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Regulation of CD44E by DARPP-32-dependent activation of SRp20 splicing factor in gastric tumorigenesis

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Abstract

Objective—CD44E is a frequently overexpressed variant of CD44 in gastric cancer. Mechanisms that regulate CD44 splicing and expression in gastric cancer remain unknown. Herein, we investigated the role of DARPP-32 (dopamine and cAMP-regulated phosphoprotein, Mr 32000) in promoting tumor growth through regulation of CD44 splicing.

Design—Quantitative luciferase reporter, quantitative real-time RT-PCR (qRT-PCR), Western blot, co-immunoprecipitation, ubiquitination, and tumor xenograft experiments were performed.

Results—Western blot and qRT-PCR results indicated that knockdown of endogenous DARPP-32 markedly reduces expression of CD44 V8-V10 (CD44E). Using a quantitative splicing luciferase reporter system, we detected a significant increase in the reporter activity following DARPP-32 overexpression ($p < 0.001$). Conversely, knocking down endogenous DARPP-32 significantly attenuated the splicing activity ($p < 0.001$). Further experiments showed that DARPP-32 regulates the expression of SRp20 splicing factor and co-exists with it in the same protein complex. Inhibition of alternative splicing with digitoxin followed by immunoprecipitation and immunoblotting indicated that DARPP-32 plays an important role in regulating SRp20 protein stability. The knockdown of endogenous DARPP-32 confirmed that DARPP-32 regulates the SRp20-dependent CD44E splicing. Using tumor xenograft mouse model, knocking down endogenous DARPP-32 markedly reduced SRp20 and CD44E protein levels with a decreased tumor growth. The reconstitution of SRp20 expression in these cells rescued tumor growth. In addition, we also demonstrated frequent co-overexpression and positive correlation of DARPP-32, SRp20 and CD44E expression levels in human gastric primary tumors.

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Conflict of interest:

The authors declare no conflict of interest.

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Conclusion—Our novel findings establish for the first time the role of DARPP-32 in regulating splicing factors in gastric cancer cells. The DARPP-32–SRp20 axis plays a key role in regulating the CD44E splice variant that promotes gastric tumorigenesis.

Keywords

SRp20; CD44; Splicing; DARPP-32

Introduction

Gastric cancer is one of the most frequently diagnosed malignancies with poor prognosis in the world (Jemal et al 2011). We have previously shown that Dopamine and cAMP-regulated phosphoprotein, Mr 32000 (DARPP-32) is overexpressed in approximately two-thirds of gastric cancers (El-Rifai et al 2002, Mukherjee et al 2010); and associated with increased survival, drug resistance, and invasion activity of gastric cancer cells (Belkhiri et al 2005, Zhu et al 2011, Zhu et al 2012). We have reported that the overexpression of DARPP-32 occurs in the early stages of the gastric tumorigenesis (Mukherjee et al 2010). However, the mechanisms by which DARPP-32 promotes gastric carcinogenesis remain unclear.

The ubiquitously expressed CD44 is a cell surface glycoprotein, which participates in cell-cell or cell-extracellular matrix interactions (Goodison et al 1999, Pall et al 2011). CD44 expression in cancer cells promotes tumorigenicity, and enhances cell migration, invasion, and metastasis (Hiraga et al 2013). Alternative splicing of the human *CD44* gene is the main source of the diverse *CD44* isoforms (Klingbeil et al 2010). In the standard form (*CD44s*), only 10 exons are transcribed. The 10 variant exons (v1–v10) between exons 5 and 15 can be alternatively spliced in a very large number of different combinations (Screaton et al 1992). The overexpression of *CD44*v9 has been associated with invasive prostate cancer (Omara-Opyene et al 2004). In addition, a previous study indicated that the expression of *CD44* variant isoforms, specifically CD44v6, in sporadic gastric tumors is a potential marker to distinguish intestinal- and diffuse-type gastric adenocarcinomas (Heider et al 1993). The expression of CD44v8-v10 (CD44E) has been reported to be a prognostic marker in gallbladder cancer (Muramaki et al 2004).

mRNA splicing is involved in the maturation of nearly all mRNAs, and a previous study indicated that 90% of human genes can produce different isoforms through alternative splicing (Wang et al 2008). Genome-wide molecular analyses have revealed that tumorigenesis often involves alternative splicing (Xi et al 2008). The regulation of alternative splicing requires interactions between splicing factors and the pre-mRNA sequences (Long and Caceres 2009). The splicing regulatory sequences are recognized by splicing factors (e.g., SR and hnRNP proteins) (Erkelenz et al 2012) and can easily be perturbed by relatively small changes in the levels of splicing factors. SRp20 (SRSF3) is a splicing factor that regulates alternative splicing by interacting with RNA cis-elements (Cavaloc et al 1999). In fact, overexpression of SRp20 alters the RNA splicing of many genes in mammalian cells, thereby affecting the expression levels of various protein isoforms (Matlin et al 2005). The overexpression of SRp20 in many cancer types is essential for cancer cell survival and carcinogenesis (Biamonti et al 1998, Jia et al 2010).

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The objective of this study was to investigate the role of DARPP-32 in regulating CD44 and promoting gastric tumorigenesis. We have uncovered that DARPP-32 enhances CD44E expression through regulation of CD44 splicing mediated by SRp20 splicing factor. We have demonstrated that DARPP-32 interacts and stabilizes SRp20 protein, thereby increasing splicing activity and expression of CD44E. These novel findings underscore the importance of the DARPP-32-SRp20 axis in regulating the CD44E splice variant that plays a crucial role in promoting gastric tumorigenesis.

Results

Expression of CD44E splice variant is regulated by DARPP-32

To examine if modulation of DARPP-32 expression has an effect on the expression of *CD44* splice variants, we utilized MKN-45 gastric cancer cell model. The human *CD44* gene is able to produce several functional mRNAs through the combinatorial inclusion of one or multiple in-frame alternative exons in the central variable region (da Cunha et al 2010, Ponta et al 2003). We measured the mRNA expression of each variable exon (exon6 -exon14) by qRT-PCR in MKN-45 cells stably expressing DARPP-32 shRNA or control shRNA. The qRT-PCR data indicated that exons 12-14 are expressed at significantly higher levels than other exons of *CD44* gene and their expression levels were significantly reduced upon knockdown of endogenous DARPP-32 (Figure 1A, $p < 0.01$), suggesting that DARPP-32 promotes differential expression of *CD44* exons. Amplification of the entire central variable region of *CD44* by RT-PCR indicated that MKN-45 cells constitutively express one major variant transcript of *CD44*, but knockdown of DARPP-32 significantly decreased the transcript level (Figure 1B). The transcript was purified, cloned, and identified by DNA sequencing as human *CD44E*, which is standard *CD44* spliced together with exon12-14 (Figure 1B). Western blot analysis confirmed the expression of CD44E protein (110 kDa) band with higher molecular weight than standard CD44 (80 kDa). Of note, knockdown of endogenous DARPP-32 in MKN-45 cells led to a remarkable decrease in CD44E protein level with a modest effect on the CD44s form (Figure 1C). We also confirmed by qRT-PCR with different sets of primers that knocking down DARPP-32 decreased splicing of exon12-14 in MKN-45 cells (Figure 1D, $p < 0.01$, $p < 0.05$). These results indicated that knockdown of DARPP-32 expression significantly down-regulated *CD44E* splice variant expression. Taken together, the data suggested a possible important role for DARPP-32 in regulating alternative splicing of *CD44*.

