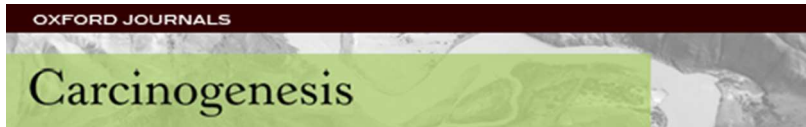




Title	Downregulation of ASPP2 in choriocarcinoma contributes to increased migratory potential through Src signaling pathway activation
Author(s)	Mak, CY; Lee, L; Siu, KY; Wong, GW; Lu, X; Ngan, HYS; Wong, ES; Cheung, ANY
Citation	Carcinogenesis, 2013, v. 34 n. 9, p. 2170-2177
Issued Date	2013
URL	http://hdl.handle.net/10722/189574
Rights	This is a pre-copy-editing, author-produced PDF of an article accepted for publication in Carcinogenesis following peer review. The definitive publisher-authenticated version Carcinogenesis, 2013, v. 34 n. 9, p. 2170-2177 is available online at: http://carcin.oxfordjournals.org/content/34/9/2170



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

Journal:	<i>Carcinogenesis</i>
Manuscript ID:	CARCIN-2012-01361.R1
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	29-Apr-2013
Complete List of Authors:	Mak, Victor CY; The University of Hong Kong, Pathology Lee, Lee; The University of Hong Kong, Pathology Siu, Michelle KY; The University of Hong Kong, Obstetrics and Gynaecology Wong, Oscar GW; The University of Hong Kong, Pathology Lu, Xin; University of Oxford, Ludwig Institute for Cancer Research Ltd., Nuffield Department of Clinical Medicine Ngan, Hextan Y.S.; The University of Hong Kong, Obstetrics and Gynaecology Wong, Esther; The University of Hong Kong, Pathology Cheung, Annie; The University of Hong Kong, Pathology
Keywords:	Choriocarcinoma, ASPP2, Gestational trophoblastic disease, Src

SCHOLARONE™
Manuscripts

1
2
3 **Downregulation of ASPP2 in choriocarcinoma contributes to**
4 **increased migratory potential through Src signaling pathway**
5 **activation**
6
7
8
9

10 Victor C. Y. Mak¹, Lee Lee¹, Michelle K. Y. Siu³, Oscar G. W. Wong¹, Xin Lu², Hextan Y. S. Ngan³,
11 Esther S. Y. Wong¹, Annie N. Y. Cheung^{1,*}
12

13
14
15 ¹ Departments of Pathology, The University of Hong Kong, HKSAR, China

16 ² The Ludwig Institute for Cancer Research Ltd., Nuffield Department of Clinical Medicine, University
17 of Oxford, UK

18 ³ Departments of Obstetrics and Gynaecology, The University of Hong Kong, HKSAR, China
19

20
21 * To whom correspondence should be addressed: Annie N.Y. Cheung, MD., FRCPath., Department of Pathology,
22 The University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Hong Kong, China. Tel: +852 22554876;
23 Fax: +852 28725197; Email: anycheun@.hkucc.hku.hk
24

25
26 Short title: ASPP2 controls cell migration in choriocarcinoma
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 choriocarcinoma [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

1
2
3 members have been identified in *C. elegans* (ape-1) and *Drosophila* (dASPP) [15,16]. Surprisingly, a
4 number of ASPP2 binding partners that are involved in biological pathways other than apoptosis have
5 also been identified, suggesting that ASPP2 function is far more complex than simply enhancing p53-
6 mediated apoptosis [17]. Abnormal activation of Src-family kinases has been implicated in a wide
7 variety of cancers and is associated with tumour metastasis [18]. A recent study has found that
8 *Drosophila* ASPP (dASPP) could maintain epithelial integrity through physical interaction with C-
9 terminal Src kinase (Csk) to augment the inhibitory phosphorylation of *Drosophila* Src (dSrc) [16].
10 However, little is known about these interactions in human and the corresponding biological
11 significance in cancer cells has not been reported.

