Carcinogenesis Advance Access published May 13, 2013

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Journal:	*Carcinogenesis*
Manuscript ID:	CARCIN-2012-01361.R1
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	29-Apr-2013
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Keywords:	Choriocarcinoma, ASPP2, Gestational trophoblastic disease, Src

Downregulation of ASPP2 in choriocarcinoma contributes to increased migratory potential through Src signaling pathway activation

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Short title: ASPP2 controls cell migration in choriocarcinoma

Abstract

Gestational choriocarcinoma is a malignant tumour derived from placental trophoblast and the most aggressive member of gestational trophoblastic disease (GTD). Apoptotic stimulating protein of p53-2 (ASPP2) is a member of ASPP family that transactivates p53 and thereby functions as a tumour 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 mRNA 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 TUNEL 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 tumour progression. Transfection of ASPP2 but not ASPP1, another tumour 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 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 tumour suppressive ASPP2 in choriocarcinoma with effects on cell migration as well as 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 tumours like choriocarcinoma, placental site trophoblastic tumour (PSTT) and epithelioid trophoblastic tumour (ETT). 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 haemorrhagic metastasis [4,5].

Unlike other solid tumours, classical tumour suppressor genes p53, RB1 and p21 were found to be upregulated in choriocarcnoma [6]. The increased expression of these tumour 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 tumour suppressors has also been reported. For example, p16, E-cadherin, and TIMP3 were downregulated in choriocarcinoma through promoter hypermethylation [7,8]. Restoration of certain tumour suppressor in choriocarcinoma may alter the tumour cell phenotype. For instance, ectopic NECC1 (not expressed in choriocarcinoma clone 1) could suppress the tumorigenicity and induced differentiation of choriocarcinoma cells [9]. Thus, a unique profile of alternations in tumour 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 shares 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 uncovers 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 seems to be evolutionarily conserved. Homologs of ASPP family

members have been identified in *C. elegan* (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 tumour 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 (dSrc) [16]. However, little is known about these interactions in human 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 the Src signaling pathway.

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, 12 choriocarcinomas were retrieved from the archives of Department of Pathology, Queen Mary Hospital, The University of Hong Kong. All tissue sections were histologically reviewed using generally agreed and accepted diagnostic criteria [1]. First trimester and term placenta 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 evacuate and/or hysterectomy.

Ethics 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 (hCG) level for 4 weeks or a further rise in hCG for three consecutive 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, German) [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, USA) were cultured in Minimum Essential Eagle's Medium (Sigma, St Louis, MO, USA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS, USA), and 100 U/ml penicillin and streptomycin (Invitrogen, San Diego, CA, USA)[23]. Normal trophoblast cell lines B6 and PE4 are kind gifts from Professor George S. W. Tsao (the Department of anatomy, the University of Hong Kong).

Immunohistochemical study

Paraffin sections 5 μm thick 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 (HRP) Polymer (LabVision, Fremont, CA, USA)[24]. A monoclonal mouse anti-human antibody of ASPP2 (Clone DX 54.10) (Sigma, St.Louis, MO) was applied in 1:1500 dilution and incubated overnight at room temperature. Freshly-prepared 3.3'-diaminobenzidine tetrahydrochloride (DAB) (Amresco, Solon, Ohio) in PBS with hydrogen peroxide was applied as chromagen and sections were counter-stained with hematoxylin. Negative controls were prepared by replacing the primary antibody with PBS. 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; 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 cDNA was synthesized from 2.5 μg total RNA by SuperScript Reverse Transcriptase system (Invitrogen). Primers used were as followed: ASPP2 (Forward: 5'-GTG CTG CCT CAT GTA ACA AC-3'; Reverse: 5'-TAT GCC CAT CTT CTC CTG AAC-3') and GAPDH (Forward: 5'-TCC ATG ACA ACT TTGGTA TCG CG-3'; Reverse: 5'-ACA GTC TTC TGG GTG GCA GTG-3'). Quantitative PCR (qPCR) was performed on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The expression of ASPP2 determined by $2^{-\Delta\Delta CT}$ method was normalized with respected to that of GAPDH.

Transfection and Western blot analysis

JEG-3 and JAR culture in 6-well plates were transfected with either 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 with lysis buffer (0.125 M Tris, pH 6.8 at 22ºC containing 1% NP-40 [v/v], 2 mM EDTA, 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). Twenty μg of protein was resolved by SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difuoride 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 previously described [21]. The number of TUNEL-positive cells in different controls and in JEG-3 and JAR after ASPP2 transient transfection was counted in three different fields at X40 magnification by fluorescence microscopy.

