Overexpression of Protease-Activated Receptor-1 Contributes to Melanoma Metastasis via Regulation of Connexin 43

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Abstract
Protease-activated receptor-1 (PAR-1) is a key player in melanoma metastasis with higher expression seen in metastatic melanoma cell lines and tissue specimens. cDNA microarray and Western blot analyses reveal that the gap junctional intracellular communication molecule connexin 43 (Cx-43), known to be involved in tumor cell diapedesis and attachment to endothelial cells, is significantly decreased after PAR-1 silencing in metastatic melanoma cell lines. Furthermore, Cx-43 promoter activity was significantly inhibited in PAR-1–silenced cells, suggesting that PAR-1 regulates Cx-43 at the transcriptional level. Chromatin immunoprecipitation studies showed a reduction in the binding of SP-1 and AP-1 transcription factors to the promoter of Cx-43. Both transcription factors have been shown previously to be required for maximal Cx-43 promoter activity. These results were corroborated by mutating the AP-1 and SP-1 binding sites resulting in decreased Cx-43 promoter activity in PAR-1–positive cells. Moreover, as Cx-43 has been shown to facilitate arrest of circulating tumor cells at the vascular endothelium, melanoma cell attachment to endothelial cells was significantly decreased in PAR-1–silenced cells, with this effect being abrogated after PAR-1 rescue. Herein, we report that up-regulation of PAR-1 expression, seen in melanoma progression, mediates high levels of Cx-43 expression. As both SP-1 and AP-1 transcription factors act as positive regulators of Cx-43, our data provide a novel mechanism for the regulation of Cx-43 expression by PAR-1. Indeed, Cx-43 expression was restored following PAR-1 rescue in PAR-1–silenced cells. Taken together, our data support the tumor promoting function of Cx-43 in melanoma. [Cancer Res 2009;69(16):6730–7]

Introduction
The thrombin receptor, protease-activated receptor-1 (PAR-1), is a transmembrane G-protein–coupled receptor that has been found to be involved in many types of cancers. PAR-1 can be activated by coagulation factor Xa, granzyme A, trypsin, and matrix metalloprotease-1, although thrombin is the most potent activator (1–4). PAR-1 activation leads to induction of G proteins that trigger various downstream molecules and signal transduction pathways such as Rho kinase, phosphoinositol 3-kinase, and mitogen-activated protein kinase, which have been shown to be involved in cell growth, tumor promotion, and carcinogenesis (5, 6).

PAR-1 overexpression has been found in various cancers, including those of breast, lung, and prostate (7–11). Our laboratory has found PAR-1 to be a key player in the progression of melanoma. PAR-1 is overexpressed in metastatic melanoma cell lines compared with nonmetastatic cell lines (12). Furthermore, melanoma tumors have increased PAR-1 expression compared with dysplastic nevi (13, 14). Recently, we have shown that silencing PAR-1 via lentiviral short hairpin RNA (shRNA) or through PAR-1 small interfering RNA-DOPC delivery significantly decreases both melanoma tumor growth and metastasis (7). Based on these results, we sought to determine, via cDNA microarray studies, novel downstream gene targets regulated by PAR-1 that contribute to the metastatic phenotype of melanoma. This led us to identify connexin 43 (Cx-43) as a target gene of PAR-1.

Increased Cx-43 expression has been observed in several cancers, including breast cancer, hepatocellular carcinoma, and gliomas (15–18). Connexin gap junctions are intracellular membrane channels that form when six connexin subunits are arranged to form a pore. They align with complementary connexins on the plasma membrane of adjacent cells. This allows for small molecules (<1.2 kDa) such as Ca2+, secondary messengers, and metabolic products to pass between neighboring cells, a process known as gap junction intracellular communication. This process appears to be key for maintaining tissue regulation, growth, and proliferation (19, 20).

Unlike typical gap junctions, connexins are also considered membrane proteins with adhesive properties (21, 22). The attachment of tumor cells in transition from a primary site to a secondary organ site requires the attachment as well as the migration of tumor cells through the vascular endothelium, a process known as tumor cell diapedesis. It has been shown that, immediately following adhesion to the endothelium, tumor cells establish gap junctional channels with the endothelial cells. Several studies have found that the communication between tumor cells and endothelial cells is mediated by connexins and is critical to tumor cell extravasation at the metastatic site (23–25). In fact, it has been reported that Cx-43–mediated gap junctional communication enhances breast tumor cell diapedesis (24). Furthermore, decreased Cx-43 expression reduced adhesion of breast cancer cells to the pulmonary endothelium. Moreover, up-regulation of Cx-43 was seen in tumor cell-endothelial cell contact areas both in vivo and in vitro (26).