DARPP-32 enhances gene splicing activity

To address the question of whether DARPP-32 can regulate splicing activity, we utilized a rapid-response splicing luciferase reporter system (Younis et al 2010). We transfected multiple increasing amounts of DARPP-32 expression construct in combination with the splicing luciferase reporter into HEK-293 cells. The luciferase activity data showed that DARPP-32 significantly enhanced splicing activity in a dose-dependent manner ($p < 0.001$, Figures 2A&2B). In addition, the stable overexpression of DARPP-32 in AGS gastric cancer cells significantly increased splicing activity ($p < 0.001$, Figure 2C). Conversely, the knockdown of endogenous DARPP-32 in MKN-45 gastric cancer cells decreased cell

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splicing activity ($p < 0.001$, Figure 2D). Collectively, the luciferase data confirmed a novel role for DARPP-32 in enhancing the splicing activity in gastric cancer cells.

DARPP-32 up-regulates SRp20 protein levels through a posttranslational mechanism

To investigate whether DARPP-32 enhances *CD44E* expression through regulation of splicing factors, we utilized AGS and MKN-45 cell models. We knocked down Tra2-β1 or SRp20 splicing factors by specific siRNA in MKN-45 cells and evaluated CD44E expression by Western blot analysis. The data indicated that knocking down SRp20, but not Tra2-β1, markedly decreased CD44E expression (Supplemental Figure 1). This indicated that SRp20 is a specific splicing factor that mediates CD44E expression in gastric cancer cells. We next examined if modulation of DARPP-32 affects SRp20 expression, thereby promoting the expression of CD44E splice variant. Indeed, stable overexpression of DARPP-32 enhanced SRp20 protein level in AGS cells (Figure 3A). In contrast, knocking down endogenous DARPP-32 in MKN-45 cells significantly decreased SRp20 protein expression (Figure 3A). Of note, the qRT-PCR data showed that overexpression of DARPP-32 in AGS cells or knockdown of endogenous DARPP-32 in MKN-45 cells had no significant effects on SRp20 mRNA levels (Figure 3B). These findings indicate that DARPP-32 positively regulates SRp20 protein expression in gastric cancer cells. We investigated the potential posttranslational mechanism that may explain the DARPP-32-mediated increase in SRp20 protein expression. Accordingly, we tested the hypothesis that DARPP-32 protein binds to SRp20, thereby increasing SRp20 protein stability. Dual co-immunoprecipitation assay and Western blot analysis demonstrated the co-existence of DARPP-32 and SRp20 in the same protein complex (Figure 3C&3D).

To examine if DARPP-32-SRp20 protein interaction enhances SRp20 protein stability, AGS cells transiently expressing flag-tagged SRp20 in combination with DARPP-32 or pcDNA3 (control) were split into different plates and treated with the protein synthesis inhibitor cycloheximide (CHX) for different time points and flag-tagged SRp20 protein levels were evaluated using a flag-antibody by Western blot analysis. The results indicated that DARPP-32 prolonged the SRp20 protein half-life (20.5h) as compared with that of control cells (14.9h) (Figure 4A). In an attempt to identify the underlying mechanism, we investigated whether DARPP-32 stabilizes SRp20 protein through regulation of ubiquitination that subsequently induces protein degradation. We transiently transfected AGS cells with DARPP-32 or pcDNA3 plasmids followed by treatment with digitoxin, a cardiotonic steroid that regulates alternative splicing through ubiquitination/degradation of splicing factors including the SRp20 and Tra2-β1 (Anderson et al 2012), and SRp20 immunoprecipitation and Western blot analysis of ubiquitin were performed. The data showed that DARPP-32 expression markedly decreased digitoxin-induced ubiquitination of SRp20 in AGS cells (Figure 4B). In contrast, knocking down endogenous DARPP-32 in MKN-45 cells significantly increased ubiquitination of SRp20 with or without digitoxin treatment (Figure 4C).

To confirm that SRp20 induces splicing and determine if DARPP-32 expression enhances this effect, we utilized the Rapid-response splicing reporter system (Younis et al 2010). We transfected DARPP-32, SRp20, or DARPP-32 plus SRp20 expression constructs together

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with the splicing reporter plasmid into AGS cells. The data showed that expression of either DARPP-32 or SRp20 enhanced splicing activity relative to control ($p < 0.01$), and the combination induced higher reporter activity than SRp20 alone ($p < 0.01$, Figure 4D). In contrast, the knockdown of endogenous DARPP-32 in MKN-45 cells decreased cell splicing activity ($p < 0.01$, Figure 4E). However, in a rescue experiment, overexpression of DARPP-32, SRp20, or DARPP-32 plus SRp20 significantly attenuated the DARPP-32 shRNA-induced splicing activity reduction ($p < 0.01$, Figure 4E). Collectively, our results demonstrated that DARPP-32 up-regulates the splicing factor SRp20 protein expression, and the DARPP-32-SRp20 axis plays an important role in modulating the expression of *CD44E* splice variant in gastric cancer cells.

DARPP-32 enhances CD44E splicing through regulation of SRp20

To determine if SRp20 is a major determinant of the DARPP-32-induced *CD44E* splicing, we used SRp20 expression knockdown approach. To confirm that DARPP-32 regulates *CD44E* splicing, we performed a rescue experiment in MKN-45 cell model. Western blot data showed that knocking down DARPP-32 alone markedly decreased CD44E protein level, and the combination with DARPP-32 overexpression restored CD44E protein expression in MKN-45 cells (Figure 5A). As additional evidence that DARPP-32 regulates CD44E expression in a different gastric cancer cell line, our results indicated that knockdown of endogenous DARPP-32 significantly reduced CD44E protein expression in SNU-16 cells (Supplemental Figure 2). We next investigated if DARPP-32-induced expression of *CD44E* splice variant is mediated by SRp20 splicing factor protein expression. MKN-45 cells were transfected with control siRNA, DARPP-32 siRNA, or in combinations with SRp20 siRNA, and followed by Western blot analysis. The data showed that knockdown of DARPP-32 or SRp20 induced relatively similar reduction in CD44E protein expression. However, the data indicated that the combined knockdown completely eliminated CD44E expression (Figure 5B). The fact that knocking down DARPP-32 decreased SRp20 protein level, but knockdown of SRp20 did not affect DARPP-32 protein expression; strongly suggested that DARPP-32 regulates SRp20-mediated splicing function and the expression of CD44E splice variant (Figure 5B). We further explored the role of SRp20 in mediating DARPP-32-dependent *CD44E* splice variant expression. The knockdown of endogenous DARPP-32 by shRNA in MKN-45 cells decreased *CD44E* mRNA expression, as evaluated by qRT-PCR of exon5-12 or exon12-14 (Figure 5C&5D). Conversely, overexpression of DARPP-32, SRp20, or DARPP-32 plus SRp20, abrogated the DARPP-32 knockdown-dependent reduction in CD44E splice variant mRNA expression (Figure 5C&5D). Together, our findings indicated that DARPP-32 regulates the expression of CD44E splice variant through modulation of SRp20 protein expression and stability.

