Increased MAGEC2 Expression Renders Resistance to Apoptotic Death Through Sustained Activation of STAT3 in Suspension Cultured Tumor Cells

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Abstract

Background

Melanoma-associated antigen C2 (MAGEC2) is an oncogene associated with various cancer types. However, the biological function of MAGEC2 in circulating tumor cells is unclear. In this study, we investigated the role of MAGEC2 using adapted suspension cells (ASCs), which were previously developed to study circulating tumor cells (CTCs).

Methods

Differential gene expression between adherent cells (ADs) and ASCs was examined using RNA-seq analysis. MAGEC2 expression was assessed using RT-qPCR, immunoblotting, and ChIP-seq analysis. Depletion of MAGEC2 expression was carried out using siRNA. MAGEC2-depleted ADs and ASCs were used to investigate the change in proliferation rate and cell cycle. Then, the protein levels of STAT3, phosphorylated STAT3, and downstream of STAT3 were measured using control and MAGEC2-depleted ADs and ASCs. The direct effect of active STAT3 inhibition with Stattic in ASCs was also assessed in terms of proliferation and apoptosis. Finally, an Annexin V/7-AAD assay was performed to determine the percentage of apoptotic cells in Stattic-treated cells.

Results

MAGEC2 was highly expressed in ASCs compared to ADs. Depletion of MAGEC2 reduced the proliferation rate and viability of ASCs. To elucidate the underlying mechanism, the level of STAT3 was examined because of its oncogenic properties. Tyrosine-phosphorylated active STAT3 was highly expressed in ASCs and decreased in MAGEC2-depleted ASCs. In addition, when ASCs were treated with Stattic, an active STAT3 inhibitor, they were more sensitive to intrinsic pathway-mediated apoptosis.

Conclusions

High expression of MAGEC2 may play an important role in the survival of ASCs by maintaining the expression of activated STAT3 to prevent apoptotic cell death.

Background

Metastases and cancer recurrence are widely known to be responsible for the majority of cancer-associated deaths [1]. Circulating tumor cells (CTCs) are derived from the primary solid tumor tissues and circulate in the patient's bloodstream and their fate is death or survival at the secondary tumor sites. Thus, it is considered that survival of CTCs is a critical event for the success of metastatic spread of tumor cells [2]. Nonetheless, the underlying survival mechanism and functional characterization of CTCs are not well known due to their extreme rarity. To overcome this hurdle and to obtain continuously growing tumor cells in suspension, we previously cultured MDA-MB-468 cells in ultra-low attachment plates [3], and the cells were cultured for over 6 months and named adapted suspension cells (ASCs) [4].
In addition, MDA-MB-468 parent cells were named as adherent cells (ADs). ASCs exhibit increased expression of silent mating-type information regulation 2 homolog 1 (SIRT1), which contributes to the survival of ASCs by repressing nuclear factor kappa B (NF-κB) and maintaining low levels of reactive oxygen species (ROS). In the present study, we aimed to elucidate other molecular mechanisms that might affect the survival of ASCs.

Melanoma-associated antigen (MAGE) proteins are classified as cancer-testis antigens (CTAs), which are expressed only in germ cells of the testis and are aberrantly expressed in various human cancers. The MAGE family can be divided into type I and type II MAGEs. Both type I and type II MAGEs share a conserved MAGE homology domain [5]. Type I MAGEs are composed of three subfamilies (MAGE-A, -B, and -C subfamily members) and are all located on the X chromosome [6]. On the other hand, type II MAGEs are composed of seven subfamilies (MAGE-D, -E, -F, -G, -H, -L subfamilies, and Necdin), which are expressed in various tissues and are not limited to the X chromosome [7]. Owing to their unusual characteristics, MAGEs are thought to be ideal targets for cancer therapy [8]. However, the underlying mechanism and biological function of MAGEs remain unclear. MAGEC2, a member of type I MAGEs, is expressed in various cancer types including breast cancer, prostate cancer, and multiple myeloma. In breast cancer, MAGEC2 expression is associated with poor clinical prognosis and EMT [9, 10]. In multiple myeloma, MAGEC2 promotes proliferation independent of p53 [11]. In addition, MAGEC2 in prostate cancer has been elucidated as a predictor of recurrence and a potential target for cancer therapy [12]. A recent study performed using a melanoma cell line revealed that MAGEC2 expression induces rounded morphology in vitro and promotes metastasis in vivo via activation of signal transducer and activator of transcription 3 (STAT3) [13]. Although several studies have documented the oncogenic function of MAEC2 in cancer cells, the biological function of MAGEC2 in CTCs has not been elucidated.

