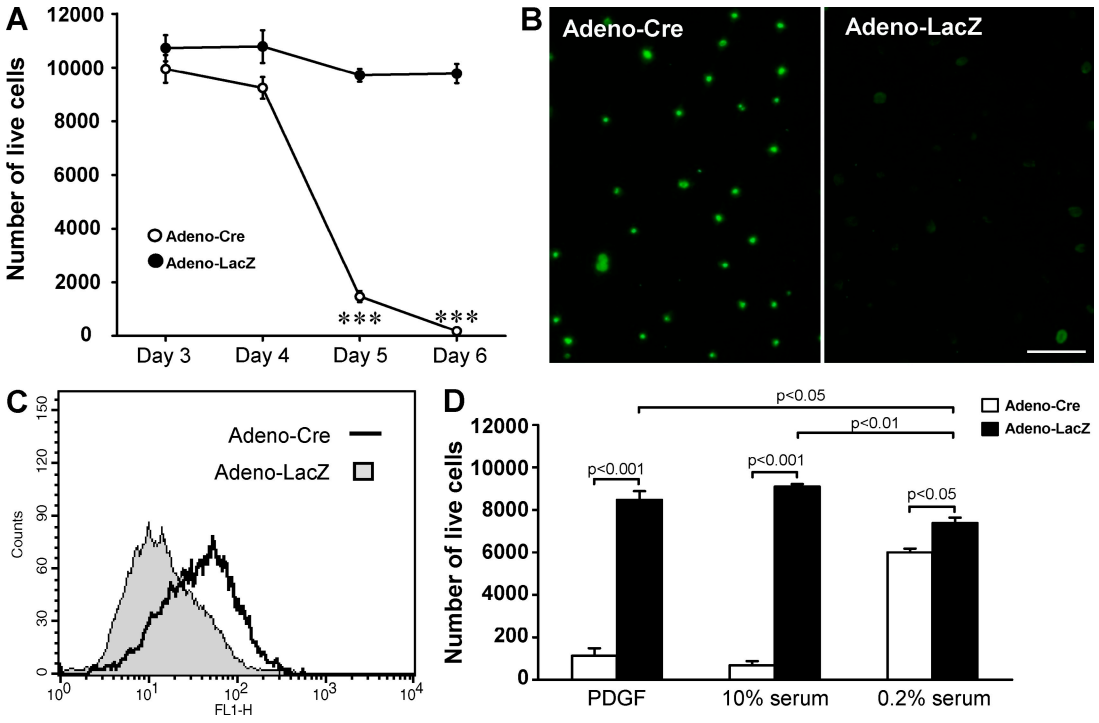

Figure 5. CD98hc is required for survival of cultured VSMCs. (A) The relative number of live *Slc3a2*$^{fl/fl}$ VSMCs estimated by flow cytometry 3, 4, 5, and 6 d after CD98hc gene deletion with Adeno-Cre. Data are presented as means ± SEM (n = 3 in each group). ***, P < 0.001. (B) TUNEL staining of *Slc3a2*$^{fl/fl}$ VSMCs 5 d after Adeno-Cre and Adeno-LacZ treatment. Bar, 100 µm. (C) Caspase-1/3 activity demonstrated by flow cytometry (CaspACE) in *Slc3a2*$^{fl/fl}$ VSMCs 4 d after adenovirus treatment. (D) Growth factor stimulation increases the loss of CD98hc-deficient VSMCs. At day 3.5, adenovirus-infected *Slc3a2*$^{fl/fl}$ VSMCs were treated with 20 ng/ml PDGF-BB, 10% serum, or 0.2% serum. At day 4, the relative number of live cells was assessed by flow cytometry. Data are presented as means ± SEM (n = 3 for each group). Data in A and D are from three experiments, and the experiments in B and C were performed twice with identical results.

is remarkably similar to the "addiction" of tumor cells to oncogenes, so-called oncogene addiction (Weinstein, 2002). For example, inhibition of Abl kinase with Gleevec leads to the death of BCR-Abl–expressing chronic myelogenous leukemia leukocytes but has no effect on the survival of normal leukocytes (Dan et al., 1998). The mechanism of oncogene addiction is uncertain, but one concept is that the presence of a given oncogene enhances proliferation/survival as well as apoptosis signals. Consequently, cancer cells may be addicted to the survival activity of the oncogene to avoid apoptosis (Weinstein and Joe, 2006). Our data show that the CD98 expression in activated VSMCs enables their survival; hence, these cells are similarly dependent on CD98hc to avoid apoptosis.