DARPP-32-regulated tumor growth *in vivo*

We investigated whether modulation of DARPP-32 and SRp20 expression could affect gastric tumor growth in a tumor xenograft mouse model. The results indicated that xenograft tumors derived from MKN-45/DARPP-32 shRNA cells grew significantly slower than tumors from MKN-45/control shRNA cells ($p < 0.05$, Figure 6A&6B). In addition, overexpression of SRp20 rescued tumor growth in MKN-45/DARPP-32 shRNA cells that grew faster than MKN-45/DARPP-32 shRNA, although slower than MKN-45 controls ($p <$

0.01, Figure 6A&6B). Accordingly, tumor weight data showed that MKN-45/DARPP-32 shRNA tumors were significantly smaller than MKN-45/control shRNA tumors ($p < 0.01$, Figure 6C). Notably, MKN-45/DARPP-32 shRNA and SRp20 overexpressing tumors were significantly larger than MKN-45/DARPP-32 shRNA but smaller than MKN-45/control shRNA tumors ($p < 0.05$, Figure 6C). Western blot analysis confirmed the lower levels of CD44E, DARPP-32, and SRp20 in DARPP-32 shRNA tumors as compared to control shRNA xenografts (Figure 6D). The reconstitution of SRp20 expression in DARPP-32 shRNA tumors rescued the decreased CD44E protein expression (Figure 6D). The xenograft tumors data confirmed that the knockdown of endogenous DARPP-32 by shRNA decreased CD44E mRNA level, and overexpression of SRp20 rescued the down-regulated CD44E mRNA expression as evaluated by qRT-PCR of exon5-12 or exon12-14 (Figure 6E&6F). In support of CD44E function in mediating DARPP-32-induced tumor growth, we demonstrated that overexpression of CD44E significantly rescued tumor growth in MKN-45/DARPP-32 shRNA ($p < 0.01$, Supplemental Figure 4A&4B). Together, our results clearly indicated that knockdown of DARPP-32 decreases CD44E expression levels *in vivo*, and DARPP-32-regulated gastric tumor growth is associated with SRp20-dependent expression of CD44E splice variant.

SRp20, CD44E and DARPP-32 are frequently co-overexpressed and positively correlated in human gastric primary tumors

To determine the clinical significance of DARPP-32 in gastric cancer, we evaluated *DARPP-32, CD44E* and *SRp20* mRNA levels in 26 gastric tumor and 24 normal tissue samples by qRT-PCR. Our data indicated that tumor samples (65.4%) expressed higher mRNA levels of DARPP-32 as compared to normal tissue samples ($p < 0.001$, Figure 7A). High *CD44E* (69.2%) and *SRp20* (76.9%) mRNA levels were detected in tumors, as compared to normal tissues ($p < 0.001$, Figure 7B&7C). The CD44E PCR products indicated a distinct DNA band accounting for the expected size of CD44E splice variant (Supplemental Figure 3). Statistical analysis using the Pearson's method indicated a significant positive correlation between CD44E and DARPP-32 expression in these gastric tissue samples ($r^2 = 0.45$, $p < 0.001$, Figure 7D). We also found that SRp20 and DARPP-32 expression have a significant positive correlation in these samples ($r^2 = 0.70$, $p < 0.001$, Figure 7E).

Discussion

DARPP-32 is a frequently overexpressed protein in several malignancies including esophageal and gastric cancers (Beckler et al 2003, Belkhiri et al 2005, Mukherjee et al 2010, Wang et al 2005). Although DARPP-32 has been shown to promote cell survival, resistance to chemotherapeutic drugs, and cancer cell invasion (Belkhiri et al 2005, Belkhiri et al 2008, Hamel et al 2009, Zhu et al 2011, Zhu et al 2012); the spectrum of its molecular functions remain poorly characterized in cancer. The expression of CD44 variant isoforms, generated by alternative splicing, has been associated with the development of cancer (Heider et al 1993, Omara-Opyene et al 2004). Recent studies have shown that CD44E is an important splice variant in carcinogenesis (Cavaloc et al 1999). In this study, we have shown for the first time that expression of CD44E splice variant is regulated by SRp20 splice factor

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through DARPP-32-dependent mechanism in gastric cancer. We propose a novel mechanism by which DARPP-32 promotes gastric tumorigenesis through enhancing CD44E expression, mediated by SRp20 splicing factor. This hypothesis is supported by several lines of evidence: 1) we showed that knockdown of DARPP-32 has a profound negative impact on CD44E splice variant mRNA and protein expression in gastric cancer cells; 2) using a rapid-response splicing reporter system, we found that overexpression of DARPP-32 significantly increases the splicing activity, whereas knockdown of endogenous DARPP-32 reverses the effect in cancer cells; 3) we demonstrated that the endogenous levels of SRp20 splicing factor protein are regulated by DARPP-32, and knockdown of endogenous SRp20 decreases DARPP-32-induced splicing and CD44E expression.

Altered expression of splicing factors has been frequently observed in various tumor types (Biamonti et al 1998, Cohen-Eliav et al 2013). SRp20 is the smallest member of the SR protein family (Caceres et al 1997), and is a splicing factor that affects alternative splicing by interacting with RNA cis-elements (Jia et al 2009). Our results demonstrate that DARPP-32 interacts with and up-regulates SRp20 protein expression by enhancing its protein stability by reducing ubiquitination and subsequent proteasomal protein degradation. We further confirmed that DARPP-32 expression markedly decreased digitoxin-induced ubiquitination/degradation of SRp20 protein. The regulation of SRp20 protein level is an important mechanism that could alter the RNA splicing of many genes in mammalian cells, thereby substantially affecting the expression levels of various protein isoforms (Matlin et al 2005). In fact, overexpression of SRp20 has been associated with the increase in cancer cell survival and oncogenesis in several types of cancer (Biamonti et al 1998). These functions overlap with the reported functions of DARPP-32 in cancer (El-Rifai et al 2002) suggesting that the DARPP-32-SRp20 axis could be an important one in gastric tumorigenesis.