12
13
14
15
16
17
18
19
20 In this study, we investigated the expression profile of ASPP2 in GTD, the effects of ASPP2 on
21 apoptosis, cell migration and the Src signaling pathway.
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 Paraffin sections 5 μ m thick were cut and deparaffinized. Antigen retrieval was carried out at
4 95 °C for 10 min in 10 mM sodium citrate buffer at pH 6.0. Immunohistochemistry was performed
5 using the UltraVision LP Value Detection System Horseradish Peroxidase (HRP) Polymer (LabVision,
6 Fremont, CA, USA)[24]. A monoclonal mouse anti-human antibody of ASPP2 (Clone DX 54.10)
7 (Sigma, St.Louis, MO) was applied in 1:1500 dilution and incubated overnight at room temperature.
8 Freshly-prepared 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Amresco, Solon, Ohio) in PBS with
9 hydrogen peroxide was applied as chromagen and sections were counter-stained with hematoxylin.
10 Negative controls were prepared by replacing the primary antibody with PBS. A known positive
11 control from a normal first trimester placenta was used. The percentage of ASPP2 immunopositive
12 cells was scored according to the following criteria: 0, negative; 1, 0.1–25.0% of cells immunopositive;
13 2, 25.1–50.0% immunopositive; 3, 50.1–75.0% immunopositive; 4, 75.1–100% of cells
14 immunopositive [24,25]. ASPP2 immunoreactivity was further correlated with p53 expression [22].
15
16
17
18
19
20
21
22
23
24
25
26

27 *Quantitative Real-time PCR*

28
29 Trizol reagent (Invitrogen, Life Technologies Inc, Rockville, MD) was used for total RNA
30 extraction according to the manufacturer's instruction. First strand cDNA was synthesized from 2.5 μ g
31 total RNA by SuperScript Reverse Transcriptase system (Invitrogen). Primers used were as followed:
32 ASPP2 (Forward: 5'-GTG CTG CCT CAT GTA ACA AC-3'; Reverse: 5'-TAT GCC CAT CTT CTC
33 CTG AAC-3') and GAPDH (Forward: 5'-TCC ATG ACA ACT TTGGTA TCG CG-3'; Reverse: 5'-ACA
34 GTC TTC TGG GTG GCA GTG-3'). Quantitative PCR (qPCR) was performed on the ABI PRISM
35 7900 Sequence Detection System (Applied Biosystems, Foster City, CA). The expression of ASPP2
36 determined by $2^{-\Delta\Delta CT}$ method was normalized with respected to that of GAPDH.
37
38
39
40
41
42
43
44
45
46

47 *Transfection and Western blot analysis*

48
49 JEG-3 and JAR culture in 6-well plates were transfected with either ASPP2 and ASPP1 construct
50 (generous gift from Prof. Xin Lu of Ludwig Institute for Cancer Research, UK) using Lipofectamine
51 2000 (Invitrogen, Carlsbad, CA). The pCDNA 3.1 vector was used as control. Total protein lysate was
52 extracted with lysis buffer (0.125 M Tris, pH 6.8 at 22°C containing 1% NP-40 [v/v], 2 mM EDTA, 2
53 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate and 0.1 μ M sodium okadate). Protein
54
55
56
57
58
59
60

1
2
3 concentration was determined by detergent compatible protein assay (Bio-Rad Laboratories, Hercules,
4 CA). Twenty μg of protein was resolved by SDS-polyacrylamide gel electrophoresis and then
5 transferred to polyvinylidene difluoride membrane and probed with corresponding antibodies [25].
6
7 Primary antibodies used in this study were listed in Table 1.
8
9

10 11 12 *TdT-Mediated dUTP Nick End Labeling Assay*

13
14 TdT-mediated dUTP nick end labeling assay (TUNEL) was performed using an In Situ Cell Death
15 Detection kit (Roche Biochemical, Indianapolis, IN) as previously described [21]. The number of
16 TUNEL-positive cells in different controls and in JEG-3 and JAR after ASPP2 transient transfection
17 was counted in three different fields at X40 magnification by fluorescence microscopy.
18
19
20
21
22
23

24 *Silencing of Csk by small interfering RNA (siRNA)*

25
26 To knockdown Csk in choriocarcinoma cell line, Silencer® select Pre-designed siRNA of Csk
27 (siCsk) (Cat: 4427037; ID:s3614) and non-targeting Negative Control #2 (scramble) siRNA (Cat:
28 43900846) were used (Ambion, Austin, TX). Co-transfection with 5 nM Csk-specific/non-targeting
29 siRNA and empty vector pCDNA3.1/ ASPP2 construct in Lipofectamine 2000 (Invitrogen, Carlsbad,
30 CA) was carried in 6-well culture plate. To assess the knockdown efficiency, Western blotting was
31 performed using the protein lysate harvested 48 h post-transfection.
32
33
34
35
36
37
38
39
40
41
42

43 *Wound healing assay*

44 Migration of the cells was determined by wound healing assay. Cells were first transfected with
45 specified plasmid constructs or siRNA oligo for one day in twelve-well plates with 90% confluence.
46 The treated cell monolayer was then scratched with a sterile 200- μl pipette tip. Fresh culturing
47 medium was added. Photos were retaken at the same position of the wound after 48 hours. Results
48 expressed in percentage to control were defined as the average percentage change in linear wound
49 closure in treatment with respect to that in control.
50
51
52
53
54
55