Signal transducer and activator of transcription (STAT) family is one of the most well-known transcription factor families [14]. Among them, the function of STAT3 has been extensively studied as a central mediator of cancer metastasis and a potent target of anticancer drugs [15]. STAT3 upregulates cyclin D1, c-Myc, and Bcl-2 to maintain the survival of tumor cells [16]. STAT3 also advances metastasis by upregulating EMT-associated genes such as twist, snail, slug, vimentin, MMP2, and MMP9. Various cytokines including interleukin-6 (IL-6) and interleukin-10 (IL-10), growth factors, and oncogenic proteins activate STAT3 [17] through the induction of tyrosine phosphorylation at the 705 residue of STAT3 (Y705) by Janus kinases (JAKs), upon binding of ligands to their receptor [18]. The tyrosine phosphorylated STAT3 promotes homodimerization and translocates to the nucleus, where it forms a complex with some coactivators and binds to the promoter region of the target genes involved in tumorigenesis and cancer progression [15]. In this study, we show that overexpression of MAGEC2 in ASCs contributes to the stable accumulation of activated STAT3, thereby contributing to the prevention of apoptotic cell death in ASCs.

**Methods**

**Cell culture**
The human breast cancer cell line, MDA-MB-468, was purchased from the American Type Culture Collection (ATCC, VA, USA). A suspension cell line was established following a previously published method [3], and the cells were cultured for over 150 passages using ultra-low attachment plates (Corning, NY, USA). Both ADs and ASCs were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (HyClone, UT, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Equitech-Bio, TX, USA) at 37°C and incubated under 5% CO₂ humidified atmosphere.

**RNA-seq analysis**

MDA-MB-468 ADs and ASCs were used for the analysis. ASCs were used at passage 155. Total RNA was extracted and sequencing of the produced library was conducted by LAS Inc. (Seoul, Republic of Korea).

**PCR and Real-time polymerase chain reaction (RT-qPCR) analysis:** Total RNA was prepared using RNAiso Plus (TaKaRa, Shiga, Japan) following the manufacturer’s instructions. Reverse transcription was performed using M-MLV Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). AccuPower PCR PreMix (Bioneer, Daejeon, Korea) was used for the PCR amplification and SYBR Green (Thermo Fisher Scientific) was used for RT-qPCR. The primers used for PCR amplification were as follows: MAGEA1 forward (5’- GCTTTACGGGACAGGCAAC-3’), MAGEA1 reverse (5’- AGGATCTGGACACCAGGCTTG-3’), MAGEC2 forward (5’- GTGACGAACCTGGGATGAGG-3’), MAGEC2 reverse (5’- TGGGATGCTGTGACATTCC-3’), β-actin forward (5’- GTGAGGCGCCACGCGACCA-3’), β-actin reverse (5’- CTCTATGGCAGGACCA-3’).

The primers used for RT-qPCR amplification were as follows: MAGEA1 forward (5’- AACTGACACCAGCCTCTGT - 3’), MAGEA1 reverse (5’- ATGAAGACCACACAGGACAT - 3’), MAGEC2 forward (5’- GTGACGAACCTGGGATGAGG-3’), MAGEC2 reverse (5’- TGGGATGCTGTGACATTCC-3’), 18srRNA forward (5’- AGCTATCAATCCTGTCAATCC-3’), 18srRNA reverse (5’- CCTAATTGACTCAACACGGGA-3’).

**Chromatin immunoprecipitation sequencing / analysis**

The cells were fixed using 1% formaldehyde for 10 min and 0.125 M glycine was used to terminate crosslinking. Nuclei were isolated using Farnham lysis buffer (5 mmol/L PIPES pH 8.0, 85 mmol/L KCl, 0.5% NP-40, and protease inhibitor) and then fragmented. Fragmented chromatin (1 mg) was immunoprecipitated with 10 µg H3K27ac, 4 µg H3K4me3 (Millipore, Temecula, CA, USA), and 10 µg RNA Pol II (Abcam, Burlingame, CA, USA). The human reference genome hg19 was used for the alignment of sequenced reads. Integrative Genomics viewer genome browser and HOMER software were used for peak calling and visualization.

