Gestational choriocarcinoma is a malignant tumor derived from placental trophoblast and the most aggressive member of gestational trophoblastic disease (GTD). Apoptosis-stimulating protein of p53-2 (ASPP2) is a member of ASPP family that transactivates p53 and thereby functions as a tumor suppressor. In this study, the expression profile of ASPP2 in choriocarcinoma was examined in comparison with normal placentas and hydatidiform moles, the latter being a type of GTD that carries malignant potential. Downregulation of ASPP2 messenger RNA and protein was demonstrated in choriocarcinoma by quantitative PCR and immunohistochemistry. ASPP2-transfected choriocarcinoma cells (JEG-3 and JAR) showed an increase in apoptosis and a decrease in cell migration as detected by TdT-mediated dUTP nick end labeling and wound healing assays, respectively, illustrating the complex action of ASPP2 on cell functions other than programmed cell death. Activated Src is known to be important in tumor progression. Transfection of ASPP2 but not ASPP1, another tumor-suppressive ASPP, was found to be related to subsequent decreased Src-pY416 phosphorylation, suggesting an inactivating effect of ASPP2 on Src. Moreover, this ASPP2-mediated inactivation of Src could be abolished by RNA interference with C-terminal Src kinase (Csk), a kinase that can inhibit Src activation. Our findings suggested that the ability of ASPP2 to attenuate Src activation was specific to ASPP2 in a Csk-dependent manner. Taken together, we demonstrated a loss of tumor-suppressive ASPP2 in choriocarcinoma with effects on cell migration and apoptosis. We also unveiled a possible mechanistic link between ASPP2 and Csk/Src signaling pathway, implicating the multiple cellular functions of ASPP2.

Introduction
Choriocarcinoma is a prominent member of gestational trophoblastic disease (GTD), which encompasses a heterogeneous family of allografts arising from placental trophoblasts with varying potential for local invasion and metastasis (1,2). GTD can be classified into premalignant hydatidiform moles and frankly malignant tumors like choriocarcinoma, placental site trophoblastic tumor (PSTT) and epithelioid trophoblastic tumor. Hydatidiform moles, including partial and complete moles, often regress after suction evacuation although around 20% of cases progress to persistent gestational trophoblastic neoplasia requiring chemotherapy (3). In contrast, choriocarcinoma, the most malignant lesion in GTD, is characterized by massive trophoblastic tissue invasion and vascular permeation leading to hemorrhagic metastasis (4,5).

Abbreviations: ASPP, apoptosis-stimulating protein of p53; Csk, C-terminal Src kinase; GTD, gestational trophoblastic disease; miRNA, messenger RNA; PSTT, placental site trophoblastic tumor; qPCR, quantitative PCR; siRNA, small interfering RNA; TUNEL, TdT-mediated dUTP nick end labeling.

Materials and methods

Clinical samples and cell lines
A total 94 trophoblastic tissues, including 20 first trimester placentas, 12 term placentas, 15 partial moles, 35 complete moles and 12 choriocarcinomas, were retrieved from the archives of Department of Pathology, Queen Mary Hospital, The University of Hong Kong, China. All tissue sections were histologically reviewed using generally agreed and accepted diagnostic criteria (1). First trimester and term placentas were collected after induced abortion by suction evacuation and normal delivery, respectively. The tissues of hydatidiform moles and choriocarcinomas were obtained from specimens of uterine evacuation and/or hysterectomy.

Ethical approval for the use of such tissues in this study has been obtained from institutional review board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster. The experimental results were delinked from subjects’ personal information and individual’s consent was considered not necessary. The need for written informed consent from the participants was waived by the institutional review board.

The clinical diagnosis of persistent gestational trophoblastic neoplasia was made if there was a plateau in human chorionic gonadotrophin level for 4 weeks or a further rise in human chorionic gonadotrophin for three consecutive

Unlike other solid tumors, classical tumor suppressor genes p53, RB1 and p21 were found to be upregulated in choriocarcinoma (6). The increased expression of these tumor suppressors may represent inherent but failed mechanisms to antagonize the overgrowth and excessive proliferative activity in the trophoblast cells. On the other hand, loss or reduced expression of a panel of tumor suppressors has also been reported. For example, p16, E-cadherin and TIMP3 were downregulated in choriocarcinoma through promoter hypermethylation (7,8). Restoration of certain tumor suppressor in choriocarcinoma may alter the tumor cell phenotype. For instance, ectopic NECCI (not expressed in choriocarcinoma clone 1) could suppress the tumorigenicity and induced differentiation of choriocarcinoma cells (9). Thus, a unique profile of alternations in tumor suppressor expression appears to contribute to the malignant phenotype of choriocarcinoma.