56 *Statistical analysis*

57
58
59
60

1
2
3 Statistical analysis was performed using the Statistical Package for Social Science (SPSS) 15.1.
4
5 Non-parametric unpaired t-test (Mann-Whitney test) was used for continuous data. Spearman's rho
6
7 test was used for correlation analysis. *P* values <0.05 were considered as statistically significant.
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

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 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 $2.6 \pm 1.9\%$ to $10.5 \pm 2.7\%$ in JEG-3 (Figure 2A) and 3.5 ± 2.9 to

1
2
3 14.0±3.7% in JAR (Figure 2B) cells in contrast to control respectively (Figure 2), suggesting that loss
4 of proapoptotic ASPP2 could attenuate apoptosis in choriocarcinoma cells.
5
6

7 By wound healing assay, slower migration rate was found in JEG-3 transfected with ASPP2 than
8 those transfected with ASPP1 or the control (Figure 3A, left panel). Our results demonstrated, for the
9 first time, that ASPP2 negatively regulate cell migration in choriocarcinoma. Furthermore, western blot
10 analysis also showed an ASPP2-specific induction of E-cadherin expression (Figure 3A, right panel).
11
12
13
14

15 16 17 18 *ASPP2-specific inactivation of Src*

19
20 In view of the multi-functional nature of ASPP2 with effect on both apoptosis and cell migration
21 in choriocarcinoma cells, possible interaction between ASPP2 and Src signaling pathway was
22 evaluated. Activation and inactivation of Src is known to be tightly regulated by phosphorylation at two
23 sites with opposite effects. Phosphorylation site at Tyr416 (Y416) in the activation loop of the kinase
24 domain contributes to augmentation of Src activity. In contrary, Csk-mediated phosphorylation of
25 Tyr527 (Y527) at C-terminal tail of Src results in a closed structure, through intramolecular interaction
26 with its SH2 domain, that diminishes the access of substrates to the kinase domain and prevent Y416
27 autophosphorylation [26]. As shown in Figure 3B, overexpression of ASPP2 in both JEG-3 and JAR
28 could reduce the expression level of Src-pY416, an activated form of Src. However, such observation
29 was not found in cells transfected with ASPP1, another member of the ASPP family exerting tumour
30 suppressive effect. Moreover, complementary increased phosphorylation at inhibitory domain Src-
31 pY527 accompanied with ectopic ASPP2 expression was also demonstrated. The total form of Src
32 remained unchanged after ASPP2 or ASPP1 transfection.
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48

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

50
51 The above findings suggested that ASPP2 may negatively regulate cell migration through
52 Csk/Src signaling pathway in choriocarcinoma cells. To unveil the mechanistic action of this potential
53 interaction, ASPP2 and Csk-specific siRNA were cotransfected in JEG-3 cells for downstream
54 analysis. As shown in figure 4A, in the presence of scramble siRNA, ectopic ASPP2 expression
55
56
57
58
59
60

1
2
3 inactivate Src as indicated by suppressing the expression of Src-pY416 and was also able to induce
4 E-cadherin expression in JEG-3. In contrast, such inactivation and induced E-cadherin expression
5 were abolished when the JEG-3 cells underwent co-transfection of ASPP2 and Csk siRNA. More
6
7 importantly, inhibitory effect on choriocarcinoma cell migration by ectopic ASPP2 was also eliminated
8
9 after Csk-specific knockdown as demonstrated in wound healing assay (Figure 4B).
10
11

12
13 As the Y527 inhibitory phosphorylation site is mediated by Csk, our results demonstrated that
14
15 Src could be inactivated specifically by ASPP2 through the action of Csk with effects of cell migration.
16
17

18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3 in both the nucleus and cytoplasm was observed, respectively. Such findings suggested a possible
4 interaction between cytoplasmic ASPP2 protein and gene products that are responsible for cellular
5 functions other than apoptosis, and may therefore account for the aggressiveness of choriocarcinoma
6 or PSTT.
7
8
9

10
11 Although *Drosophila* homolog of ASPP (dASPP) has been reported to interact with dCsk to
12 regulate dSrc kinase to maintain epithelial integrity [16], biological significance regarding interaction
13 between Src signaling pathway and ASPP family members are not available in humans. Our data on
14 RNA interference and Western blot analysis clearly showed that ectopic ASPP2 could inactivate Src
15 signaling in a Csk dependent manner in choriocarcinoma cells and reported for the first time the ability
16 of ASPP2 to negatively regulate cell migration through the Csk/Src axis. Indeed, a wide body of
17 evidences has shown that activated Src kinase endows migratory phenotype through interaction with
18 a number of downstream signaling pathways including FAK, paxillin, ERK as well as internalization of
19 E-cadherin [18,33,34]. As a result, ASPP2 mediated cell migration through Csk/Src axis is noteworthy
20 to be further investigated in cancer biology.
21
22
23
24
25
26
27
28
29