CD44 is an important stem cell marker that is expressed in several cancers. The expression of CD44 is often an indication of aggressive tumors with poor response to therapy. Recent studies have shown changes in the ratio of expression of CD44 splice variants in cancer cells (Miwa et al 1996). While the CD44 standard isoform is common in some cancers, the expression of other splice variants with different combinations of CD44 exons has been widely reported in cancer (Prochazka et al 2014). Some of the most common CD44 splice variants in cancer include CD44v6, CD44 v3–v10, and CD44E (Prochazka et al 2014). Our results have shown a selective high expression level of CD44E splice variant in gastric cancer, which is in agreement with an earlier study that reported the expression of CD44E in gastric cancer cells (Miwa et al 1996). CD44E plays a pivotal role in the onset of oncogenesis and tumor progression (Bourguignon et al 1998). Recent studies have shown that CD44E promotes carcinogenesis (Muramaki et al 2004) by driving stem cell properties, which contribute to tumor initiation (Lau et al 2014). In addition, CD44E expression was found significantly elevated in metastatic colorectal carcinomas (Takeuchi et al 1995) and its expression correlated with hematogenous and lymph node metastasis in colorectal and gallbladder cancer (Hiraga et al 2013, Muramaki et al 2004). Our data demonstrated a significant reduction of tumor xenograft growth *in vivo* following the knockdown of endogenous DARPP-32. Consistent with our in vitro data, this was accompanied with a decrease in CD44E and SRp20 protein levels. Furthermore, our results demonstrate that DARPP-32, SRp20, and CD44E are frequently co-overexpressed in human gastric cancers.

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The fact that DARPP-32 is overexpressed in approximately two-third of gastric cancers (Mukherjee et al 2010), involved in early stages of gastric tumorigenesis (Mukherjee et al 2010), and promotes the expression of CD44E splice variant through a mechanism that involves regulation of SRp20 splice variant; implies a potentially significant role of this protein in gastric tumorigenesis.

In conclusion, our findings uncover a novel mechanism by which DARPP-32 regulates SRp20 splicing factor and the expression of CD44E splice variant in gastric cancer cells. The DARPP-32–SRp20 axis provides a new paradigm in gastric tumorigenesis where its full impact on the mRNA splicing calls for additional investigations in cancer.

Materials and Methods

Cell culture and reagents

Human gastric cancer cell lines including AGS, MKN-45, and the immortalized human embryonic kidney epithelial cell line (HEK-293) were cultured in Dulbecco's modified Eagle's medium (GIBCO, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin (GIBCO). AGS and HEK-293 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). MKN-45 cells were obtained from the Riken Cell Bank (Tsukuba, Japan). All cell lines were ascertained to conform to the original in vitro morphological characteristics. Cycloheximide (CHX) was purchased from Sigma-Aldrich (St. Louis, MO). DARPP-32 (sc271111) and SRp20 (sc135) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Flag-tag antibody (F1804) was obtained from Sigma-Aldrich (St. Louis, MO). Horseradish peroxidase-conjugated mouse (7074P2) and rabbit (7062P2) secondary antibodies, ubiquitin (3933S), CD44 (3570S), and β-actin (4970) antibodies were purchased from Cell Signaling Technology (Danvers, MA).

Expression plasmids and shRNA vectors

The flag-tagged coding sequence of DARPP-32, SRp20 and CD44E were cloned in pcDNA3.1 mammalian expression plasmid (Invitrogen). AGS cells stably expressing DARPP-32 or pcDNA3.1 empty vector were generated as described previously (Belkhiri et al 2005, El-Rifai et al 2002). Lentivirus particles expressing DARPP-32 shRNA or control shRNA were produced by GeneCopoeia (Rockville, MD) and then utilized to transduce MKN-45 cells. SRp20 siRNA (sc-38338) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Cloning and identification of *CD44* isoforms

Total RNA was prepared from AGS or MKN-45 cells using an RNeasy Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instruction, followed by cDNA synthesis from 1 μg total RNA. PCR was performed using 4 μl of diluted cDNA and Platinum PCR SuperMix (Invitrogen). The following primer sets were used to amplify the open reading frame (ORF) of CD44: 5′-CCGCTATGTCCAGAAAGGAG -3′ (forward) and 5′-TTGTTCACCAAATGCACCAT-3′ (reverse). The amplified products were ligated into pCR8/GW/TOPO TA Cloning vector (Invitrogen) and verified by sequencing.

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Quantitative Real-time PCR analysis

Total RNA was isolated from cell lines by using the RNeasy Mini Kit (Qiagen, Valencia, CA). Total RNA (1 μg) was reverse transcribed by an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The quantitative real-time PCR (qRT-PCR) was performed using an iCycler (Bio-Rad), with the threshold cycle number determined by iCycler software version 3.0. The primers for SRp20 were forward: 5′-AGGAAAGCGGGAAGACTCAT-3′; reverse: 5′-CGTTCCAATTCCGTCTTGTT-3′. The primers for different CD44 exons are shown in Supplemental Table 1. The primers for *HPRT1* were forward: 5′-TTGGAAAGGGTGTTTATTCCTCA-3′; reverse: 5′-TCCAGCAGGTCAGCAAAGAA-3′. Reactions were performed in duplicate and results of three independent experiments were subjected to statistical analysis. Expression fold change was calculated using the C(t) method (Pfaffl 2001). *HPRT1* was used as normalization control.

Luciferase assay

An intron-containing and intronless luciferase reporter plasmids were a kind gift from Dr. Gideon Dreyfuss (University of Pennsylvania) (Younis et al 2010). Briefly, the intron-containing plasmid was generated by inserting a 132-nucleotide chimeric β-globin/immunoglobulin intron (Promega, Madison, WI) at nucleotide position 1344 of the firefly luciferase gene. Cells were seeded in 12-well plates and transfected with splicing luciferase reporters using the DNAfectin Transfection Reagent (Applied Biological Materials, Richmond, BC). The cells were harvested for luciferase assays 24 hours later using a luciferase assay kit (Promega) according to the manufacturer's protocol. β-galactosidase was used for normalization.

Immunoprecipitation and Western blotting

Cells were lysed with TENN buffer containing 0.5% Triton X-100, 150 mmol/L NaCl, 5 mmol/L EDTA, and 50 mmol/L Tris supplemented with protease and phosphatase inhibitors (Rockford, IL). Immunoprecipitations of equal total protein amounts were performed using Dynabeads® Protein A Immunoprecipitation Kit (Invitrogen) following the manufacturer's protocol. Briefly, protein samples were incubated with primary antibody-bound Protein A Dynabeads. The beads were washed four times and heated to 100°C for 5 min in 20 μl of sample buffer. Proteins were separated on 12% SDS-PAGE and transferred to Immobilon PVDF membrane (Millipore). Membranes were probed with specific antibodies. Proteins were then visualized by using horseradish peroxidase (HRP)-conjugated secondary antibodies and Immobilon Western Chemiluminescent HRP Substrate detection reagent (Millipore). β-actin was used as loading control.