**Immunoblotting**
Cell lysates were mixed with 5X SDS sample buffer and heated at 99 °C for 10 min. Whole cell lysis loaded samples were loaded onto 10% SDS-PAGE gels, electrophoresed at 140 V and 400 mA for 1 h, and then transferred to 0.45 µm nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK) for 2 h. The membranes were blocked using TBST buffer (20 mmol/L Tris-HCl pH 8.0, 150 mM NaCl pH 7.6, 0.05% Tween20) with 3% bovine serum albumin (BSA) for 30 min. Next, the membranes were incubated with each specific primary antibody at 4 °C overnight. The membranes were washed and incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. The protein bands were visualized using ECL solution and detected using a LAS3000 luminescent imager (Fuji Film, Tokyo, Japan). Analysis and processing of the image was carried out using FUSION software.

**Cell proliferation assay**

Each cell was seeded in triplicate in classic 6-well or ultra-low attachment plates. The cell numbers were counted during each time period manually using hemocytometer cell counting with trypan blue staining.

**Antibodies and reagents:** The following antibodies and reagents were purchased: Phospho-Stat Antibody Sampler Kit # 9914T, Death Receptor Antibody Sampler Kit #8356, Caspase-3 #9662S, cleaved caspase-3 #9664, MMP-9 #3852S, and Survivin #2808S (Cell Signaling Technology, Danvers, MA, USA); Anti-MAGEC2 antibody #ab209667 (Abcam, Cambridge, MA, USA); cyclin D1 #sc-718, BID #sc-11423, β-actin #sc-58673 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); PARP #51-6639GR (BD Biosciences, Franklin Lakes, NJ, USA); Stattic #S7947 (Sigma Aldrich, St Louis, MO, USA).

**siRNA transfection:** The cells were transfected using RNAiMax (Invitrogen, Carlsbad, CA, USA) with an siRNA concentration of 20 ng/mL. The siRNAs used for MAGEC2 knockdown were as follows: siMAGEC2-1 (5'- CGAGGAACGUAGUGUUCUU-3’) and siMAGEC2-2 (5’- GAUACCGCAGAUGCCAGUGUCA-3’).

**Cell cycle analysis**

The analysis was performed using PI/RNase staining buffer #550825 (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s protocol. The cells were incubated with 500 nM propidium iodide for 15 min and then analyzed using flow cytometry.

**Apoptosis detection by Annexin V/7-AAD assay**

Cells at a density of 5 × 10^5 cells/well were plated in a 6-well plate the day before treatment. The control group was treated with 1000X DMSO, while the experimental group was treated with a final concentration of 2 µM Stattic. The apoptosis assay was performed 48 h after Stattic treatment. The assay was performed using Annexin V, Annexin V-FITC /PI #556570 and 7-AAD #51-68981E (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol.

**Statistical analysis**
Multiple comparisons were analyzed with a one-way factorial ANOVA. Individual group mean differences in the main effect were determined using Student’s t-test. \( p < 0.05 \) was considered as the threshold. (*\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \)).

Results

ASCs express high levels of the cancer testis antigen MAGEC2

We previously reported that high SIRT1 expression is associated with the downregulation of reactive oxygen species (ROS) in ASCs, which is a key event for the survival of ASCs [4]. To systemically identify other signaling molecules responsible for the survival of ASCs, differential gene expression between ADs and ASCs was examined using RNA-seq analysis. As a result, 1352 genes were found to be differentially expressed with more than a 2-fold change. Among differentially expressed genes, several MAGE genes were observed (Table 1). Although the expression of CTA genes is restricted in reproductive organs, some genes are expressed in tumor tissues. Thus, we focused on elucidating the role of MAGE in ASCs. First, the differences in MAGEA1 and MAGEC2 expression between ADs and ASCs were measured. Consistent with the results of RNA-seq analysis, ASCs showed a significant increase in MAGEA1 and MAGEC2 expression (Fig. 1A), and the amount of protein was also increased along with the increase in mRNA (Fig. 1B), suggesting that MAGE is increased to carry out their proper role in ASCs. In this study, we focused on MAGEC2 as it is highly increased in ASCs. To examine whether promoter regional activation is a reason for the increase in expression, chip-seq analysis was performed using antibodies against the promoter opening markers H3K4me3 and H3K27Ac. While the promoter region of ADs was closed, the promoter region of MAGEC2 was highly occupied by RNA polymerase II with an opening by modification of H3K4me3 and H3K27Ac (Fig. 1C).
Table 1
The list of significantly up-regulated genes in ASCs.