30
31 Metastasis is a multi-step process and is usually initiated by detachments of tumour cells from
32 primary sites [35,36]. E-cadherin is an extensively studied cell adhesion molecule which mediates cell
33 adhesion in a homotypic manner [36]. Suppression of E-cadherin expression is frequently involved in
34 human cancers and is considered as one of the early events of tumourigenesis [37]. Our lab has
35 earlier reported that E-cadherin is down-regulated by hypermethylation in GTD and choriocarcinoma
36 has the lowest level of expression [38]. Interestingly, increased Src activity is known to reduce cell-cell
37 adhesion by promoting the internalization and ubiquitin-mediated protein degradation of E-cadherin
38 [39,40]. Moreover, Src specific inhibitor PP2 enhanced E-cadherin expression at transcript and
39 protein levels [41]. These actions exemplify the upregulation of E-cadherin through reduced protein
40 degradation and increased transcript expression upon Src inactivation. Consistent with ASPP2-
41 specific interaction with Csk/Src axis demonstrated in this study, E-cadherin expression could be
42 specifically induced by ectopic ASPP2 in JEG-3 cells whilst this induction was abolished under Csk-
43 knockdown (Figure 4A). Hence, transfected ASPP2 inactivated Src activity, through which E-cadherin
44 was upregulated and subsequently accumulated in the cells. Taken together, loss of ASPP2 could
45 work as an additional mechanism to further down-regulate E-cadherin expression, leading to loss of
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3 cell-cell adhesions and, at least in part, contributing to the aggressive and malignant phenotype of
4
5 choriocarcinoma.

6
7 Unlike ASPP2, another ASPP family member ASPP1 failed to inactivate Src as illustrated by the
8
9 decreased expression of Src-pY416. This observation was consistently demonstrated in both JEG-3
10
11 and JAR cell lines. While it has been proposed that the interaction between ASPP proteins and Src is
12
13 commonly evolutionary conserved from *Drosophila* to humans [16,42], our *in vitro* study clearly
14
15 indicated that ASPP2, but not ASPP1, interact with Csk/Src axis and highlighted an ASPP2-specific
16
17 regulation of cell migration through this signal transduction pathway. Indeed, a recent study showed
18
19 that knockdown of ASPP2 was more effective in promoting the growth of hepatocellular carcinoma
20
21 cells both in soft-agar transformation assay and in nude mice, when compared with that of ASPP1
22
23 [14]. Thus, the loss of ASPP2 might exert a more potent effect than ASPP1 in tumour development
24
25 with respect to the pleiotropic nature of this tumour suppressor.

26
27 In conclusion, we found loss of expression of the multi-functional ASPP2 in choriocarcinoma
28
29 but not the premalignant hydatidiform moles, suggesting its crucial role in tumourigenesis through its
30
31 effect on inhibiting cell migration and promoting apoptosis. An ASPP2-specific inactivation of the
32
33 oncogenic Src in a Csk dependent manner was further demonstrated, which provides a mechanistic
34
35 link between loss of ASPP2 and the aggressive phenotype of choriocarcinoma at molecular basis.

36 37 38 **Funding**

39
40 Areas of Excellence Scheme, Research Grants Council of the Hong Kong Special Administrative
41
42 Region
43
44

45
46 *Conflict of Interest Statement:* None declared.
47
48

49 50 **References**

- 51
52
53 1. Shih, I., et al. (2002) Gestational trophoblastic disease and related lesions. In Kurman, R.
54 (ed.), *Blaustein's pathology of the female genital tract*. Springer, New York, pp. 1193-1247.
55 2. Altieri, A., et al. (2003) Epidemiology and aetiology of gestational trophoblastic diseases.
56 *Lancet Oncol*, **4**, 670-8.
57 3. Shih le, M. (2007) Gestational trophoblastic neoplasia--pathogenesis and potential
58 therapeutic targets. *Lancet Oncol*, **8**, 642-50.
59
60