Determination of SRp20 protein half-life

AGS cells transiently expressing flag-tagged SRp20 in combination with DARPP-32 or pcDNA3 (control) were split into six-well plates and treated with the protein synthesis inhibitor cycloheximide (CHX, 50 μmol/L, Sigma-Aldrich). Cell extracts were isolated from individual wells at 0, 6, 9, 15 and 24 hours post CHX treatment. Flag-tagged SRp20 protein levels were evaluated using a flag-antibody by Western blot analysis. Images were acquired using a Gel Doc™ XR+ and ChemiDoc™ XRS+ gel documentation systems (Bio-Rad,

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Hercules, CA), and quantified using Image Lab software (Bio-Rad). The levels of flag-SRp20 were normalized against the corresponding Actin level.

Ubiquitination assays for SRp20

Following transfection, cells were washed and cultured in fresh DMEM medium supplemented with 100 nmol/L of digitoxin overnight. We then rapidly washed cells on ice with cold PBS, and collected cell lysates. The lysates were incubated with an anti-SRp20 polyclonal antibody and the immunoprecipitates were subjected to Western blotting using an anti-ubiquitin antibody.

In vivo experiments

Five-week-old female Sprague Dawley nude mice were purchased from Harlan Laboratories, Inc. (Frederick, MD) and maintained under specific pathogen-free conditions. Based on statistical analysis, 8 – 10 successful tumor xenografts were utilized in each group. Animals were randomized in four groups. MKN-45 cells stably expressing control shRNA, DARPP-32-shRNA or DARPP-32-shRNA in combination with SRp20 or CD44E were injected subcutaneously (2×10^6 cells per site) into the flanks. To determine tumor volume by external digital caliper, the greatest longitudinal diameter (length) and the greatest transverse diameter (width) were measured twice weekly. Tumor volume was calculated by the formula: Tumor volume = 1/2 (length × width^2). All mice were sacrificed on day 35, and tumors were collected. The Vanderbilt Institutional Animal Care and Use Committee approved all animal work.

Tissue samples

All tissue samples were obtained from the archives of pathology at Vanderbilt University (Nashville, Tennessee, USA) and the National Cancer Institute Cooperative Human Tissue Network in accordance with approved protocols. All samples were coded and de-identified and are considered as non-human subjects according to NIH guideline. The use of de-identified specimens from the tissue repository was approved by the Vanderbilt institutional review board.

Statistical analyses

Data were expressed as mean ± SD of three independent experiments. Statistical significance of the *in vitro* studies was analyzed by Student's *t* test and Pearson's method. Differences with p values 0.05 are considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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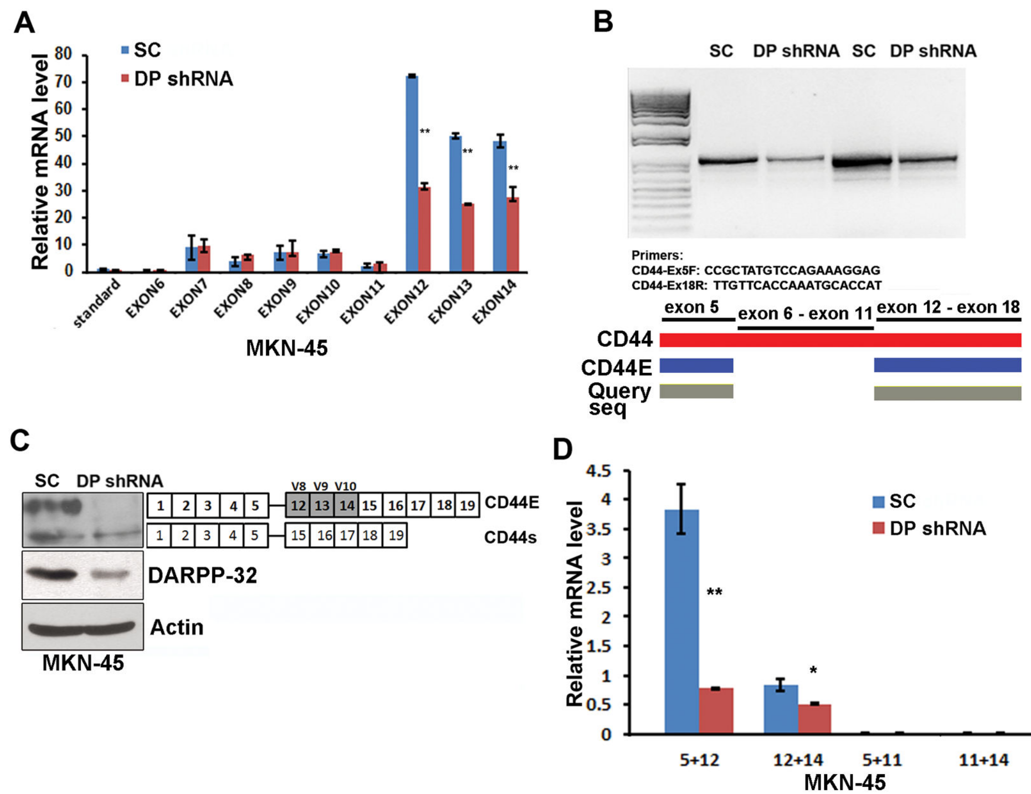

Figure 1. DARPP-32 regulates *CD44* alternative splicing

A) The qRT-PCR analysis of each variable exon of CD44 was performed in MKN-45/SC shRNA and MKN-45/DARPP-32 shRNA stable cells. The results are shown as mean ± SD. **B)** Amplification of the human *CD44* mRNA in MKN-45 stable cells. The transcript was purified, cloned, and identified by DNA sequencing as human *CD44E*, which is standard *CD44* spliced together with exon12-14. **C)** Western blot analysis of DARPP-32 and CD44 proteins (CD44s and CD44E) in MKN-45 cells stably expression shRNA control or DARPP-32 shRNA. **D)** The qRT-PCR of exon12-14 or exon11-14 of CD44 was performed in MKN-45/SC shRNA and MKN-45/DARPP-32 shRNA stable cells.

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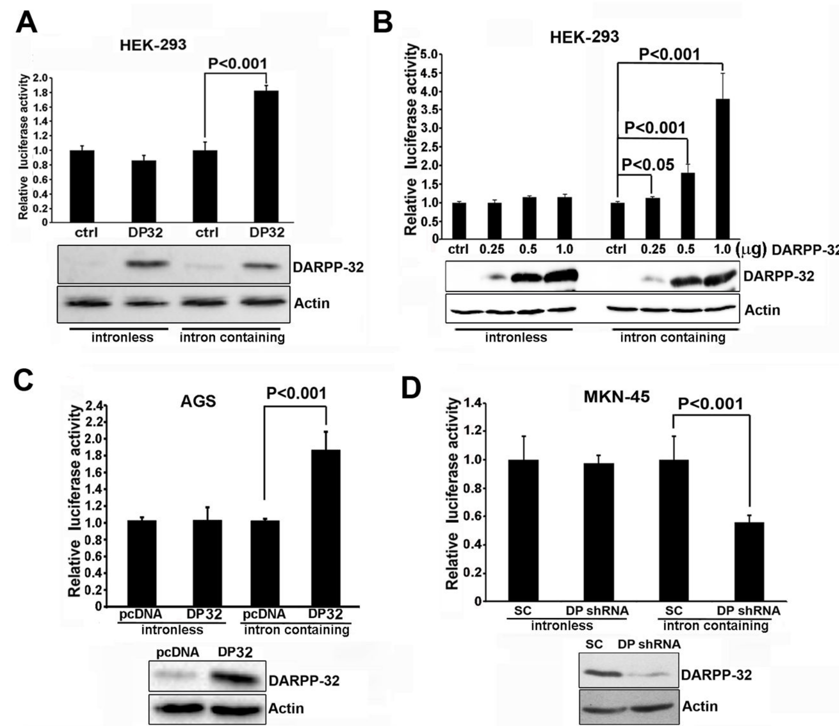