CD98hc performs two cellular functions: amino acid transport and integrin signaling. The extracellular domain of CD98hc is responsible for amino acid transport through interaction with one of several CD98 light chains (Fenczik et al., 2001; Wagner et al., 2001), and the transmembrane and cytoplasmic domains of CD98hc mediate interaction with β integrin subunits and the generation of survival signals such as activation of Akt (Fenczik et al., 1997, 2001; Féral et al., 2005). Reconstitution studies with CD98hc-CD69 chimeras showed that the integrin-binding moiety of CD98hc was essential for the survival of VSMCs. In contrast, the CD98hc chimeras that can interact with CD98 light chains to promote amino acid transport failed to rescue survival in vitro. Nevertheless, the interaction with CD98 light chains might be important in the functions of metabolically active VSMCs with a high proliferative rate and extensive protein synthesis. Indeed, others have reported that blockade of LAT1 and LAT2 function, two of the six CD98 light chains, can lead to apoptosis of PDGF-BB–stimulated VSMCs (Liu et al., 2004). This might explain the accelerated cell death among CD98-deficient VSMCs treated with PDGF-BB or serum in our study. On the other hand, PDGF-BB also reduces VSMC adhesion

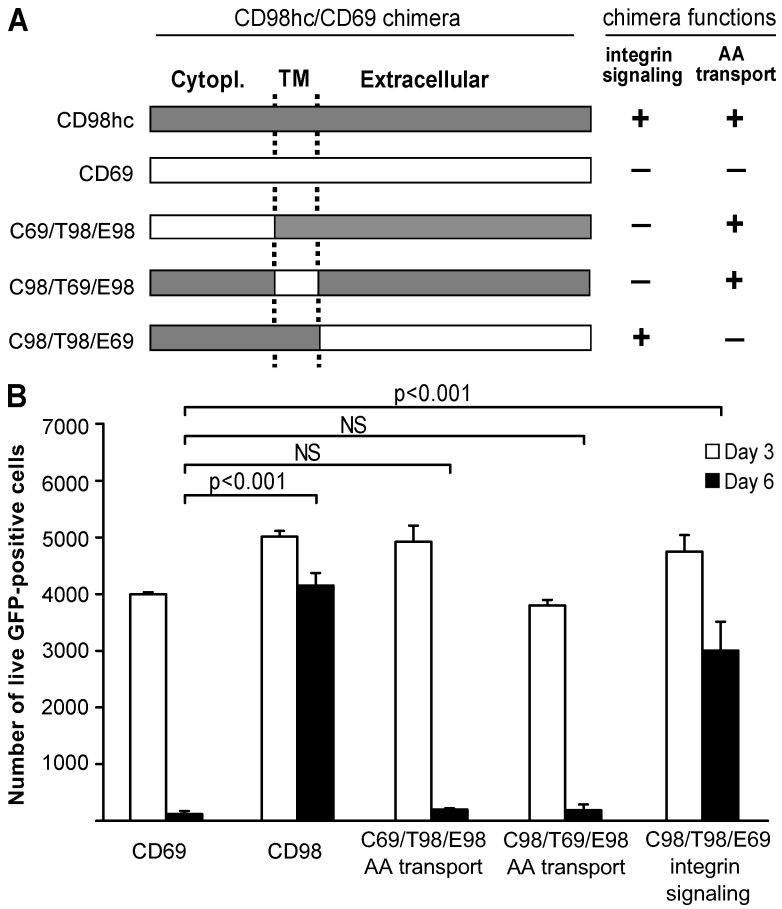

Figure 6. The integrin-binding function of CD98hc mediates VSMC survival. (A) Illustration of human CD98hc-CD69 chimeras and their interaction with integrins (integrin signaling) or amino acid transporters (AA transport). CD69 is a type II transmembrane protein not known to bind to integrins or CD98 light chains. Each chimera is defined by its cytoplasmic (C), transmembrane (T), and extracellular (E) domain derived from either CD98hc (98; gray) or CD69 (69; white). (B) The integrin-binding CD98 chimera rescues CD98hc-deficient VSMCs from cell death. *Slc3a2*$^{fl/fl}$ VSMCs were infected with retrovirus coding for GFP and any of the five human CD98hc-CD69 constructs described in A. The endogenous mouse CD98hc was deleted with Adeno-Cre. The relative number of reconstituted live cells (GFP positive and PI negative) was assessed by flow cytometry at days 3 and 6 after Adeno-Cre treatment. Data are presented as means ± SEM from three independent experiments.

to fibronectin (Berrou and Bryckaert, 2001), and hence, the PDGF-BB–treated cells might have become more susceptible to apoptosis as a result of reduced attachment to the extracellular matrix. Clearly, the possible importance of CD98hc in VSMC amino acid transport function awaits additional study. Interestingly, the LAT1/2 inhibition did not have any effect on VSMC proliferation (Liu et al., 2004), suggesting that the integrin function and not the amino acid transport function of CD98hc regulates VSMC proliferation. In support of this, Féral et al. (2005) have shown in teratocarcinomas that the integrin-binding portion of CD98hc promotes cell proliferation. Collectively, the integrin-binding function of CD98hc seems to be an important regulator of both VSMC survival and proliferation.