- 1
- 2
- 3
4. Cheung, A.N., et al. (2009) Pathogenesis of choriocarcinoma: clinical, genetic and stem cell perspectives. *Future Oncol*, **5**, 217-31.
5. Xue, W.C., et al. (2003) Minichromosome maintenance protein 7 expression in gestational trophoblastic disease: correlation with Ki67, PCNA and clinicopathological parameters. *Histopathology*, **43**, 485-90.
6. Li, H.W., et al. (2002) Current understandings of the molecular genetics of gestational trophoblastic diseases. *Placenta*, **23**, 20-31.
7. Feng, H., et al. (2004) Down-regulation and promoter methylation of tissue inhibitor of metalloproteinase 3 in choriocarcinoma. *Gynecol Oncol*, **94**, 375-82.
8. Xue, W.C., et al. (2004) Promoter hypermethylation of multiple genes in hydatidiform mole and choriocarcinoma. *J Mol Diagn*, **6**, 326-34.
9. Asanoma, K., et al. (2003) NECC1, a candidate choriocarcinoma suppressor gene that encodes a homeodomain consensus motif. *Genomics*, **81**, 15-25.
10. Samuels-Lev, Y., et al. (2001) ASPP proteins specifically stimulate the apoptotic function of p53. *Mol Cell*, **8**, 781-94.
11. Mak, V.C., et al. (2011) Downregulation of ASPP1 in gestational trophoblastic disease: correlation with hypermethylation, apoptotic activity and clinical outcome. *Mod Pathol*, **24**, 522-32.
12. Bergamaschi, D., et al. (2004) ASPP1 and ASPP2: common activators of p53 family members. *Mol Cell Biol*, **24**, 1341-50.
13. Lossos, I.S., et al. (2002) Apoptosis stimulating protein of p53 (ASPP2) expression differs in diffuse large B-cell and follicular center lymphoma: correlation with clinical outcome. *Leuk Lymphoma*, **43**, 2309-17.
14. Zhao, J., et al. (2010) Epigenetic silence of ankyrin-repeat-containing, SH3-domain-containing, and proline-rich-region- containing protein 1 (ASPP1) and ASPP2 genes promotes tumor growth in hepatitis B virus-positive hepatocellular carcinoma. *Hepatology*, **51**, 142-53.
15. Bergamaschi, D., et al. (2003) iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. *Nat Genet*, **33**, 162-7.
16. Langton, P.F., et al. (2007) Drosophila ASPP regulates C-terminal Src kinase activity. *Dev Cell*, **13**, 773-82.
17. Kampa, K.M., et al. (2009) New insights into the expanding complexity of the tumor suppressor ASPP2. *Cell Cycle*, **8**, 2871-6.
18. Yeatman, T.J. (2004) A renaissance for SRC. *Nat Rev Cancer*, **4**, 470-80.
19. Cheung, A.N., et al. (2004) Metastatic trophoblastic disease after an initial diagnosis of partial hydatidiform mole: genotyping and chromosome in situ hybridization analysis. *Cancer*, **100**, 1411-7.
20. Lai, C.Y., et al. (2004) Analysis of gestational trophoblastic disease by genotyping and chromosome in situ hybridization. *Mod Pathol*, **17**, 40-8.
21. Chiu, P.M., et al. (2001) Apoptotic activity in gestational trophoblastic disease correlates with clinical outcome: assessment by the caspase-related M30 CytoDeath antibody. *Histopathology*, **38**, 243-9.
22. Cheung, A.N., et al. (1999) Immunohistochemical and mutational analysis of p53 tumor suppressor gene in gestational trophoblastic disease: correlation with mdm2, proliferation index, and clinicopathologic parameters. *Int J Gynecol Cancer*, **9**, 123-130.
23. Siu, M.K., et al. (2010) p21-Activated kinase-1 promotes aggressive phenotype, cell proliferation, and invasion in gestational trophoblastic disease. *Am J Pathol*, **176**, 3015-22.
24. Chan, H.Y., et al. (2008) Activated Stat3 expression in gestational trophoblastic disease: correlation with clinicopathological parameters and apoptotic indices. *Histopathology*, **53**, 139-46.
25. Siu, M.K., et al. (2008) Overexpression of NANOG in gestational trophoblastic diseases: effect on apoptosis, cell invasion, and clinical outcome. *Am J Pathol*, **173**, 1165-72.
26. Bjorge, J.D., et al. (2000) Selected glimpses into the activation and function of Src kinase. *Oncogene*, **19**, 5620-35.
27. Knofler, M. (2010) Critical growth factors and signalling pathways controlling human trophoblast invasion. *Int J Dev Biol*, **54**, 269-80.
28. Lunghi, L., et al. (2007) Control of human trophoblast function. *Reprod Biol Endocrinol*, **5**, 6.
29. Wong, S.Y., et al. (1999) Apoptosis in gestational trophoblastic disease is correlated with clinical outcome and Bcl-2 expression but not Bax expression. *Mod Pathol*, **12**, 1025-33.
30. Fong, P.Y., et al. (2005) Mcl-1 expression in gestational trophoblastic disease correlates with clinical outcome: a differential expression study. *Cancer*, **103**, 268-76.