Figure 2. DARPP-32 promotes cell splicing activity

A) Rapid splicing activity luciferase reporter assay for intron-containing and intronless plasmids transiently co-transfected separately with pcDNA3 or DARPP-32 in HEK-293 cells. **B)** Luciferase assay for intron-containing and intronless luciferase plasmids co-transfected separately with pcDNA3 or different amounts of DARPP-32 in HEK-293 cells. **C)** Luciferase assay for intron-containing and intronless luciferase constructs co-transfected separately with pcDNA3 or DARPP-32 in AGS cells. **D)** Luciferase assay for intron-containing and intronless luciferase plasmids transfected separately in MKN-45/SC shRNA or MKN-45/DARPP-32 shRNA stable cells.

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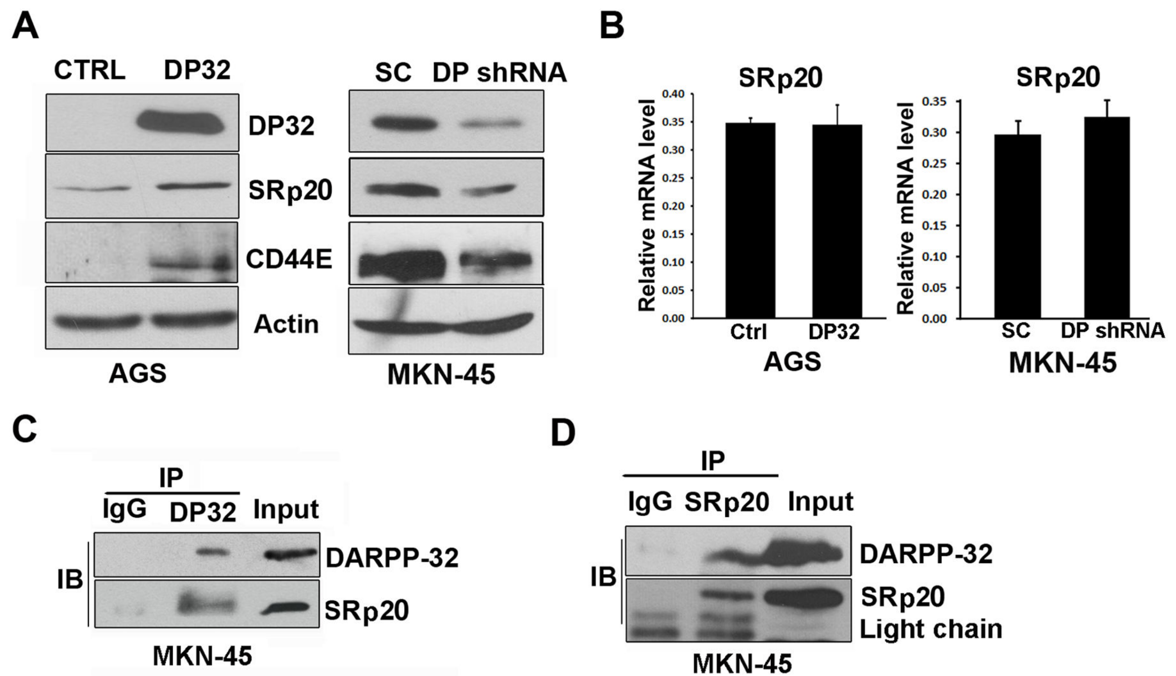

Figure 3. DARPP-32 interacts with SRp20 and enhances its protein expression

A) Western blot analysis of SRp20 and DARPP-32 in AGS cells stably expressing DARPP-32 or empty vector (left panel). SRp20 and DARPP-32 protein levels were determined by immunoblot analysis in MKN-45/SC shRNA and MKN-45/DARPP-32 shRNA stable cells (Right panel). **B)** The qRT-PCR of SRp20 was performed in AGS/pcDNA3, AGS/DARPP-32, MKN-45/SC shRNA or MKN-45/DARPP-32 shRNA stable cells. **C–D)** The protein interaction of endogenous DARPP-32 and SRp20 was evaluated by co-immunoprecipitation in MKN-45 cells using specific antibodies.

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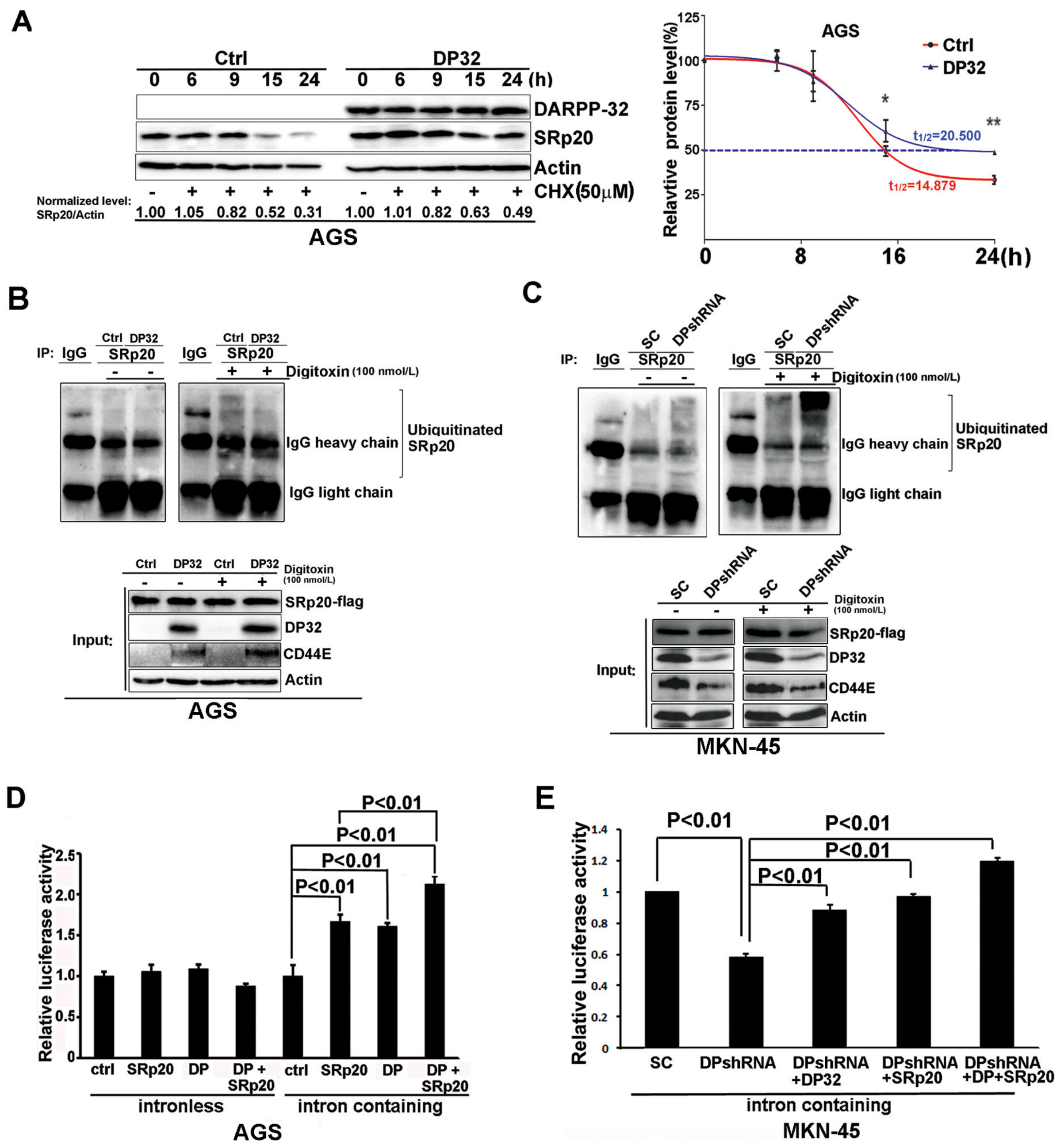