VSMC αVβ3 and α5β1, two integrins that couple to CD98hc, (Zent et al., 2000) are key players in the formation of intimal hyperplasia. Inhibition of αVβ3 reduces neointima formation in several studies (for review see Kokubo et al., 2007), and α5β1 is responsible for neointima fibronectin assembly after vascular injury (Pickering et al., 2000). Binding of α5β1 integrin to fibronectin induces a synthetic VSMC phenotype and promotes VSMC proliferation and survival (Hedin et al., 1997; Mercurius and Morla, 1998; Freyer et al., 2001; Taylor et al., 2001). CD98hc interaction with integrins is important for $pp125^{FAK}$-dependent phosphoinositide 3-kinase activation with downstream activation of Akt and phosphorylation of $p130^{CAS}$ leading to Rac activation (Féral et al., 2005), and these pathways have been implicated in the survival and proliferation of VSMCs (Bai et al., 1999; Stabile et al., 2003; Allard et al., 2008; Bond et al., 2008). Thus, suppressed proliferation and increased apoptosis in CD98hc-deleted VSMCs might be a result of impaired phosphoinositide 3-kinase–Akt signaling and Rac activation. Deletion of VSMC CD98 in vitro led to degradation of $pp125^{FAK}$ before death (unpublished data), precluding study of the effects of CD98 on signaling events linked to $pp125^{FAK}$. However, our results underscore that only activated VSMCs require CD98hc, and the CD98hc domains that support integrin function are crucial for VSMC survival, suggesting that activation of VSMCs leads cells to become dependent on enhanced integrin signaling for survival.

Our data suggest that inhibition of VSMC CD98hc function could be therapeutically useful. In particular, we show that activated VSMCs with high CD98 expression require CD98hc for survival and proliferation, whereas quiescent normal VSMCs with low CD98 expression do not. Most vascular interventions enable local postangioplastic treatments applied in a perivascular gel or attached to an endoluminal stent. Local application of small interfering RNA, antisense oligonucleotides, or lentiviral-delivered short hairpin RNA against CD98hc is a potential therapeutic strategy (Crooke, 2004; Banno et al., 2006; Akhtar and Benter, 2007). It may also be possible to develop blocking antibodies against CD98hc that could be locally administered in connection with vascular surgery (Hashimoto et al., 1983). Finally, our data suggest that the integrin–CD98hc interaction is a potential target for a small molecular inhibitor that could selectively kill activated VSMCs. The interaction sites for CD98hc on the β1 and β3 integrin tails have been mapped (Prager et al., 2007), and recent studies have established the feasibility of developing cell-permeable inhibitors of integrin cytoplasmic domain interactions (Ambroise et al., 2002; Su et al., 2008). RGT, a synthetic peptide corresponding to the β3 integrin cytoplasmic C-terminal sequence, selectively inhibits outside-in signaling in human platelets by disrupting the interaction of αIIbβ3 integrin with Src kinase (Su et al., 2008). Thus, our studies establish the selective importance of CD98hc for the survival and proliferation of activated VSMCs, show that the connection to integrins is important for this activity, and identify CD98hc as a potential therapeutic target in vaso-occlusive disorders.