- 1
- 2
- 3 31. Kobayashi, S., et al. (2005) 53BP2 induces apoptosis through the mitochondrial death
- 4 pathway. *Genes Cells*, **10**, 253-60.
- 5 32. James, J.L., et al. (2005) Cytotrophoblast differentiation in the first trimester of pregnancy:
- 6 evidence for separate progenitors of extravillous trophoblasts and syncytiotrophoblast.
- 7 *Reproduction*, **130**, 95-103.
- 8 33. Guarino, M. (2010) Src signaling in cancer invasion. *J Cell Physiol*, **223**, 14-26.
- 9 34. Webb, D.J., et al. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates
- 10 adhesion disassembly. *Nat Cell Biol*, **6**, 154-61.
- 11 35. Geiger, T.R., et al. (2009) Metastasis mechanisms. *Biochim Biophys Acta*, **1796**, 293-308.
- 12 36. Makrilia, N., et al. (2009) Cell adhesion molecules: role and clinical significance in cancer.
- 13 *Cancer Invest*, **27**, 1023-37.
- 14 37. Hirohashi, S. (1998) Inactivation of the E-cadherin-mediated cell adhesion system in human
- 15 cancers. *Am J Pathol*, **153**, 333-9.
- 16 38. Xue, W.C., et al. (2003) Methylation status and expression of E-cadherin and cadherin-11 in
- 17 gestational trophoblastic diseases. *Int J Gynecol Cancer*, **13**, 879-88.
- 18 39. Fujita, Y., et al. (2002) Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of
- 19 the E-cadherin complex. *Nat Cell Biol*, **4**, 222-31.
- 20 40. Palacios, F., et al. (2005) Lysosomal targeting of E-cadherin: a unique mechanism for the
- 21 down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions. *Mol Cell*
- 22 *Biol*, **25**, 389-402.
- 23 41. Nam, J.S., et al. (2002) Src family kinase inhibitor PP2 restores the E-cadherin/catenin cell
- 24 adhesion system in human cancer cells and reduces cancer metastasis. *Clin Cancer Res*, **8**,
- 25 2430-6.
- 26 42. Langton, P.F., et al. (2009) The dASPP-dRASSF8 complex regulates cell-cell adhesion
- 27 during *Drosophila* retinal morphogenesis. *Curr Biol*, **19**, 1969-78.
- 28
- 29
- 30
- 31
- 32
- 33
- 34
- 35
- 36
- 37
- 38
- 39
- 40
- 41
- 42
- 43
- 44
- 45
- 46
- 47
- 48
- 49
- 50
- 51
- 52
- 53
- 54
- 55
- 56
- 57
- 58
- 59
- 60

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): 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) 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 μ m. (G): Bar chart illustrating the reduced nuclear immunohistoscoring 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

1
2
3 analysis on the effect of ectopic ASPP1 and ASPP2 expression on Src phosphorylation at
4 autophosphorylation domain Y416 (activated state) and inhibitory domain Y527 (closed structure).
5
6
7
8
9

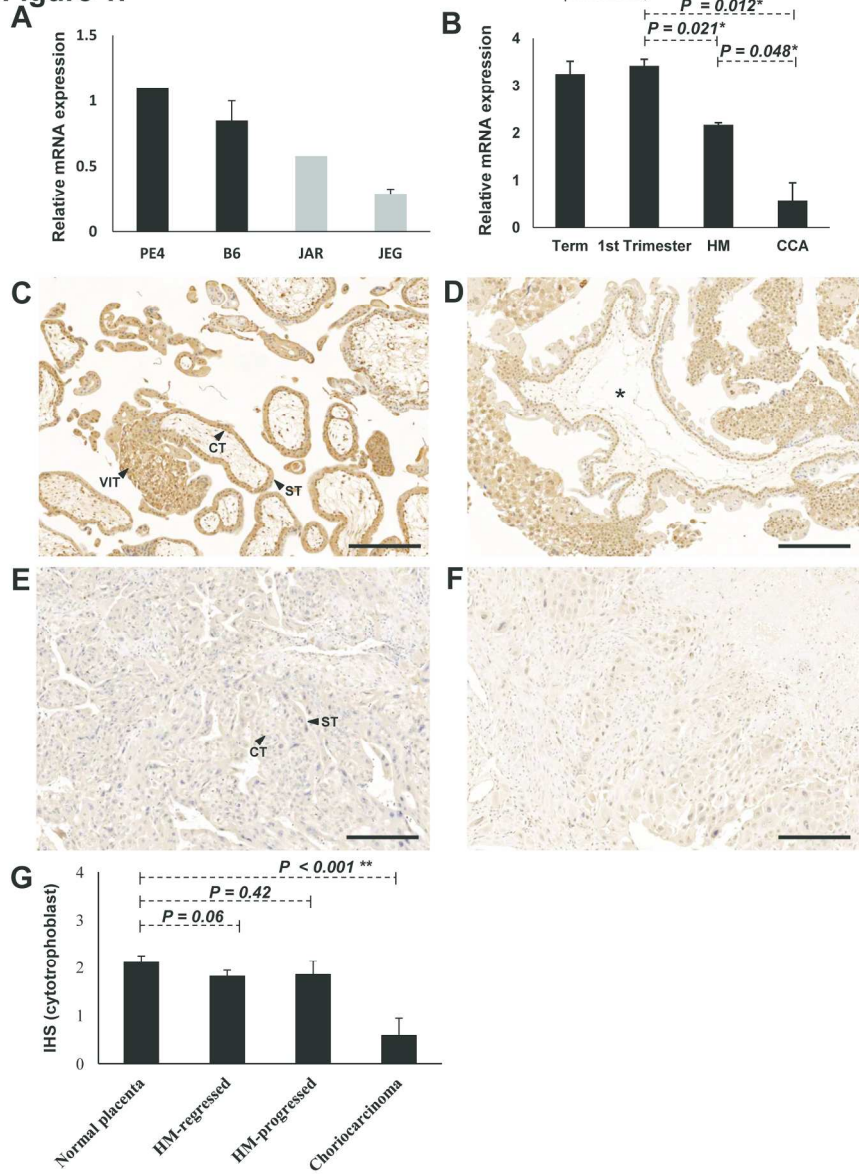
10 **Figure 4** ASPP2 inactivates Src through Csk. (A): Csk-knockdown abolished the effect of ectopic
11 ASPP2 on Src inactivation. (B): Bar chart showing effect of Csk-knockdown on cell migration of
12 ASPP2 transfected JEG-3 cells as determined by wound healing assay.
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