Silencing of Csk by small interfering RNA (siRNA)

To knockdown Csk in choriocarcinoma cell line, Silencer® select Pre-designed siRNA of Csk (siCsk) (Cat: 4427037; ID:s3614) and non-targeting Negative Control #2 (scramble) siRNA (Cat: 43900846) were used (Ambion, Austin, TX). Co-transfection with 5 nM Csk-specific/non-targeting siRNA and empty vector pCDNA3.1/ ASPP2 construct in Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was carried in 6-well culture plate. To assess the knockdown efficiency, Western blotting was performed using the protein lysate harvested 48 h post-transfection.

Wound healing assay

Migration of the cells was determined by wound healing assay. Cells were first transfected with specified plasmid constructs or siRNA oligo for one day in twelve-well plates with 90% confluence. The treated cell monolayer was then scratched with a sterile 200-μl pipette tip. Fresh culturing medium was added. Photos were retaken at the same position of the wound after 48 hours. Results expressed in percentage to control were defined as the average percentage change in linear wound closure in treatment with respect to that in control.

Statistical analysis

Statistical analysis was performed using the Statistical Package for Social Science (SPSS) 15.1. Non-parametric unpaired t-test (Mann-Whitney test) was used for continuous data. Spearman's rho test was used for correlation analysis. *P* values <0.05 were considered as statistically significant.

Results

Downregulated expression of ASPP2 mRNA and protein in choriocarcinoma

By qPCR analysis, ASPP2 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).

By 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 GTN (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).

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 choriocarinoma could modify p53 activity leading to difference in apoptotic activity among GTD. The effect of ASPP2 on apoptosis in choriocarinoma 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 those transfected with ASPP1 or the control (Figure 3A, left panel). Our results demonstrated, for the first time, that ASPP2 negatively regulate 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 multi-functional 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 is 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 tumour 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 mechanistic action of this potential interaction, ASPP2 and Csk-specific siRNA were cotransfected in JEG-3 cells for downstream analysis. As shown in figure 4A, in the presence of scramble siRNA, ectopic ASPP2 expression

inactivate 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 placentas and GTD subtypes and a pleiotropic nature of ASPP2 which, in addition to stimulating apoptosis, negatively regulate 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 identifies a critical role of ASPP2 in tumourigenesis 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 placental chorionic 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. Since cytotrophoblast is the progenitor of villous trophoblasts and syncytiotrophoblast [32] and is regarded as critical for neoplastic transformation in trophoblastic tumours [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

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 dSrc kinase to maintain epithelial integrity [16], biological significance regarding interaction between Src signaling pathway and ASPP family members are 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, ERK as well as 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 multi-step process and is usually initiated by detachments of tumour cells from primary sites [35,36]. E-cadherin is an extensively studied cell adhesion molecule which mediates cell adhesion 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 tumourigenesis [37]. Our lab has earlier reported that E-cadherin is down-regulated 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 whilst 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 down-regulate E-cadherin 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. While 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, interact 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 tumour development with respect to the pleiotropic nature of this tumour suppressor.

In conclusion, we found loss of expression of the multi-functional ASPP2 in choriocarcinoma but not the premalignant hydatidiform moles, suggesting its crucial role in tumourigenesis 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.

Funding

Areas of Excellence Scheme, Research Grants Council of the Hong Kong Special Administrative Region

Conflict of Interest Statement: None declared.

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TABLE

Table 1. Primary Antibodies Used for Immunoblotting

FIGURE LEGENDS

Figure 1 Downregulation of ASPP2 in choriocarcinoma. (A)&(B): Quantitative real-time PCR analysis on mRNA expression of ASPP2 between (A) normal trophoblast (PE4, B6) and choriocarcinoma (JAR, JEG) cells as well as (B) in normal placentas (1st trimester and Term), hydatidiform moles (HM) and choriocarcinoma (CCA) clinical samples. (C)-(G): Immunreactivity 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) 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 choricarcinoma (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 immunohistoscore of ASPP2 in cytotrophoblast of choriocarcinoma when compared with placenta and hydatidiform moles.

Figure 2 ASPP2 induced apoptosis in choriocarcinoma cells. A-B: Photographs of representative fields of TUNEL assay in choriocarcinoma cell lines JEG-3 (A) and JAR (B) after empty vector (control) and ASPP2 transfection. (C): Percentage of apoptotic cells (apoptotic cells/total cells counted) in both cell lines after empty vector (control) and ASPP2 transfection. Approximately 80-100 cells were analyzed per random high power field of view.