MATERIALS AND METHODS

Animals and carotid injury. All surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego. CD98hc conditional null mice (*Slc3a2*$^{fl/fl}$; Féral et al., 2007) were crossed with *SM22α-Cre* transgenic mice (Lepore et al., 2005) to delete the CD98hc gene (*Slc3a2*) in SMCs. For all in vivo and ex vivo experiments, *Slc3a2*$^{fl/fl}$*SM22α-Cre* mice and sex-matched *Slc3a2*$^{fl/fl}$ littermate controls aged 7–9 wk were used. The carotid injury model (Fig. 1 B) was based on a previously published study (Simon et al., 2000). The CA was dilated at 91 Pa for 20 s, and the endothelium was denuded with a nylon wire (0.19 mm in diameter). To facilitate neointima formation, the postangioplastic blood flow was reduced by leaving only the occipital artery open at the carotid bifurcation (Korshunov and Berk, 2003). Injured arteries were analyzed 450–550 μm proximal to the carotid bifurcation. Neointimal area was quantified (pixels) using the National Institutes of Health (NIH) Image J software (available at http://rsbweb.nih.gov/ij/) on cross sections visualized by autofluorescence (excitation wavelength = 488 nm; 530 nm band-pass filter; Fig. 3 A).

Histology and immunohistochemistry. Tissue samples were processed and prepared for standard histological and immunohistochemical procedures (Fogelstrand et al., 2005). Formalin-fixed tissues were pressure perfused at 100 mmHg. Formalin-fixed paraffin sections were used for all histology except for CD98 immunostaining, which was performed on frozen sections. The following antibodies and kits were used: rabbit anti–human SM22α (Proteintech Group, Inc.), horseradish peroxidase–conjugated goat anti–rabbit antibody (SouthernBiotech), rat anti–mouse CD98 (clone RL388; eBioscience), rat IgG2a isotype control (eBioscience), Alexa Fluor 488–conjugated goat anti–rat (Invitrogen), the In Situ Cell Death Detection Kit (Roche), and the BrdU Labeling and Detection Kit I (Roche).

Flow cytometry. Immunolabeled cells were analyzed on a flow cytometer (FACSCalibur; BD) with CellQuest software (BD). For all vessels, the adventitia was removed after a 5-min treatment with 10 mg/ml collagenase at room temperature, and the remaining intima + media were digested to a single-cell suspension at 37°C in a 10 mg/ml collagenase and 2.5 mg/ml elastase solution (Worthington Biochemical Corp.). Endothelial cells (CD31 positive) and leukocytes (CD18 positive) were excluded by gating during the data analyses. The following antibodies were used: PE-conjugated anti–mouse CD98 (clone RL388; eBioscience), PE-conjugated rat IgG2a control antibody (eBioscience), FITC-conjugated anti–mouse CD31 (clone MEC 13.3; BD), and FITC-conjugated anti–mouse CD18 antibodies (clone M18/2; eBioscience). For cell counting, cells were trypsinized and resuspended in a fixed volume of PBS with 5 μg/ml PI (Invitrogen). The relative number of live cells (PI negative) was determined by collecting resuspended cells for 60 s on the flow cytometer.

Aortic media explants. Approximately 1 × 1 mm media explants were prepared from the thoracic aorta (Choi et al., 2004). Endothelial cells were

killed by drying the lumen with 2 × 20 ml of air from a syringe. To facilitate the removal of the adventitia, the isolated vessels were treated for 5 min with 10 mg/ml collagenase at 37°C. The isolated media explants were cultured luminal side down in DMEM with 10% serum, under the weight of a culture plate insert. At day 4, all outgrown cells from each explant were counted manually under a phase contrast inverted tissue culture microscope (model CK2; Olympus). For flow cytometry analyses, all outgrown cells at day 4 were collected by trypsinization, and the remaining explant tissues were digested to a single-cell suspension. For proliferation analyses, BrdU was added to the culture media (10-μM final concentration) 2 h before harvest.