| Gene        | Description                                                                 | log$_2$FC | p-value     |
|-------------|------------------------------------------------------------------------------|-----------|-------------|
| SERPINB10   | serpin peptidase inhibitor, clade B (ovalbumin), member 10                   | 13.7      | 0.1868566  |
| UGT1A10     | UDP glucuronosyltransferase 1 family, polypeptide A10                        | 10.7      | 0.4226497  |
| DCAF4L2     | DDB1 and CUL4 associated factor 4-like 2                                     | 9.63      | 0.00275    |
| SOHLH2      | spermatogenesis and oogenesis specific basic helix-loop-helix 2             | 9.22      | 0.0003949  |
| MAGEC2      | melanoma antigen family C, 2                                                | 8.91      | 0.0006793  |
| GULP1       | GULP, engulfment adaptor PTB domain containing 1                             | 8.5       | 0.0124106  |
| EPHB1       | EPH receptor B1                                                              | 8.18      | 0.0005359  |
| GPRIN3      | GPRIN family member 3                                                        | 8.01      | 0.0011616  |
| ERMN        | ermin, ERM-like protein                                                     | 7.73      | 0.0121122  |
| SOD3        | superoxide dismutase 3, extracellular                                       | 7.34      | 0.333986   |
| MAGEA1      | melanoma antigen family A, 1                                                | 4.96      | 2.37E-04   |

Gene expression was compared between ADs and ASCs by RNA-seq analysis. Listed genes are highly up-regulated in ASCs as described. FC = Fold Change

Deficiency of MAGEC2 induces apoptosis in ASCs

To understand the role of increased MAGEC2 expression, it was reduced by siRNA treatment. Both the designed siMAGEC2 efficiently reduced MAGEC2 expression (Fig. 2A). When the cell number was measured after treatment with siMAGEC2, the ASCs showed a 50% decrease in cell number and the ADs also showed a decrease in cell number. However, the ratio of decrease in ASCs was higher than that in ADs (Fig. 2B). It is likely that the increase in MAGEC2 is more highly associated with the proliferation rate of ASCs than that of ADs. To investigate whether the decrease in the proliferation rate of siMAGEC2-treated ASCs was due to changes in the cell cycle, siMAGEC2-treated ASCs were stained with propidium iodide and then analyzed to examine the distribution of each phase of the cell cycle using flow cytometry. Interestingly, ASCs showed an abnormally high level of cell population with polyploidy, in which the cells have increased DNA contents of more than 4N (Fig. 3A) and the ratio of the subG1 phase, which represents apoptosis-derived fractional DNA content, markedly increased in MAGEC2-depleted ASCs (Fig. 3B), indicating that reduction of MAGEC2 expression results in apoptosis in ASCs. As reported in previous studies [19], it appears that polyploidy cells in the absence of MAGEC2 expression underwent apoptosis, resulting in an increase in the subG1 phase. Taken together, these data show that the
suspension culture could induce polyploidization, and the reduction of MAGEC2 expression results in the induction of apoptosis.

**MAGEC2 is involved in the activation of STAT3 in ASCs**

To further investigate the molecular mechanisms by which MAGEC2 plays a role in preventing ASCs from apoptosis, the binding partners of MAGEC2 were searched in the PubMed database. Among them, STAT3 is generally known as an essential mediator of cancer metastasis and invasion. Therefore, the basal protein levels of total STAT3 and tyrosine 705-phosphorylated STAT3 (pY-STAT3), which is the active form of STAT3, were examined in ADs and ASCs. No difference was observed in the total amount of STAT3, while pY-STAT3 expression was significantly increased in ASCs (Fig. 4A). To examine whether MAGEC2 affects the phosphorylation of STAT3, protein levels of STAT3, pY-STAT3, and serine 727-phosphorylated STAT3 (pS-STAT3) were determined in ASCs treated with siMAGEC2. MAGEC2 downregulation resulted in a comparable reduction in the level of pY-STAT3 (Fig. 4B), but not in pS-STAT3, confirming the previous study that MAGEC2 accumulates active STAT3. Next, the protein levels of the STAT3 downstream target genes, cyclin D1, MMP-9, and survivin, were measured in MAGEC2-depleted ADs and ASCs. Although STAT3 is activated in ASCs, no difference in the expression pattern of target genes between ADs and ASCs was observed (Fig. 4C). The reason why the expression levels of STAT3 target genes were similarly detected in Ads, in which MAGEC2 expression was not observed, was intriguing. To solve this discrepancy, cell lysates of only ADs were analyzed through over-exposure of immunoblot to determine whether MAGEC2 expression was detected. As a result, it was found that ADs express low amounts of MAGEC2, and treatment with siMAGEC2 effectively reduced the level of MAGEC2 (Fig. 4D). Taken together, these data suggest that other target genes of STAT3 may be involved in MAGEC2-associated STAT3 function in ASCs.