For Peer Review

Table 1 Primary Antibodies Used for Immunoblotting

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)

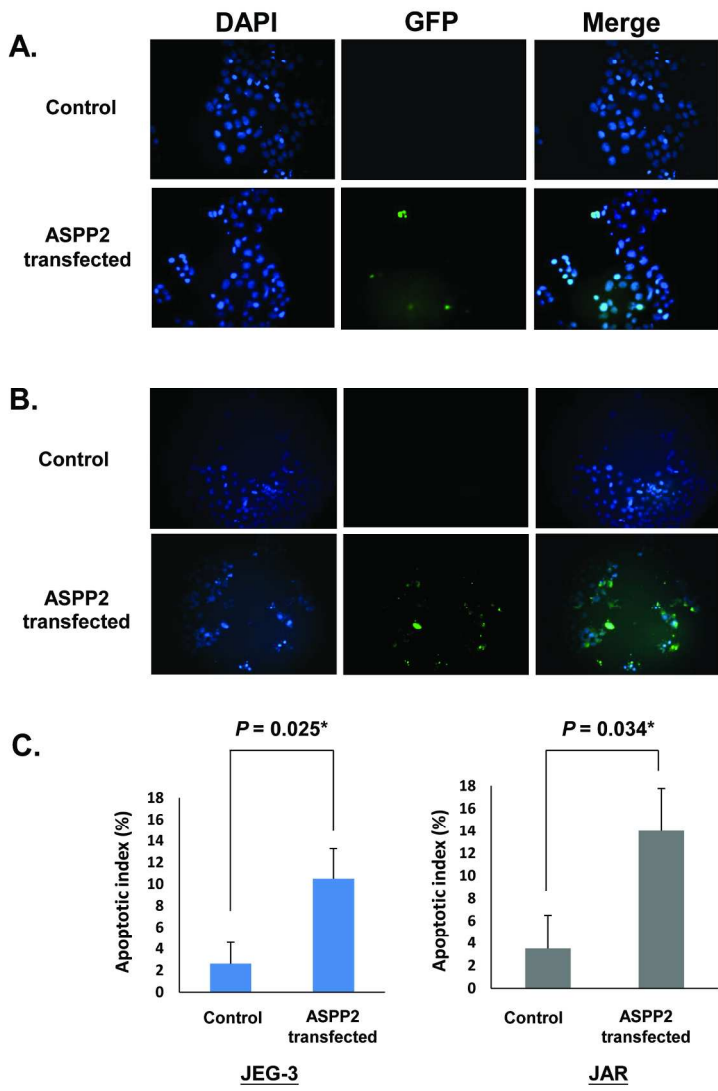
Figure 1.