Figure 4. DARPP-32 promotes SRp20 protein stability

A) AGS cells transiently overexpressing flag-SRp20 in combination with DARPP-32 or empty vector were treated with CHX (50 μM) for the indicated time points, and SRp20 protein levels were evaluated by Western blot analysis. The protein degradation data show that DARPP-32 stabilizes SRp20 protein (right panel). **B)** AGS cells co-transfected with flag-SRp20, His-ubiquitin, and pcDNA3 or DARPP-32 were treated with 100 nM of digitoxin or vehicle for 24 h. SRp20 immunoprecipitates were analyzed by Western blotting of ubiquitin. **C)** MKN-45 cells stably expressing DARPP-32 shRNA or control shRNA co-transfected with flag-SRp20, His-ubiquitin were treated with 100 nM digitoxin or vehicle for

24 h. SRp20 immunoprecipitates were subjected to Western blot analysis of ubiquitin. **D)** Intron-containing and intronless luciferase reporter assays in AGS cells co-transfected with pcDNA3, DARPP-32, SRp20, or DARPP-32 plus SRp20 expression constructs. **E)** Intron-containing luciferase reporter in MKN-45/SC shRNA or MKN-45/DARPP-32 shRNA cells co-transfected with DARPP-32, SRp20, or DARPP-32 plus SRp20 expression constructs.

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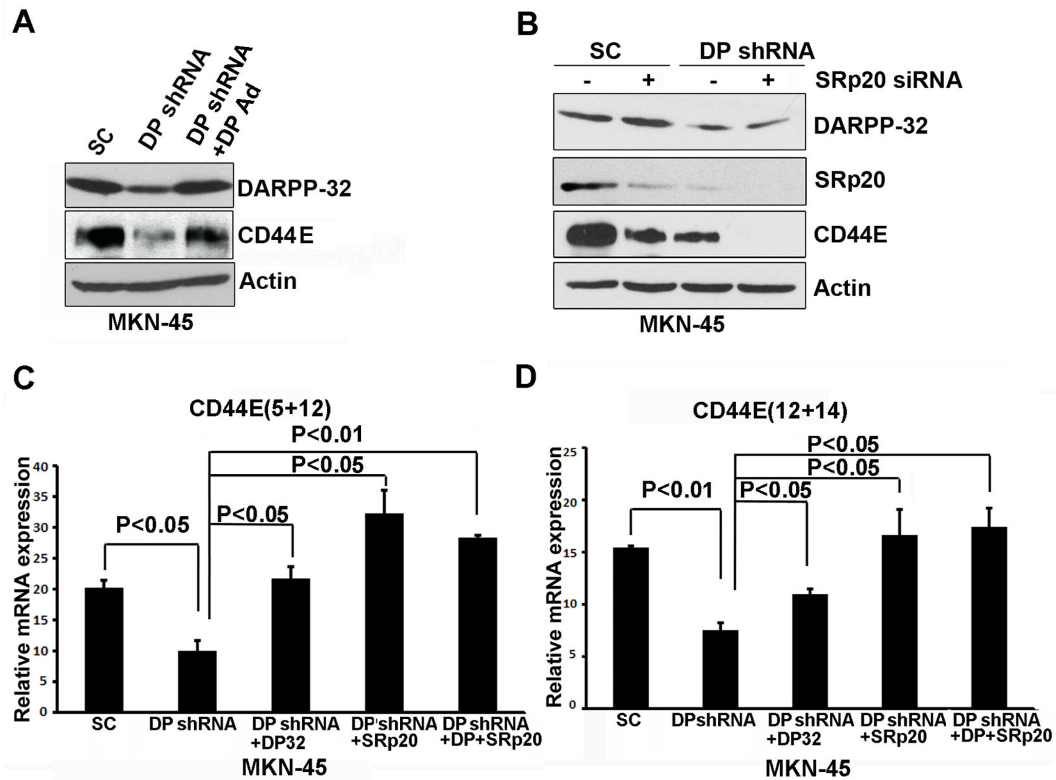

Figure 5. SRp20 mediates DARPP-32 regulated CD44E splicing

A) CD44E protein levels were determined by immunoblot analysis in MKN-45/SC shRNA, MKN-45/DARPP-32 shRNA, and MKN-45/DARPP-32 shRNA infected with DARPP-32 adenovirus. **B)** SRp20 and CD44E protein levels were determined by Western blot analysis following knockdown of DARPP-32 alone, SRp20 alone, or in combination in MKN-45 cells. **C–D)** The qRT-PCR of CD44E was performed with different sets of primers in MKN-45/SC shRNA, MKN-45/DARPP-32 shRNA, or DARPP-32 shRNA cells co-transfected with DARPP-32, SRp20, or DARPP-32 plus SRp20 expression constructs.