Apoptosis-stimulating protein of p53 (ASPP) is a family of p53 binding proteins, which share a common feature containing an ankyrin repeat domain, a SH3 domain and a poly-proline-rich domain at the C-terminus (10). To date, three family members, namely ASPP1, ASPP2 and iASPP, have been identified. Our recent report on downregulation of proapoptotic ASPP1 in association with clinical progression of GTD uncovered the importance of ASPP family in the disease development (11).

Similar to ASPP1, ASPP2 is a proapoptotic regulator that belongs to ASPP family. The expression of ASPP2 is frequently suppressed in many cancers in relation to enhanced apoptosis through the binding to p53 for transcriptional transactivation (12-14). The interaction and regulation of p53 by the ASPP family members seem to be evolutionarily conserved. Homologs of ASPP family members have been identified in Caenorhabditis elegans (ape-1) and Drosophila (dASPP) (15,16). Surprisingly, a number of ASPP2 binding partners that are involved in biological pathways other than apoptosis have also been identified, suggesting that ASPP2 function is far more complex than simply enhancing p53-mediated apoptosis (17). Abnormal activation of Src-family kinases has been implicated in a wide variety of cancers and is associated with tumor metastasis (18). A recent study has found that Drosophila ASPP (dASPP) could maintain epithelial integrity through physical interaction with C-terminal Src kinase (Csk) to augment the inhibitory phosphorylation of Drosophila Src (16). However, little is known about these interactions in humans and the corresponding biological significance in cancer cells has not been reported.

In this study, we investigated the expression profile of ASPP2 in GTD, the effects of ASPP2 on apoptosis, cell migration and Src signaling pathway.
weeks after evacuation. In most of the cases, the diagnosis of hydatidiform moles had been confirmed by fluorescent microsatellite genotyping after microdissection and chromosome in situ hybridization (19,20). These trophoblastic tissues have also been assessed earlier by immunohistochemical studies using M30 Cytodeath (Boehringer Mannheim, Mannheim, Germany) (21) and p53 (DO-7, Novocastra Laboratories Ltd, Newcastle, UK) antibodies (22).

For in vitro studies, two choriocarcinoma cell lines (JEG-3 and JAR; American Type Culture Collection, Manassas, VA) were cultured in minimum essential Eagle’s medium (Sigma, St Louis, MO) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS), and 100 U/mL penicillin and streptomycin (Invitrogen, San Diego, CA) (23). Normal trophoblast cell lines B6 and PE4 are kind gifts from Prof. George S.W. Tsao (Department of Anatomy, The University of Hong Kong).

### Immunohistochemical study

About 5 μm thick paraffin sections were cut and deparaffinized. Antigen retrieval was carried out at 95°C for 10 min in 10 mM sodium citrate buffer at pH 6.0. Immunohistochemistry was performed using the UltraVision LP Value Detection System Horseradish Peroxidase Polymer (LabVision, Fremont, CA) (24). A monoclonal mouse anti-human antibody of ASPP2 (Clone DN 54.10; Sigma) was applied in 1:1500 dilution and incubated overnight at room temperature. Freshly prepared 3,3′-diaminobenzidine tetrahydrochloride (Amresco, Solon, OH) in phosphate-buffered saline with hydrogen peroxide was used as chromagen and sections were counterstained with hematoxylin. Negative controls were prepared by replacing the primary antibody with phosphate-buffered saline. A known positive control from a normal first trimester placenta was used. The percentage of ASPP2 immunopositive cells was scored according to the following criteria: 0, negative; 1, 0.1–25.0% of cells immunopositive; 2, 25.1–50.0% immunopositive; 3, 50.1–75.0% immunopositive and 4, 75.1–100% of cells immunopositive (24,25). ASPP2 immunoreactivity was further correlated with p53 expression (22).