Figure 3 Wound healing assay and ASPP2-specific inactivation of Src. (A): Left panel: Effects of ectopic ASPP1 and ASPP2 expression on cell migration in choriocarcinoma cell JEG-3. Right panel: Enhanced E-cadherin expression in ASPP1 and ASPP2 transfected JEG-3 cells. (B): Western blot

analysis on the effect of ectopic ASPP1 and ASPP2 expression on Src phorspholyation at autophosphorylation domain Y416 (activated state) and inhibitory domain Y527 (closed structure).

Figure 4 ASPP2 inactivates Src through Csk. (A): Csk-knockdwon abolished the effect of ectopic ASPP2 on Src inactivation. (B): Bar chart showing effect of Csk-knockdwon on cell migration of ASPP2 transfected JEG-3 cells as determined by wound healing assay.

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Target Protein	Animal Source	Catalog#	Working dilution	Vendor
ASPP2	Mouse	A4480	1:500	Sigma (St. Louis, MO)
Actin	Rabbit	A5060	1:1000	Sigma
Csk	Rabbit	sc-286	1:1000	Santa Cruz biotechnology, Inc (Santa Cruz, CA)
Src Total	Mouse	sc-8056	1:1000	Santa Cruz biotechnology, Inc
Phospho-Src (Tyr416)	Rabbit	2101	1:1000	Cell Signaling (Beverly, MA)
Phospho-Src (Tyr527)	Rabbit	2105	1:1000	Cell Signaling
E-cadherin	Mouse	610181	1:5000	BD Biosciences (San Diego, CA)

Figure 1.