**ASCs are highly sensitive to apoptosis by inhibiting active STAT3**

Since no difference in the expression of the target protein of STAT3 between ADs and ASCs was observed, active STAT3 was suppressed by treatment with the active STAT3 inhibitor Stattic to investigate the role of active STAT3 in ASCs. When ADs and ASCs were treated with 2 μM of Stattic, a significant difference in the cell number was detected. On day 1 after the treatment, proliferation of Stattic-treated ASCs decreased by over 50% compared to the control ASCs, while Stattic-treated ADs decreased by approximately 25% compared to that of the control ADs (Fig. 5A). On day 2, the viability of Stattic-treated ASCs decreased dramatically, indicating that ASC is more sensitively affected by the inhibition of STAT3 activation compared to ADs. Since the inhibition of STAT3 activation results in a dramatic decrease in cell number, it was determined whether the treatment with Stattic induces apoptosis. ASCs treated with Stattic showed highly increased protein levels of cleaved caspase-3, caspase-7, and PARP compared to ADs (Fig. 5B). In addition, the level of inhibitor of apoptosis protein XIAP was significantly reduced in Stattic-treated ASCs, indicating that ASCs are more susceptible to
apoptosis induction compared to ADs when active STAT3 is inhibited. Next, the levels of anti-apoptotic proteins were assessed in ASCs treated with Stattic. It was found that the Bcl-2 family including Bcl-2, Bcl-XL, and Mcl-1 is decreased in Stattic-treated ASCs but not in Stattic-treated ADs (Fig. 5C). Then, the levels of proteins related to extrinsic apoptotic pathway signals were examined and no significant differences were observed (Fig. 5D). Although FAS is increased in ASCs, treatment with Stattic did not affect the level of FAS. Finally, early and late apoptosis were assessed after treatment with Stattic using the Annexin V/7-AAD staining assay. Flow cytometry analysis revealed that the ratio of both early and late apoptotic cells significantly increased in 2 µM Stattic-treated ASCs. The percentage of apoptotic cells increased from 2.2–21.2%, while there was only a slight increase in apoptotic cells in Stattic-treated ADs (Fig. 5E). Conclusively, these results indicate that active STAT3 contributes to resistance to apoptotic signals in ASCs.

Discussion

Metastasis and cancer recurrence remain the major causes of cancer-associated deaths. During EMT and MET, which are prerequisite processes of metastasis, CTCs originating from primary tumor sites are present in the patient’s blood. Thus, a clear characterization of CTCs is necessary to prevent patient death. Several studies have demonstrated that CTCs generally have a short survival time in the patient’s blood [21]. However, CTCs are widely known to be heterogeneous [22], indicating that not all subsets have the same phenotype. Some subsets of CTCs exhibit stemness properties, which are termed as circulating cancer stem cells (CSCs). CSCs possess high invasiveness and metastatic potential [23]. In the present study, we showed that ASCs have characteristics of both CSCs and CTCs. Some subsets of ASCs may also be CSCs. Thus, we believe that ASCs are an excellent culture model to study CTCs containing CSCs.

Cancer testis antigens (CTAs) are suggested as unique potential biomarkers of CSCs because they are more frequently expressed in CSCs compared to that of differentiated cells [24, 25]. We revealed that ASCs show high expression levels of CTAs, including the MAGE family. The MAGE family member showing the highest expression level was MAGEC2 in ASCs derived from MDA-MB-468 cells. It was considered that MAGEC2 overexpression may contribute to the stemness features of ASCs and extend survival in the suspension culture condition. To generalize this concept, we obtained ASCs from several breast cancer cell lines. However, not all ASCs showed high expression levels of MAGEC2 (Additional file 4: Figure S4). In further studies, ASCs derived from luminal A and B types, basal-like type, and triple negative types should be used to more clearly define the role of MAGE family expression.