### Quantitative real-time PCR

Trizol reagent (Invitrogen, Life Technologies Inc., Rockville, MD) was used for total RNA extraction according to the manufacturer’s instruction. First-strand complementary DNA was synthesized from 2.5 μg total RNA by SuperScript Reverse Transcriptase system (Invitrogen). Following primers were used: ASPP2 (forward: 5′-GTG CTG CCT CAT GTA ACA AC-3′; reverse: 5′-TAT GCC CAT CTT CCT CTG AAC-3′) and glyceraldehyde 3-phosphate dehydrogenase (forward: 5′-TCC ATG ACA TTG TTG GCG-3′; reverse: 5′-ACA GCC TCC TGG GTG GCA GTG-3′). Quantitative PCR (qPCR) was performed on ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The expression of ASPP2 determined by 2−ΔΔCT method was normalized with respect to that of glyceraldehyde 3-phosphate dehydrogenase.

### Transfection and western blot analysis

JEG-3 and JAR culture in six-well plates were transfected with ASPP2 and ASPP1 construct (generous gift from Prof. Xin Lu of Ludwig Institute for Cancer Research, UK) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The pCDNA 3.1 vector was used as control. Total protein lysate was extracted by lysis buffer (0.125 M Tris pH 6.8 at 22°C containing 1% NP-40 [v/v], 2 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 0.1 μM sodium okadate). Protein concentration was determined by detergent compatible protein assay (Bio-Rad Laboratories, Hercules, CA). About 20 μg of protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane and probed with corresponding antibodies (25). Primary antibodies used in this study were listed in Table 1.

### TdT-mediated dUTP nick end labeling assay

TdT-mediated dUTP nick end labeling assay (TUNEL) was performed using an In Situ Cell Death Detection kit (Roche Biochemical, Indianapolis, IN) as described previously (21). The number of TUNEL-positive cells in different fields observed at ×40 magnification by fluorescence microscopy.

### Wound healing assay

Wound healing assay was used for continuous data. Spearman’s ρ test was used for correlation analysis. P values <0.05 were considered as statistically significant.

### Results

#### Downregulated expression of ASPP2 messenger RNA and protein in choriocarcinoma

By qPCR analysis, ASPP2 messenger RNA (mRNA) was found to be downregulated in choriocarcinoma cell lines, JEG-3 and JAR, when compared with normal trophoblast cell lines, B6 and PE (Figure 1A). Significantly lower ASPP2 mRNA was also detected in choriocarcinoma samples compared with normal first trimester placenta (P = 0.012). Hydatidiform moles showed an intermediate ASPP2 mRNA expression between normal placentas and choriocarcinomas (Figure 1B).

### Immunohistochemistry, both nuclear and cytoplasmic ASPP2 expression was detected in first trimester samples. ASPP2 was found to be expressed predominantly in the nucleus and moderately in the cytoplasm of cytotrophoblasts and villous intermediate trophoblasts of normal placenta and hydatidiform moles in contrast to the absence of expression in choriocarcinoma (Figure 1C–F). Immunoreactivity to ASPP2 in PSTT was also weak. Moreover, no nuclear immunoreactivity could be detected in the syncytiotrophoblasts. Concurring with the qPCR findings, significantly lower ASPP2 immunoreactivity was found in the choriocarcinoma compared with normal first trimester samples (P < 0.001, Mann–Whitney test; Figure 1G). There was no significant difference in immunoreactivity between hydatidiform moles that subsequently developed gestational trophoblastic neoplasm (progressed mole) and those that spontaneously regressed (P = 0.786, Mann–Whitney test). Although both regressed and persistent hydatidiform moles showed a lower nuclear immunoreactivity than normal first trimester placenta, no statistical significance was reached (P = 0.06 and 0.42, respectively; Figure 1G).