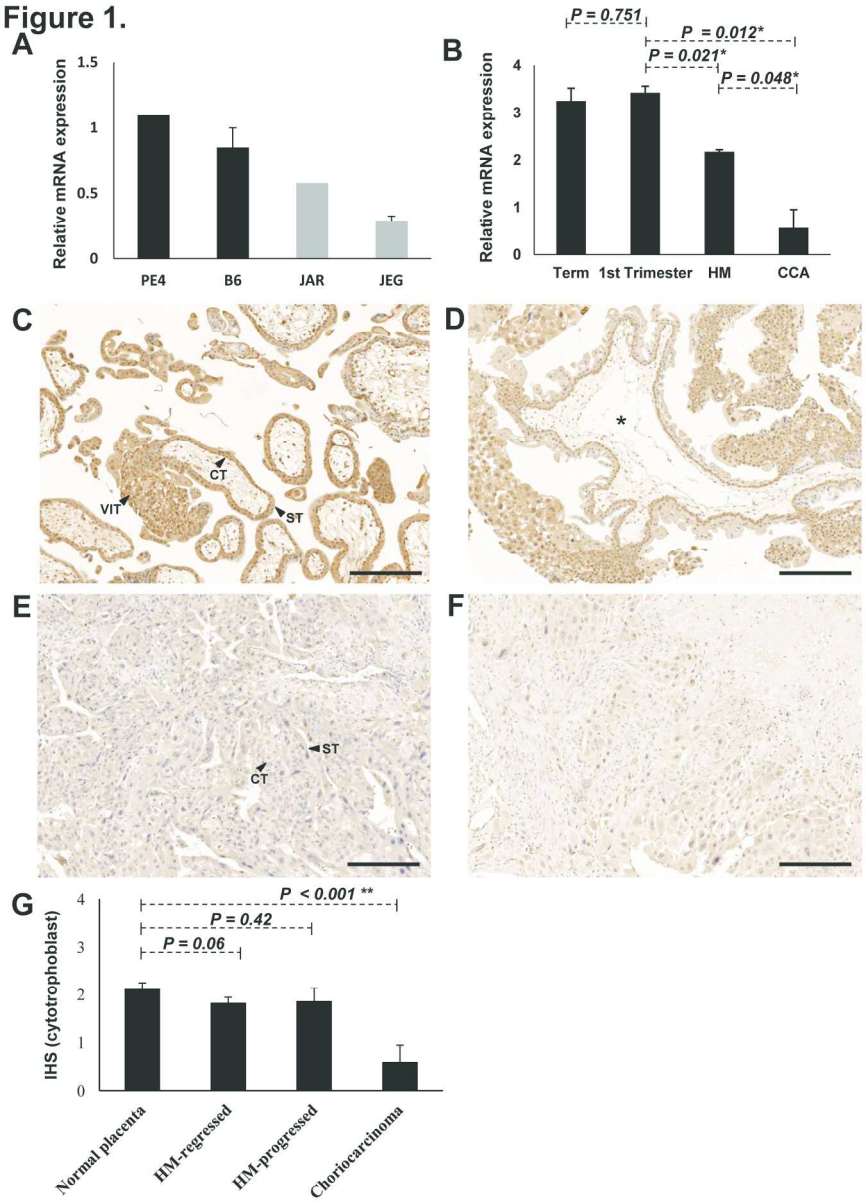

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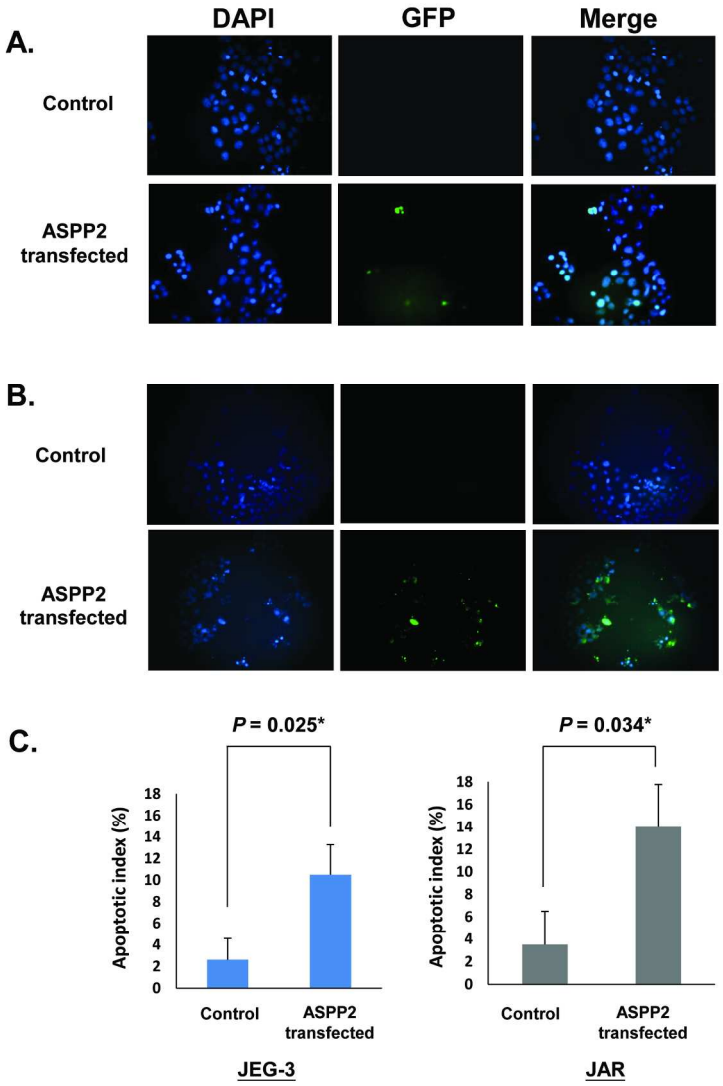
Figure 2.