In melanoma, increased expression of Cx-43 has been implicated in establishing a crucial link between melanoma cells and endothelial cells, which enhances tumor metastasis (15, 17, 24–26). However, the exact mechanism by which Cx-43 is regulated in melanoma cells is unknown. Herein, we describe that decreased Cx-43 expression occurs through differential binding of AP-1 and SP-1 transcription factors to the Cx-43 promoter mediated by...
PAR-1. Furthermore, this results in a decrease in melanoma cell attachment to endothelial cells. This is the first report to identify PAR-1 as a regulator of Cx-43 expression, thus adding an alternative mechanism by which PAR-1 contributes to the malignant phenotype of melanoma.

Materials and Methods

Cell lines and culture conditions. A375SM human melanoma cell line was maintained in Eagle’s MEM supplemented with 10% fetal bovine serum as described previously (27). C8161 human melanoma cell line was maintained in DMEM/F-12 supplemented with 5% fetal bovine serum as described previously (28). Human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection. HUVECs were plated on 0.5% gelatin-coated flasks and maintained in DMEM supplemented with 15% fetal bovine serum and 10 ng/mL basic fibroblast growth factor as described previously (29). Human dermal microvascular endothelial cells (HDMEC) were purchased from PromoCell and maintained in endothelial cell growth medium.

Antibodies. ATAP2 was purchased from Santa Cruz Biotechnology. The PAR-1 antibody used for immunoprecipitation studies was purchased from Biodesign International. The phycocerythrin (anti-mouse) antibody was purchased from Jackson ImmunolResearch. Cx-43 antibody was purchased from BD Pharmingen. SP-1, c-Jun, c-Fos, and IgG antibodies used for chromatin immunoprecipitation and Western blot assays were purchased from Santa Cruz Biotechnology. Peroxidase-conjugated anti-mouse IgG antibodies for immunoprecipitation studies were purchased from GE Healthcare.

Lentiviral shRNA to PAR-1 and Cx-43. PAR-1 shRNA (target sequence: AGAGTGTCCTCACTGAAAT, Cx-43 shRNA (target sequence: CCCTTTGTCATCTTCACTCAT), and a nontargeting (NT) shRNA (target sequence: TTCTCAGGACCTGTTCAGG) were used with the lentiviral system developed and kindly provided to us by Didier Trono (Ecole Polytechnique Fédérale de Lausanne) as described previously (7).

Flow cytometry. Flow cytometry was done as described previously (7).

Western blot analysis. Cx-43 was detected in total cell extracts by 10% SDS-PAGE as described previously (7). SP-1 (1:1,000), c-Jun (1:1,000), and c-Fos (1:1,000) were detected in nuclear extracts by using the Nuclear Extraction Kit from Panomics as per the manufacturer’s instructions.

cDNA microarray. Microarray analysis was done by using a human Genome U133 Plus 2.0 Array (Affymetrix). The microarrays were produced and kindly provided to us by Didier Trono (Ecole Polytechnique Fédérale de Lausanne) as described previously (7).

Attachment of melanoma cells to endothelial cells. HUVECs or HDMECs were plated in 24-well plates and allowed to reach confluency. A thin overlay of 2% bovine serum albumin was placed in each well and incubated for 10 h at 37 °C to prevent melanoma cell attachment to tissue culture plate. Then, 5 × 10^4 A375SM or C8161 (NT, PAR-1 shRNA, or Cx-43 shRNA) cells were added to each well and incubated overnight at 37 °C. Wells were rinsed twice with PBS, fixed with methanol, and stained with crystal violet. For experiments using HDMECs, melanoma cells were identified by green fluorescence protein. Five fields per well were counted and averaged. Results are presented as average number of cells adhered per field.