### Table 1. Primary antibodies used for immunoblotting

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<td>Sigma</td>
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Fig. 1. Downregulation of ASPP2 in choriocarcinoma. (A and B) Real-time–qPCR analysis on mRNA expression of ASPP2 between (A) normal trophectoderm (PE4 and B6) and choriocarcinoma (JAR and JEG) cells as well as (B) in normal placentas (first trimester and term), hydatidiform moles (HM) and choriocarcinoma (CCA) clinical samples. (C–G) Immunoreactivity of ASPP2 in trophoblastic tissues. In normal first trimester placenta (C) and hydatidiform mole (D), both nuclear and cytoplasmic staining could be observed in the cytotrophoblast (CT) and villous intermediate trophoblast (VIT),
**Ectopic ASPP2 increased apoptosis but reduced cell migration in choriocarcinoma cells**

Retrieval of data from corresponding cases showed that nuclear immunoreactivity of ASPP2 correlated inversely with p53 (P = 0.032, coefficient = -0.301, Spearman’s ρ test) (22). Such correlation suggested that loss of ASPP2 in choriocarcinoma could modify p53 activity leading to difference in apoptotic activity among GTD. The effect of ASPP2 on apoptosis in choriocarcinoma was assessed by ASPP2 transfection and TUNEL assay. Our results showed that ectopic ASPP2 expression increased percentage of apoptotic cells from 2.6 ± 1.9% to 10.5 ± 2.7% in JEG-3 (Figure 2A) and 3.5 ± 2.9 to 14.0 ± 3.7% in JAR (Figure 2B) cells in contrast to control, respectively (Figure 2), suggesting that loss of proapoptotic ASPP2 could attenuate apoptosis in choriocarcinoma cells.

By wound healing assay, slower migration rate was found in JEG-3 transfected with ASPP2 than in control transfected with ASPP1 or the control (Figure 3A, left panel). Our results demonstrated, for the first time, that ASPP2 negatively regulates cell migration in choriocarcinoma. Furthermore, western blot analysis also showed an ASPP2-specific induction of E-cadherin expression (Figure 3A, right panel).

**ASPP2-specific inactivation of Src**

In view of the multifunctional nature of ASPP2 with effect on both apoptosis and cell migration in choriocarcinoma cells, possible interaction between ASPP2 and Src signaling pathway was evaluated. Activation and inactivation of Src are known to be tightly regulated by phosphorylation at two sites with opposite effects. Phosphorylation site at Tyr416 (Y416) in the activation loop of the kinase domain contributes to augmentation of Src activity. In contrary, Csk-mediated phosphorylation of Tyr527 (Y527) at C-terminal tail of Src results in a closed structure, through intramolecular interaction with its SH2 domain, that diminishes the access of substrates to the kinase domain and prevent Y416 autophosphorylation (26). As shown in Figure 3B, overexpression of ASPP2 in both JEG-3 and JAR could reduce the expression level of Src-pY416, an activated form of Src. However, such observation was not found in cells transfected with ASPP1, another member of the ASPP family exerting tumor-suppressive effect. Moreover, complementary increased phosphorylation at inhibitory domain Src-pY527 accompanied with ectopic ASPP2 expression was also demonstrated. The total form of Src remained unchanged after ASPP2 or ASPP1 transfection.

**Csk knockdown abolished the ASPP2 effect on Src inactivation and cell migration inhibition**

The above findings suggested that ASPP2 may negatively regulate cell migration through Csk/Src signaling pathway in choriocarcinoma cells. To unveil the mechanismic action of this potential interaction, ASPP2 and Csk-specific siRNA were co-transfected in JEG-3 cells for downstream analysis. As shown in Figure 4A, in the presence of scramble siRNA, ectopic ASPP2 expression inactivates Src as indicated by suppressing the expression of Src-pY416 and was also able to induce E-cadherin expression in JEG-3. In contrast, such inactivation and induced E-cadherin expression were abolished when the JEG-3 cells underwent co-transfection of ASPP2 and Csk siRNA. More importantly, inhibitory effect on choriocarcinoma cell migration by ectopic ASPP2 was also eliminated after Csk-specific knockdown as demonstrated in wound healing assay (Figure 4B).

As the Y527 inhibitory phosphorylation site is mediated by Csk, our results demonstrated that Src could be inactivated specifically by ASPP2 through the action of Csk with effects of cell migration.