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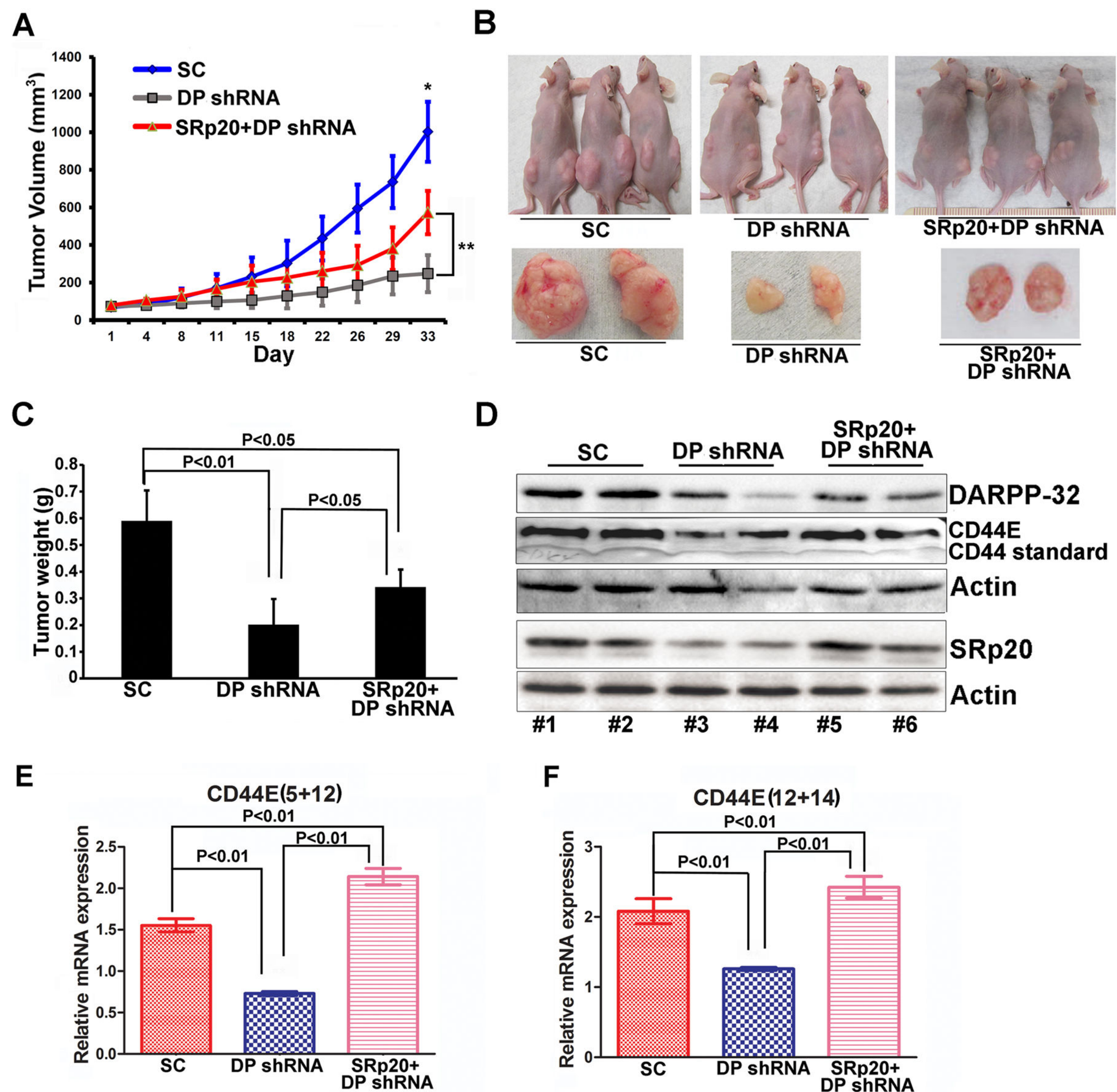

Figure 6. DARPP-32 mediates gastric tumor growth in a xenografted mouse model

A) MKN-45/SC shRNA, MKN-45/DARPP-32 shRNA or MKN-45/DARPP-32 shRNA and SRp20 overexpression cells were injected s.c. into nude mice. Tumor volume was measured at the indicated times; each data point represents the mean ±SD for 10 xenografts. **B)** Representative sacrificed mice and xenograft tumors. **C)** Quantification of tumor weight at the end of experiment. The tumor weight is indicated by mean ±SD ($p < 0.05$). **D)** Protein expression of SRp20, CD44E, and DARPP-32 in xenograft tumors were analyzed by Western blot. **E–F)** The qRT-PCR of CD44E was performed with different sets of primers in xenograft tumors.

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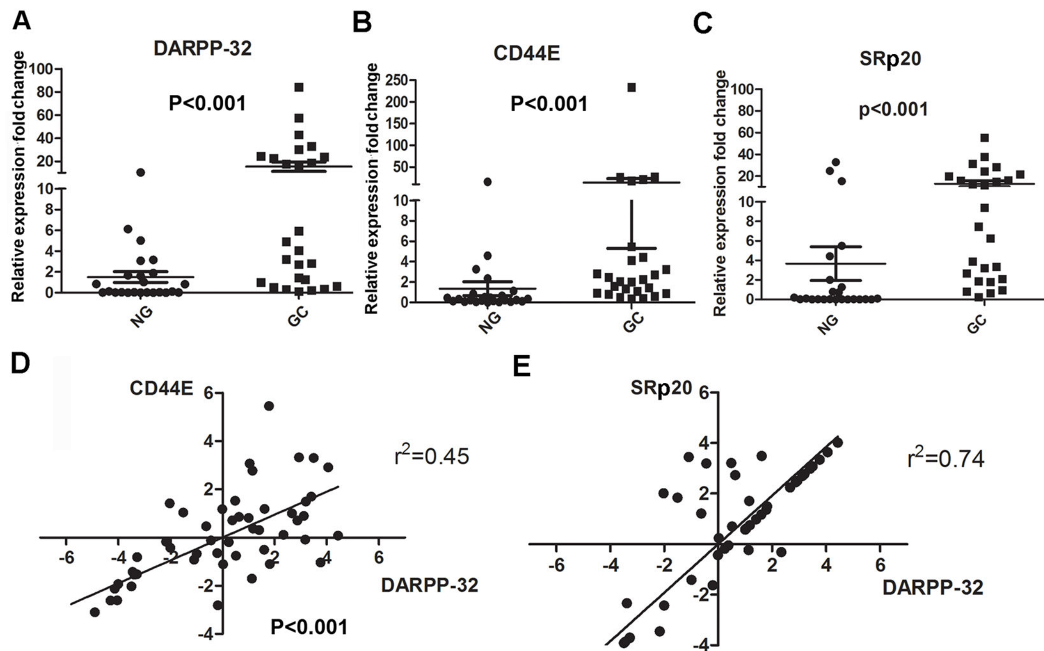

Figure 7. Positive correlation between DARPP-32 and CD44E mRNA expression

A–C) The mRNA expression levels of DARPP-32, CD44E and SRp20 were assessed by qRT-PCR in human gastric cancer samples (n=26) and adjacent histologically normal tissue samples (n=24). NG: adjacent normal tissues, GC: gastric cancer tissues. **D–E**) A statistically significant positive correlation between the CD44E and DARPP-32 and SRp20 and DARPP-32 mRNA levels were detected ($r^2 = 0.45$, $p < 0.001$, $r^2 = 0.70$, $p < 0.001$, respectively).