PAR-1 rescue experiments. PAR-1 constructs with a NGF-terminal prolactin signal peptide and flag tag (kindly provided by Shaun R. Coughlin, University of California-San Francisco) was combined with nontargetable PAR-1 coding region (7-bp silent mutations that will not be recognized by PAR-1 shRNA). The resulting open reading frame insert was then ligated into the pLVX-DsRed-Monomer-C1 vector (Clontech) replacing the red protein coding sequence of DsRed and the final rescue lentiviral vector was obtained. The recombinant lentivirus was produced as described previously (7).

Immunoprecipitation. C8161 cells were plated on 10 cm plates and allowed to reach ~85% confluence. The wells were washed twice in PBS and lysed using passive lysis buffer [10 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100, 5 mmol/L EDTA, 10% glycerol (pH 7.6)]. Total cell lysate (300 µg) was incubated with 1 µg anti–PAR-1 (ATAP2) or IgG isotype control at 4 °C overnight under rotation. Protein G Plus-agarose beads (30 µL; Santa Cruz Biotechnology) were added to each sample and incubated at 4 °C for 4 h under rotation. Samples were collected by centrifugation at 7,000 × g for 5 min at 4 °C and washed twice with TBS-Tween 20 and once with PBS. Proteins were eluted in 1× SDS gel loading buffer and subjected to 10% SDS-PAGE, blotted onto nitrocellulose membranes, and probed with the anti–PAR-1 antibody (Biodesign International). Peroxidase-conjugated anti-mouse antibody (GE) was used as secondary antibody and visualized with an enhanced chemiluminescence kit (Amersham) according to the manufacturer’s instructions.

Statistics. The Student’s t test was used to evaluate the data. P values <0.05 are considered statistically significant.

Results

Identification of Cx-43 as a downstream target gene of PAR-1. Previously, we have shown that silencing PAR-1 in metastatic melanoma cells resulted in an inhibition of tumor growth and metastasis (7). PAR-1 has been shown to regulate the expression of proangiogenic molecules such as vascular endothelial growth factor, platelet-derived growth factor, interleukin-8, and urokinase-type plasminogen activator, thus contributing to melanoma growth and metastasis. To further investigate how PAR-1 contributes to the malignant phenotype, we sought herein to identify other potential downstream PAR-1 target genes.

A375SM and C8161 metastatic melanoma cell lines are both high expressors of PAR-1 (8, 12). These cells were stably transduced with PAR-1 shRNA and NT lentiviral constructs. The NT construct has no known homology to any human gene. Fluorescence-activated
cell sorting analyses were used to determine the levels of PAR-1 expression on the cell surface. Figure 1A and B show a significant decrease in PAR-1 expression in both cell lines after transduction (7). These stably silenced PAR-1 melanoma cell lines were subjected to cDNA microarrays to determine downstream gene targets of PAR-1. Among the genes that were differentially expressed, Cx-43 was found to be down-regulated by ~6-fold in PAR-1–silenced cells compared with NT-transduced cells. Furthermore, Western blot analyses revealed an 82% and 72% decrease in Cx-43 expression in A375SM and C8161, respectively, after PAR-1 silencing (Fig. 1C), thereby validating the results obtained from the cDNA microarrays.

**Figure 1.** Cx-43 protein expression after PAR-1 silencing. Flow cytometry analysis reveals decreased PAR-1 expression [less phycoerythrin (PE) intensity] in (A) A375SM and (B) C8161 cells after transduction with PAR-1 shRNA compared with NT shRNA-transduced cells. Mouse IgG was used as an isotype control. As a negative control, only secondary phycoerythrin antibody was used without adding PAR-1 antibody. C. Western blot of PAR-1–silenced A375SM and C8161 melanoma cell lines depicting ~82% and 72% decrease in Cx-43 expression, respectively, compared with NT-transduced cells. Actin is used as a loading control. The ratio of Cx-43 to actin was calculated using Image J software to determine band intensities.

**Regulation of Cx-43 by PAR-1 at the transcriptional level.** To further determine the mechanism by which PAR-1 regulates Cx-43, the Cx-43 promoter (~360 to +150) was cloned in front of the luciferase reporter gene. The luciferase activity driven by the Cx-43 promoter was significantly inhibited by 3- and 2.5-fold ($P < 0.001$) by PAR-1 silencing in A375SM and C8161 cell lines compared with NT-transduced cells, respectively (Fig. 2A and B). These results indicate a possible transcriptional regulation of Cx-43 by PAR-1.