**Discussion**

In this study, we have revealed a choriocarcinoma specific loss of ASPP2 compared with normal placenta and GTD subtypes and a pleiotropic nature of ASPP2 which, in addition to stimulating apoptosis, negatively regulates cell migration through Src signaling pathway in a Csk-dependent manner. Human trophoblasts exert a crucial role in implantation and placentation of pregnancy and fetal development. Alterations in molecular mechanism and signal transduction pathways on trophoblast cell migration and its invasiveness may lead to pathological conditions (27,28). As choriocarcinoma is the malignant extreme of the spectrum of GTD, our present data identify a critical role of ASPP2 in tumorigenesis of choriocarcinoma with its effects in apoptosis and cell migration.

Apoptosis is an important process in pathogenesis in GTD (21,24,29,30). Studies on p53-related genes and modulation of p53 activity may help understand the development of GTD. ASPP2 is originally identified as a p53 binding protein. It has been demonstrated that ASPP2 is able to induce apoptosis through mitochondrial pathway associated with activation of caspase-9 (31). Our results in apoptosis in choriocarcinoma cells concur with the central role of ASPP2 in the regulation of apoptosis as described in these studies.

Real-time qPCR showed a downregulation of ASPP2 mRNA level in hydatidiform mole and choriocarcinoma, respectively, compared with normal placenta (Figure 1B). Normal placent alchorionic villi are composed of a complex and heterogeneous population of trophoblast cells, including cytotrophoblast, syncytiotrophoblast and villous intermediate trophoblast. Specific spatial and subcellular alteration of ASPP2 expression was further evaluated by immunohistochemistry. As cytotrophoblast is the progenitor of villous trophoblasts and syncytiotrophoblast (32) and is regarded as critical for neoplastic transformation in trophoblastic tumors (3), we specifically assessed the immunoreactivity of nuclear ASPP2 in cytotrophoblast as a function of p53 transactivation (Figure 1G). Loss of nuclear ASPP2 immunoreactivity particularly in the truly malignant choriocarcinoma concurs with the fact that choriocarcinoma has a lower apoptotic activity than premalignant hydatidiform moles as we reported previously (21,29). Besides the expression in nuclei, cytoplasmic immunoreactivity of ASPP2 was also detected in both first trimester placenta and hydatidiform mole samples. In contrast, in choriocarcinoma and PSTT, the malignant forms of GTD, total loss or very weak ASPP2 expression was observed in both the nucleus and cytoplasm was observed, respectively. Such findings suggested a possible interaction between cytoplasmic ASPP2 protein and gene products that are responsible for cellular functions other than apoptosis and may therefore account for the aggressiveness of choriocarcinoma or PSTT.

Although Drosophila homolog of ASPP (dASPP) has been reported to interact with dCsk to regulate Drosophila Src kinase to maintain epithelial integrity (16), biological significance regarding interaction between Src signaling pathway and ASPP family members is not available in humans. Our data on RNA interference and western blot analysis clearly showed that ectopic ASPP2 could inactivate Src signaling in a Csk-dependent manner in choriocarcinoma cells and reported for the first time the ability of ASPP2 to negatively regulate cell migration through the Csk/Src axis. Indeed, a wide body of evidences has shown that activated Src kinase endows migratory phenotype through interaction with a number of downstream signaling pathways including FAK, paxillin, extracellular signal-regulated kinase and internalization of E-cadherin (18,33,34). As a result, ASPP2-mediated cell migration through Csk/Src axis is noteworthy to be further investigated in cancer biology.