Accumulating evidence suggests that STAT3 plays a key role in the survival of cancer cells. Recently, a new biological function of STAT3 in breast CSCs has been uncovered. STAT3 activation is required for the maintenance, promotion, regulation, and expansion of CSCs [26, 27]. Moreover, activation of STAT3 protects CTCs by secretion of immunosuppressive factors, thereby protecting CTCs from immune surveillance. Therefore, it is conceivable that STAT3 activation contributes to the survival of CTCs. In this study, the level of active STAT3 significantly increased in ASCs compared to ADs. Inhibition of active STAT3 dramatically induced apoptotic cell death in ASCs. This implies that a higher level of active STAT3
should be maintained in ASCs than in ADs. Thus, an increase in active STAT3 is an important tactic for the survival of cancer cells in suspended status.

A previous study showed that MAGEC2 interacts with pY-STAT3 in the nucleus of cancer cells and inhibits polyubiquitination and proteasome degradation, thereby supporting the maintenance of highly activated STAT3, and that MAGEC2 expression is associated with increased metastasis [13]. In the present study, MAGEC2 inhibition blocked the activation of STAT3 and the expression of STAT3 downstream targets. This result is consistent with a previous study performed using melanoma cell lines. In addition, there was a noticeable difference between ADs and ASCs when treated with an active STAT3 inhibitor. The apoptosis of ASCs was more highly induced by a low dose of inhibitor compared to ADs. Therefore, it can be explained that the increase in MAGEC2 expression in ASCs is essential to maintain increased STAT3 activation for survival.

ASCs have an abnormally high proportion of polyploid populations. Previous studies have shown that polyploid CTCs are frequently detected in diverse types of cancers, and that the number of patients with polyploid CTCs is more distributed to stages III and IV than stages I and II [28]. MAGEC2-depleted ASCs resulted in a reduced polyploidy ratio compared to control ASCs (Fig. 3A). It is known that polyploidy cells undergo apoptosis at a high rate [19]. In a previous study, we found that ASCs undergo rapid apoptosis and proliferation. Therefore, we believe that a high proportion of polyploidy cells in ASCs is associated with rapid apoptosis. Furthermore, the inhibition of apoptosis accumulates polyploid population and apoptosis induced by the intrinsic pathway contributes to the elimination of polyploidy cells [29]. In this study, we demonstrated that high expression of MAGEC2 prevents ASCs from apoptosis, which may be a reason for the accumulated polyploidy population, and depletion of MAGEC2 resulted in the elimination of polyploidy cells, consistent with previous studies.

Furthermore, TCGA (Cancer Genome Atlas) data were evaluated to systematically determine whether MAGEC2 expression is related to the overall survival of breast cancer patients. The survival of the MAGEC2-low patient group was far longer than that of the MAGEC2-high patient group (Additional file 5: Figure S5), indicating that elevated expression of MAGEC2 is associated with poor prognosis in breast cancer. These findings highlight the importance of investigating MAGEC2-associated pathways and suggest that MAGEC2 is a potential target for breast cancer treatment.

**Conclusions**

Conclusively, ASCs exhibit a short survival time, but due to the presence of CSCs in ASCs, ASCs can continue to proliferate. In this study, we demonstrated that MAGEC2 and active STAT3 are highly expressed in ASCs. High expression of MAGEC2 contributes to the maintenance of hyperactivated STAT3 in ASCs, which is an essential survival strategy in the growth environment of ASCs. Therefore, we propose MAGEC2 as a potential therapeutic target for treating breast cancer metastasis.

**List of abbreviations**
CTC, circulating tumor cell; AD, adherent cell; SC, suspension cell; ASC, adapted suspension cell; CSC, cancer stem cell; MAGE, Melanoma-associated antigens; CTA, Cancer-testis antigens; STAT, Signal transducer and activator of transcription; TF, Transcription factor; EMT, Epithelial-mesenchymal transition; MET, Mesenchymal-epithelial transition; ROS, Reactive oxygen species; SIRT, Silent mating-type information regulation; siRNA, Small interfering RNA; pY-STAT3, Tyrosine 705-phosphorylated STAT3; pS-STAT3, Serine 727-phosphorylated STAT3; MMP-9, matrix metalloproteinase-9; XIAP, X-linked inhibitor of apoptosis protein.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used for the present study will be made available from the corresponding author upon reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

DP conceived and designed the study, performed experiments, analyzed data, and prepared the manuscript. SH, HJJ, JYP contributed to the development of cell line used in the study, and study design. HYK, SJS participated in the interpretation of data. YY designed the concept of the study, interpreted the data and finally approved the manuscript. All authors read and approved the final manuscript.