Promoter analyses revealed four SP-1 binding sites and one AP-1 (c-Fos or c-Jun) binding site within 100 bp of the transcriptional initiation site (refs. 34, 35; Fig. 3A). Previous studies have shown that both SP-1 and c-Jun are required for maximal Cx-43 promoter activity (35, 36). Western blot analyses were done to determine whether expression levels of these transcription factors were affected by PAR-1 silencing. No differences in protein expression for c-Fos, c-Jun, or SP-1 were seen between NT and PAR-1 shRNA-transduced cells (data not shown). Therefore, we determined whether there was differential binding of these transcription factors to the Cx-43 promoter in PAR-1 shRNA-transduced metastatic melanoma cell lines. Chromatin immunoprecipitation

**Figure 2.** Cx-43 promoter activity after PAR-1 silencing. The Cx-43 promoter region (~360 to +150) was amplified from genomic DNA and cloned into the pGL3-basic firefly luciferase vector. The luciferase activity driven by the Cx-43 promoter was significantly inhibited ($P < 0.001$) by PAR-1 silencing in both (A) A375SM and (B) C8161 cell lines compared with NT-transduced cells. The ratio of firefly luciferase activity to CMV-driven Renilla luciferase activity was used to normalize for differences in transfection efficiency among samples. *, $P < 0.001$. 

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Furthermore, to determine if this decrease in attachment was compared with both cell lines transduced with NT shRNA (Fig. 5A), compared with NT-transduced cell lines. IgG antibodies were used as negative controls. Input DNA is used to determine equal amount of chromatin in each condition used.

To further establish the link between PAR-1 regulation of Cx-43 expression via SP-1 and AP-1, point mutations in the binding sites of SP-1 and AP-1 were made in the Cx-43 promoter. Site-directed mutagenesis for SP-1 elements were created by dinucleotide substitutions (italicized) in the SP-1 binding motif (GGGAGG to GGTAGG) as described previously (31). Site-directed mutagenesis for the AP-1 element was created by single-base-pair mutation (italicized) in the AP-1 binding motif (TGAGTCA to TGAGCCA) as described previously (32). These mutants were cloned in front of the luciferase reporter gene. Figure 4 depicts significant decreases in luciferase expression driven by the Cx-43 promoter in the NT shRNA-transduced cells in all mutated SP-1 sites and in the AP-1 site compared with NT cells transfected with nonmutated Cx-43 promoter. Site-directed mutagenesis studies depict decreased binding of (B) SP-1, (C) c-Jun, and (D) c-Fos transcription factors to the promoter of Cx-43 in both PAR-1–silenced cell lines (A375SM and C8161) compared with NT-transduced cell lines. IgG antibodies were used as negative controls. Input DNA is used to determine equal amount of chromatin in each condition used.

Decreased Cx-43 expression after PAR-1 silencing affects melanoma cell attachment to endothelial cells. Previous studies have shown that Cx-43 have adhesive properties and can mediate cell-cell interactions (21, 22). Cx-43 expression has also been shown to increase attachment of breast cancer cells to the pulmonary endothelium, thereby augmenting metastasis (23–26). Therefore, attachment assays were done to determine if decreases in Cx-43 expression, caused by PAR-1 silencing, affects melanoma cell attachment to HUVECs or HDMECs. Results from the attachment assays reveal that there is less binding of PAR-1–silenced A375SM (P < 0.001) and C8161 (P < 0.001) cells to the endothelial cells when compared with both cell lines transduced with NT shRNA (Fig. 5A). Furthermore, to determine if this decrease in attachment was specifically due to the decrease in Cx-43 expression after PAR-1 silencing, Cx-43 was silenced via lentiviral shRNA in A375SM and C8161 cell lines (Fig. 5B), both of which are high expressers of Cx-43. Attachment assays again reveal less binding of Cx-43–silenced melanoma cells compared with NT-transduced cells (P < 0.001; Fig. 5C), similar to the results obtained with the PAR-1–silenced cells.