Metastasis is a multistep process and is usually initiated by detachment of tumor cells from primary sites (35,36). E-cadherin is an extensively studied cell adhesion molecule, which mediates cell adhesion whereas predominantly cytoplasmic staining was detected in syncytiotrophoblast (ST) (indicated by arrows). Central cistern in enlarged villi in hydatidiform mole was marked by asterisk (*). In choriocarcinoma (E), which was composed of both CT and ST, loss of ASPP2 immunoreactivity was observed. A much weak expression of ASPP2 was also detected in PSTT, another trophoblastic malignancy (F). Scale bar, 200 µm. (G) Bar chart illustrating the reduced nuclear immunoreactivity of ASPP2 in cytotrophoblast of choriocarcinoma when compared with normal placenta and hydatidiform moles.
in a homotypic manner (36). Suppression of E-cadherin expression is frequently involved in human cancers and is considered as one of the early events of tumorigenesis (37). Our laboratory has earlier reported that E-cadherin is downregulated by hypermethylation in GTD and choriocarcinoma has the lowest level of expression (38). Interestingly, increased Src activity is known to reduce cell–cell adhesion by promoting the internalization and ubiquitin-mediated protein degradation of E-cadherin (39,40). Moreover, Src-specific inhibitor PP2 enhanced E-cadherin expression at transcript and protein levels (41). These actions exemplify the upregulation of E-cadherin through reduced protein degradation and increased transcript expression upon Src inactivation. Consistent with ASPP2-specific interaction with Csk/Src axis demonstrated in this study, E-cadherin expression could be specifically induced by ectopic ASPP2 in JEG-3 cells, whereas this induction was abolished under Csk knockdown (Figure 4A). Hence, transfected ASPP2 inactivated Src activity, through which E-cadherin was upregulated and subsequently accumulated in the cells. Taken together, loss of ASPP2 could work as an additional mechanism to further downregulate E-cadherin expression in a homotypic manner (36). Suppression of E-cadherin expression is frequently involved in human cancers and is considered as one of the early events of tumorigenesis (37). Our laboratory has earlier reported that E-cadherin is downregulated by hypermethylation in GTD and choriocarcinoma has the lowest level of expression (38). Interestingly, increased Src activity is known to reduce cell–cell adhesion by promoting the internalization and ubiquitin-mediated protein degradation of E-cadherin (39,40). Moreover, Src-specific inhibitor PP2 enhanced E-cadherin expression at transcript and protein levels (41). These actions exemplify the upregulation of E-cadherin through reduced protein degradation and increased transcript expression upon Src inactivation. Consistent with ASPP2-specific interaction with Csk/Src axis demonstrated in this study, E-cadherin expression could be specifically induced by ectopic ASPP2 in JEG-3 cells, whereas this induction was abolished under Csk knockdown (Figure 4A). Hence, transfected ASPP2 inactivated Src activity, through which E-cadherin was upregulated and subsequently accumulated in the cells. Taken together, loss of ASPP2 could work as an additional mechanism to further downregulate E-cadherin expression.
expression, leading to loss of cell–cell adhesions and, at least in part, contributing to the aggressive and malignant phenotype of choriocarcinoma.

Unlike ASPP2, another ASPP family member ASPP1 failed to inactivate Src as illustrated by the decreased expression of Src-pY416. This observation was consistently demonstrated in both JEG-3 and JAR cell lines. Although it has been proposed that the interaction between ASPP proteins and Src is commonly evolutionary conserved from Drosophila to humans (16,42), our in vitro study clearly indicated that ASPP2, but not ASPP1, interacts with Csk/Src axis and highlighted an ASPP2-specific regulation of cell migration through this signal transduction pathway. Indeed, a recent study showed that knockdown of ASPP2 was more effective in promoting the growth of hepatocellular carcinoma cells both in soft-agar transformation assay and in nude mice, when compared with that of ASPP1 (14). Thus, the loss of ASPP2 might exert a more potent effect than ASPP1 in tumor development with respect to the pleiotropic nature of this tumor suppressor.

In conclusion, we found loss of expression of the multifunctional ASPP2 in choriocarcinoma but not the premalignant hydatidiform
moles, suggesting its crucial role in tumorigenesis through its effect on inhibiting cell migration and promoting apoptosis. An ASPP2-specific inactivation of the oncogenic Src in a Csk-dependent manner was further demonstrated, which provides a mechanistic link between loss of ASPP2 and the aggressive phenotype of choriocarcinoma at molecular basis.

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**Conflict of Interest Statement:** None declared.

**References**


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**Fig. 4.** ASPP2 inactivates Src through Csk. (A) Csk knockdown abolished the effect of ectopic ASPP2 on Src inactivation. (B) Bar chart showing effect of Csk knockdown on cell migration of ASPP2-transfected JEG-3 cells as determined by wound healing assay.
ASPP2 controls cell migration in choriocarcinoma


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