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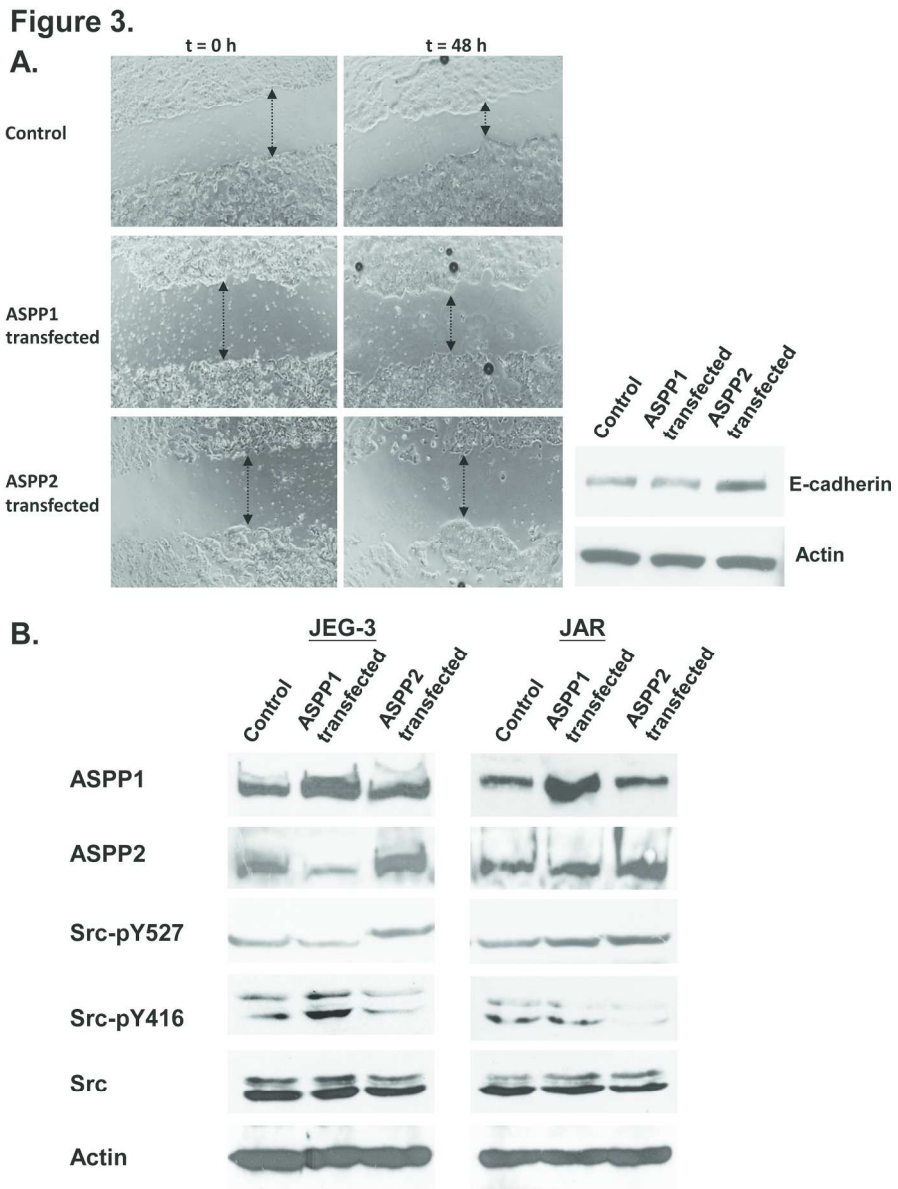

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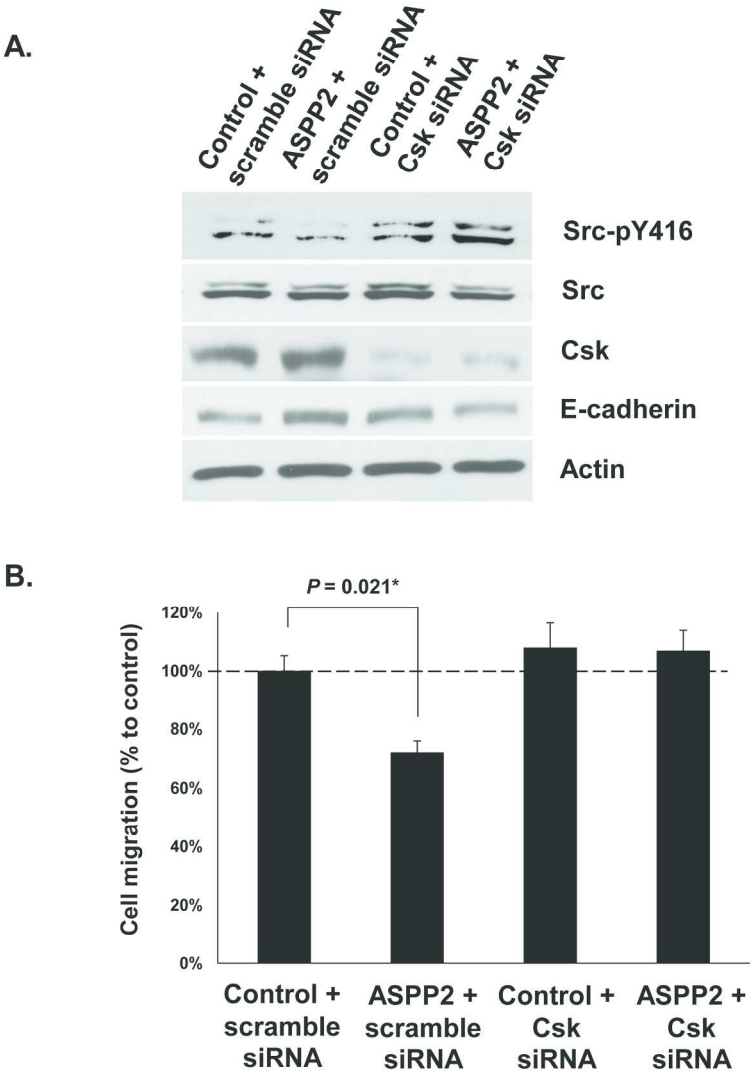
Figure 4.



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