As a further proof-of-principle, PAR-1 was rescued in PAR-1–silenced C8161 melanoma cell line (Fig. 6A). This results in increased Cx-43 protein levels similar to that of NT shRNA-transduced melanoma cells (Fig. 6B). Reexpressing PAR-1 also reverts the decrease seen in melanoma cell attachment to endothelial cells after PAR-1 silencing (Fig. 6C and D). Furthermore, to ascertain that functional gap junctions were indeed occurring between melanoma cells and endothelial cells, dye transfer assays were done, thereby corroborating functional gap junction formation (Supplementary Fig. S1).

In summary, our results show that silencing PAR-1 decreases Cx-43 expression. This decrease is mediated via reduced binding of SP-1 and AP-1 transcription factors to the Cx-43 promoter after PAR-1 silencing. We further show that decreasing Cx-43, via both PAR-1 shRNA and Cx-43 shRNA, results in decreased binding of these cells to endothelial cells, thereby attesting to the important role PAR-1 plays in tumor cell diapedesis via regulation of Cx-43.

**Discussion**

PAR-1 plays a major role in the progression of melanoma. We and others have shown that PAR-1 is overexpressed in metastatic melanoma cell lines and tumors compared with nonmetastatic cell lines and dysplastic nevi (8, 12–14). We, therefore, silenced PAR-1 by using both shRNA and in vivo liposomal delivery of small interfering RNA and found a significant decrease in both tumor growth and metastasis, further establishing the central role PAR-1 plays in melanoma progression (7). Interestingly, the addition of thrombin or hirudin in our studies revealed no differences in the effects of PAR-1 activity on melanoma cells beyond that of basal levels probably due to activation of PAR-1 by means other than
thrombin. We cannot exclude the possibility that activation of PAR-1 in our system could occur in an autocrine manner.

To fully understand how PAR-1 is involved in melanoma growth and metastasis, we used cDNA microarray profiling on PAR-1–silenced cells. Based on these results, we have found a link between PAR-1 expression and the gap junctional intracellular communication molecule, Cx-43, in melanoma. This is the first time that Cx-43 has been identified to be regulated by PAR-1 in human melanoma.

The role of Cx-43 in cancer is controversial. Studies have shown that, in several cancers, Cx-43 acts as a tumor suppressor gene with loss of Cx-43 contributing to metastasis (37–39). Conversely, expression of Cx-43 has also been shown to increase tumor metastasis in breast cancer as well as in gliomas through increased attachment and communication with the vascular endothelium (15, 17, 21–26).

Previous studies using murine melanoma cells report increased coupling of melanoma cells expressing higher levels of Cx-43 to the vascular endothelial cells (23). In fact, the ability of tumor cells to metastasize appears to correlate with the ability of tumor cells to communicate with endothelial cells (24). Nevertheless, studies analyzing the early steps in melanoma progression found a decrease in Cx-43 in human melanoma cells (19, 40). Our findings, however, show high levels of Cx-43 protein expression in metastatic melanoma cell lines and that loss of PAR-1 expression results in the loss of Cx-43. Our finding does not support the role of Cx-43 acting as a tumor suppressor gene in malignant melanoma. Its expression level was high in the metastatic A375SM and C8161 cell lines but was dramatically reduced in these cells transduced with PAR-1 shRNA. Our data suggest another possible mechanism by which PAR-1 contributes to invasion and metastasis in melanoma, that is, by regulating Cx-43.

After identifying Cx-43 as a possible gene target of PAR-1, we validated our cDNA microarray results to determine if there was indeed a decrease in Cx-43 protein levels. This validation is essential, as cDNA microarrays only analyze the mRNA, thereby necessitating further studies to determine if actual protein levels are decreased. Therefore, Western blot analyses on both PAR-1–silenced metastatic melanoma cell lines were done. These studies revealed a significant decrease in Cx-43 protein levels after PAR-1 silencing, thus confirming that Cx-43 was being regulated by PAR-1 at the protein level. We, therefore, sought to determine the mechanism by which PAR-1 was affecting Cx-43 expression. Promoter analyses revealed that the Cx-43 promoter activity was significantly inhibited by PAR-1 shRNA, suggesting that PAR-1 regulates Cx-43 expression at the transcriptional level.