180x240mm (300 x 300 DPI)

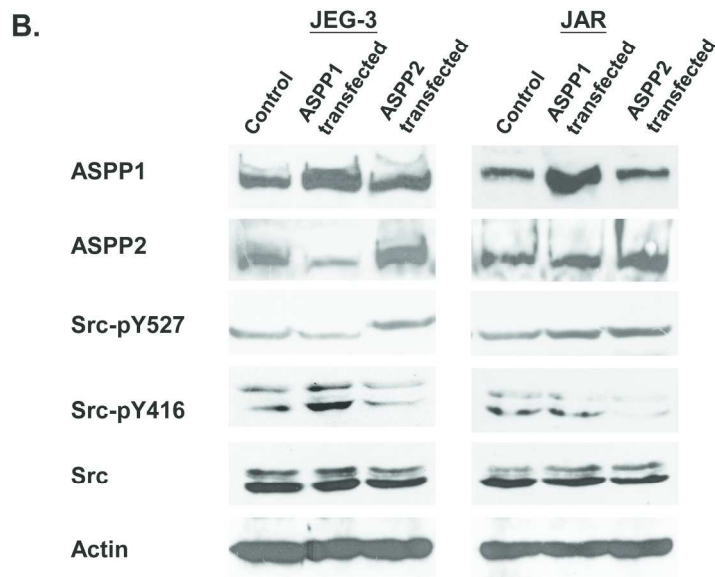
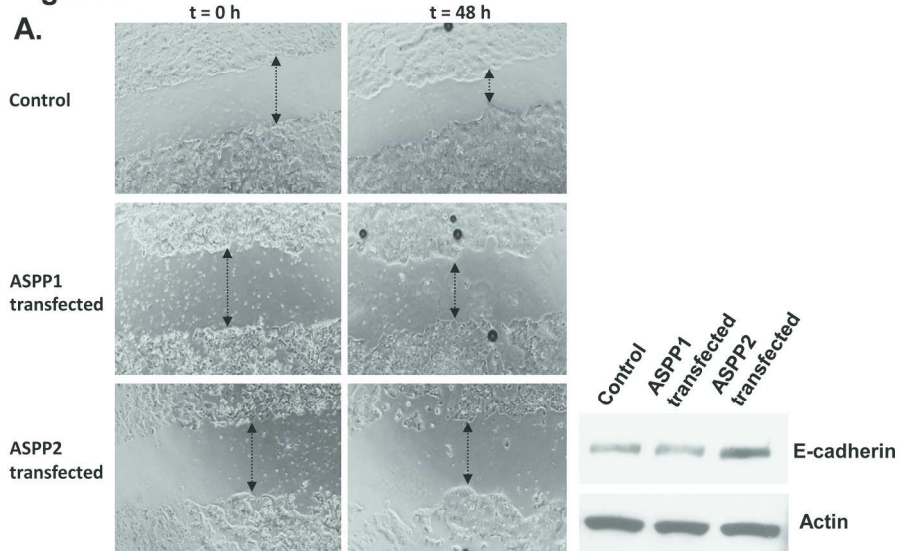
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 2.



180x240mm (300 x 300 DPI)

Figure 3.

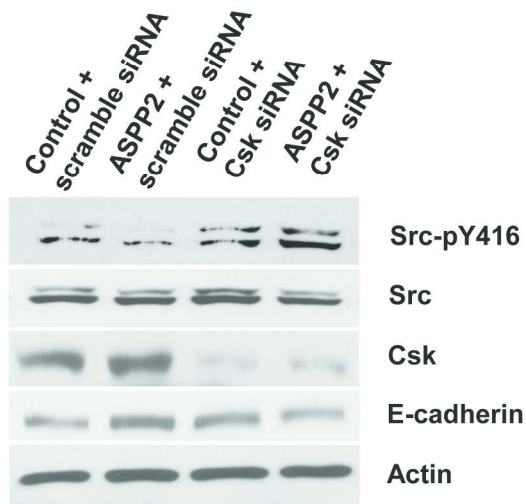


180x240mm (300 x 300 DPI)

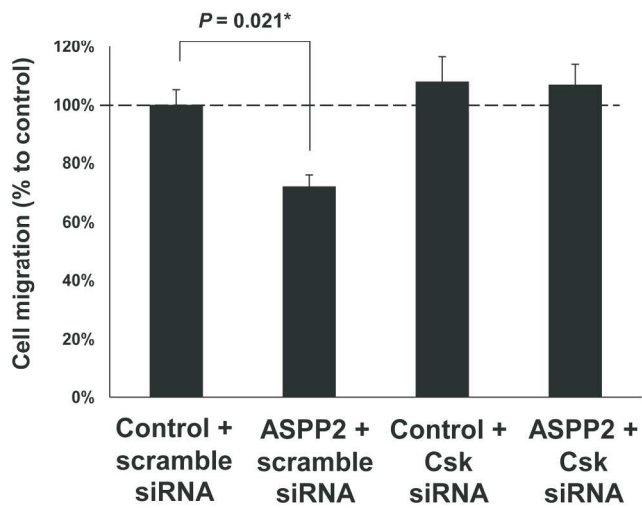
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

Figure 4.

A.



B.



180x240mm (300 x 300 DPI)