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Figures
Figure 1

MAGEC2 expression in ADs and ASCs. A, Expression levels of type I MAGEs in ADs and ASCs were determined by PCR and RT-PCR. β-actin and 18S rRNA were used for the normalization of the data. B, Cells were plated in a 6-well plate at a density of 5 x 10^5 per well. Protein levels of MAGEA1 and MAGEC2 in ADs and ASCs were analyzed by immunoblot assay. Full-length gels and blots are presented in additional file 1: Figure S1. Visualization of H3K4me3, H3K27ac and RNA Pol II ChIP-seq near MAGEC2 gene.

Figure 2

MAGEC2 inhibition suppresses proliferation of ASCs. A, Both ADs and ASCs were transfected with siControl or siMAGEC2 in the concentration of 20 ng/mL for 48 h. The efficiency was evaluated by immunoblot assay. Full-length blots are presented in additional file 1: Figure S1. B, Cells were plated in a 6-well plate at a density of 2 x 10^5 per well. Numbers of cells were counted at 24 h and 48 h post treatment of siRNAs (20 ng/mL). Cell counting was carried out in a triplicate. Bars represent the mean ±
SD. One way ANOVA and Student’s t-test were performed to assess the statistical significance. (p=0.004139234 at the time point of 24 h, while p=1.37929E-11 at 48 h, One way ANOVA. *p < .05, **p < .01, ***p < .0001, Student’s t-test.)

Figure 3

Downregulation of MAGEC2 promotes apoptosis in ASCs. A, ASCs were transfected with siControl of siMAGEC2 and harvested after 48 h. Harvested cells were stained with propidium iodide and subjected to flow cytometry analysis. Representative data are shown in the figure. B, The graph indicates the percentage distribution of subG1 population in ASCs. Bars represent the mean ± SD. (p=2.88E-05, One way ANOVA. **p < .01, ***p < .0001 Student’s t-test)
Figure 4

MAGEC2 contributes to the hyper activation of STAT3 in ASCs. A, Levels of total STAT3 and pY-STAT3 in the basal ADs and ASCs were evaluated by immunoblot assay. B, Cells were transfected with siControl or siMAGEC2 for 48 h and levels of STAT3, pY-STAT3 and pS-STAT3 were evaluated. C, Protein levels of STAT3 downstream target genes, Cyclin D1, MMP-9 and Survivin, were examined by immunoblot assays in cells treated with siControl and siMAGEC2. D, Depletion of efficiency of siMAGEC2 in ADs was evaluated by over-exposure immunoblotting. Full-length blots are presented in additional file 2: Figure S2.
Figure 5

Inhibition of active STAT3 results in induction of apoptotic cell death in ASCs. A, Cells were plated in a 6-well plate at a density of 2 x 10^5 per well. 2 μM of stattic or DMSO were treated to each well and the number of cells was counted at 24 h and 48 h after treatment. Cell proliferation assay was carried out in triplicate. (p= 1.52E-08 at the time point of 24 h, while p= 1.65891E-10 at 48 h, One way ANOVA. **p < .01, ***p < .0001, Student’s t-test.) B, Levels of cleaved caspase-3, -7, PARP, cleaved PARP and XIAP in cells treated with 2 μM and 5 μM of Statistic were determined by immunoblot assay. C, Levels of Bcl-2 family members, Bcl-2, Bcl-X, and Mcl-1 in ADs and ASCs treated with the indicated concentration of Statistic were examined by immunoblot assay. D, Levels of death receptor signaling related proteins in stattic-treated cells were investigated by immunoblot assay. Full-length blots are presented in additional file 3: Figure S3. E, ADs and ASCs were plated in a 6-well plate at a density of 5 x 10^5 per well and treated with 2 μM of Statistic for 48 h. Harvested cells were stained with Annexin V/7-AAD for 15 min and subjected to flow cytometry analysis.

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