Based on these results, we determined whether PAR-1 was affecting the binding of transcription factors that were shown previously to be required for maximal promoter activity, that is, AP-1 and SP-1 (35, 36). Although PAR-1 did not affect the expression of these transcription factors, it did affect binding of SP-1, c-Jun, and c-Fos to the promoter of Cx-43. As both transcription factors...
(SP-1 and AP-1) act as positive regulators of Cx-43, our data provide a novel mechanism for the regulation of Cx-43 expression by PAR-1.

The link between PAR-1 affecting binding of AP-1 and SP-1 to the Cx-43 promoter was further strengthened by performing mutation analyses on these transcription factor binding sites and determining the effects on Cx-43 promoter activity after PAR-1 silencing.

With mutations in both SP-1 and AP-1 sites, there is significantly less PAR-1 induction of the Cx-43 promoter as seen in the NT-transduced cells (PAR-1 positive) after the transcription factor binding sites were mutated. Furthermore, the promoter activity in PAR-1–silenced cells is further decreased after promoter mutagenesis. This allowed us to conclude that the mechanism for the regulation of Cx-43 by PAR-1 occurs through differential binding of AP-1 and SP-1 to the promoter of Cx-43.

Previous studies have found that increased Cx-43 levels have an effect on tumor cell attachment to certain cell types including endothelial cells (21–26). These studies support our findings showing that, with decreased Cx-43 expression via PAR-1 shRNA, there was a significant decrease in melanoma cell attachment to endothelial cells. To ascertain that the changes seen in attachment of PAR-1–silenced cells to endothelial cells was truly a result of decreased Cx-43 expression, lentiviral Cx-43 shRNA was used to silence Cx-43 in A375SM and C8161 cell lines. As with PAR-1–silenced cells, silencing of Cx-43 also caused a reduction in binding of melanoma cells to HUVEC. Further proof that PAR-1 was regulating attachment of melanoma cells to endothelial cells via Cx-43 was obtained when PAR-1 was reexpressed in PAR-1–silenced C8161 melanoma cells. Rescuing PAR-1 resulted in an increase in Cx-43 expression. This also resulted in an increase of melanoma cell attachment to endothelial cells compared with PAR-1–silenced cells or control. HDMECs were used in these experiments to illustrate that these effects on attachment were also seen in microvessel endothelial cells and was, therefore, not an artifact of using HUVECs. Finally, to illustrate that Cx-43 gap junctions are present between melanoma cells and microvessel endothelial cells, dye transfer assays were used to show functional gap junction formations (Supplementary Fig. S1). We, therefore, concluded that attachment of melanoma cells to endothelial cells was in part due to the expression of Cx-43.

PAR-1 regulation of Cx-43 in melanoma cells might have direct effects on melanoma metastasis. The ability of tumor cells to metastasize appears to correlate with the ability of tumor cells to communicate with endothelial cells (24). The metastatic cascade is complex and involves expression and silencing of a myriad of genes. It has been argued that Cx-43 is lost in the early phases of melanoma progression in which melanocytes, but not melanoma cells, were able to communicate with keratinocytes through connexins (19, 40). However, these findings did not include studies on melanoma cells en route to the metastatic organ. Once melanoma cells have reached the vasculature, they must arrest and extravasate through the vascular endothelium in the metastatic organ. In this process (tumor cell diapedesis), Cx-43 plays an important role in melanoma progression. Previous studies have found that this increase in Cx-43 is not only crucial for communication between tumor cells and endothelial cells but also plays a role in tumor cell adherence and diapedesis (21, 22). Studies have also shown the importance of Cx-43 in enhancing angiogenesis in vivo (41, 42), which correlate with our previous findings of decreased blood vessel formation and angiogenesis after treating tumor-bearing mice with liposomal-delivered PAR-1 small interfering RNA (7).

Our data indicate that Cx-43 is not a tumor suppressor gene in melanoma. Rather, it functions to enhance attachment and diapedesis of circulating melanoma cells to the vascular endothelium. Moreover, up-regulation of Cx-43 allow for the establishment of intracellular communication between the tumor microenvironment and the metastatic tumor cells, allowing for the passage of ions and second messengers, which further enhances the...
metastatic process. Herein, we report that up-regulation of PAR-1 expression seen in melanoma progression mediates high levels of Cx-43 expression through increased binding of SP-1 and AP-1 transcription factors to the Cx-43 promoter.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


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