VSMC isolation and CD98hc gene deletion. VSMCs were isolated from the thoracic aorta from *Slc3a2*$^{fl/fl}$ mice. The cultured VSMCs were characterized by morphological (Fig. S4 A) and immunological criteria (flow cytometry quantification of smooth muscle α-actin; Fig. S4 B). For all in vitro experiments, VSMCs were used at passages 5–7. All culture plates were coated with a mix of 1 μg/ml fibronectin and 0.02% gelatin for 30 min at room temperature. The CD98hc gene was deleted using Adeno-Cre (600 particles/cell). Adeno-LacZ was used as control (Vector Laboratories).

Regulation of VSMC CD98 by extracellular matrix and PDGF-BB. 700,000 *Slc3a2*$^{fl/fl}$ VSMCs were plated in a 10-cm petri dish coated with 200 μl Matrigel (BD) in low-glucose DMEM supplemented with 1% serum and 2% Matrigel. The medium was changed on day 1 to low-glucose DMEM supplemented with 0.2% serum and 2% Matrigel. On day 2, the cells were detached with dispase (BD) and replated in a 12-well plate (20,000 cells/well) coated with either 20 μg/ml of mouse laminin-1 (BD) or 10 μg/ml of bovine fibronectin (Sigma-Aldrich). 10 μg/ml laminin-1 or 10 μg/ml fibronectin was also added to the media. 20 ng/ml PDGF-BB was added to half of the cells. On day 3, the VSMC CD98 expression was analyzed using a flow cytometer.

TUNEL assay and caspase-1/3 activity on cultured VSMCs. DNA fragmentation on Adeno-Cre–treated VSMCs was analyzed with In Situ Cell Death Detection Kit, and caspase-1/3 activity was demonstrated with flow cytometry using FITC-zVAD-FMK (CaspACE; Promega) according to manufacturer's instructions.

Reconstitution with human CD98hc-CD69 chimeras. Reconstituted cells were generated by infecting *Slc3a2*$^{fl/fl}$ VSMCs with MSCV-IRES-GFP retrovirus (Cherry et al., 2000) encoding human CD98hc, human CD69, or CD98hc-CD69 chimeras (Fig. 6 A) and GFP (Fenczik et al., 2001). Viruses were generated in the Phoenix EcoPack mouse ecotropic packaging cell line (Imgenex) by transfection (Lipofectamine Plus; Invitrogen) of the MSCV-IRES-GFP constructs together with a pCL-Eco mouse ecotropic packaging plasmid (Imgenex). On days 2 and 3 after transfection, the virus-containing cell-culture media were filtered (0.45-μm SFCA filter; Corning), supplemented with 6 μg/ml polybrene (Sigma-Aldrich), and added to the VSMCs. 2 d later, the VSMCs were replated in 12-well plates and infected with Adeno-Cre to delete the endogenous mouse CD98hc gene. At days 3, 4, 5, and 6 after Adeno-Cre treatment, the number of PI-negative/GFP-positive cells was counted using a flow cytometer.

Statistics. Data were analyzed with a two-tailed Student's *t* test and a two-way analysis of variance with the Bonferonni posthoc test using Prism 5 software (GraphPad Software, Inc.).

Online supplemental material. Fig. S1 shows flow cytometry data analyses on injured CAs. Fig. S2 shows the histology of different vessels and organs from *Slc3a2*$^{fl/fl}$*SM22α-Cre* and *Slc3a2*$^{fl/fl}$ mice. Fig. S3 shows CD98 immunostaining of the dorsal aorta from *Slc3a2*$^{fl/fl}$*SM22α-Cre* and *Slc3a2*$^{fl/fl}$ embryos at embryonic day 13.5. Fig. S4 shows morphological and immunological characterization of isolated VSMCs. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20082845/DC1.

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