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Research Article

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How mesenchymal stem cell conditioned media affect the HeLa cells on Wnt/beta-catenin signaling, Notch-1 signaling, and apoptosis?

Mezenşimal kök hücre süpernatanı HeLa hücrelerinde Wnt/beta-katenin, Notch-1 sinyal yollarını ve Apoptozisi nasıl etkiler?

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ABSTRACT

This study aims to investigate the influence of mesenchymal stem cells (MSCs) cell-conditioned media (MSCs-CM) on the Wnt/beta-catenin and Notch-1 signaling as well as the apoptosis in cervical cancer cells. Conditioned media of characterized MSCs were freshly collected and filtered before use. HeLa cells cultured standard conditions and treated with MSCs-CM 24, 48, 72 hours. Untreated cells serve as a control. Cell viability measured with MTT assay for all incubation periods. Immunocytochemical staining of beta-catenin, Notch-1 and cleaved caspase 3 were performed for each time-point. MTT cell viability, AO/PI, and immunocytochemical staining of cleaved caspase 3 results showed that through all incubation periods, there was no statistically significant difference between the MSCs-CM treated HeLa cells and the controls ($p>0.05$). Beta-catenin immunoreactivity was upregulated following treatment from 24 hours to 48 and 72 hours ($p<0.001$), however a significant decrease in Notch-1 receptor expression after MSCs-CM treatment independent of time ($p<0.001$). This study demonstrated that MSCs-CM has no cytotoxic and proliferative effects on HeLa cells. However, treatment with MSCs-CM enhanced the activity of Wnt/beta-catenin signaling via the accumulation of beta-catenin and decreased Notch-1 signaling in HeLa cells. Further analyses that identify regulatory factors of these pathways may provide promising opportunities for cervical cancer therapy.

Key Words

Mesenchymal stem cell, beta-catenin, notch-1, cancer therapy, cleaved caspase 3, apoptosis.

ÖZ

Çalışmamızda, mezenşimal kök hücre süpernatanının (MSCs-CM) Wnt/beta-katenin ve Notch-1 sinyal yolu ile apoptosis üzerindeki etkilerini servikal kanser hücrelerinde (HeLa) araştırmayı amaçlanmıştır. Karakterize edilmiş mezenşimal kök hücreler (MSCs)'den elde edilen MSCs-CM, kullanımdan önce taze olarak toplanmış ve süzülmüştür. HeLa hücreleri standart koşullarda MSCs-CM ile 24, 48, 72. saat kültüre edilmiştir. Uygulama yapılmayan hücreler kontrol olarak kullanılmıştır. Tüm inkübasyon dönemleri için hücre canlılığı MTT analizi ile yapılmıştır. Hücreler, her inkübasyon periyodunda beta-katenin, Notch-1 ve aktif kaspaz 3 ile immünositokimyasal olarak işaretlenmiş ve analiz edilmiştir. MTT hücre canlılığı analizi, AO/PI ve aktif kaspaz 3'ün immünositokimyasal boyaması ile elde edilen sonuçlar, tüm inkübasyon süreleri boyunca, MSCs-CM ile tedavi edilen HeLa hücreleri ve kontrol arasında istatistiksel olarak anlamlı bir fark olmadığını göstermiştir ($p>0,05$). Beta-katenin immünoreaktivitesi, tedaviyi takiben 24. saatten 48 ve 72 saate artış gösterirken ($p<0,001$), bununla birlikte, MSCs-CM tedavisinden sonra Notch-1 reseptör ekspresyonunda zamandan bağımsız olarak önemli bir azalma olduğu belirlenmiştir ($p<0,001$). Bu çalışma, MSCs-CM'nin HeLa hücreleri üzerinde sitotoksik ve proliferatif etkileri olmadığını göstermiştir. Bununla birlikte, MSCs-CM ile tedavi, beta-katenin birikimi yoluyla Wnt/beta-katenin sinyal yolu aktivitesini arttırdığı ve HeLa hücrelerinde Notch-1 sinyalini azalttığı gözlemlenmiştir. Bu yolların düzenleyici faktörlerini tanımlayan daha ileri analizler, serviks kanseri tedavisi için umut verici fırsatlar sağlayabilir.

Anahtar Kelimeler

Mezenşimal kök hücre, beta-katenin, notch-1, kanser terapi, aktif kaspaz 3, apoptozis.

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INTRODUCTION

Cervical cancer is the fourth most common malignancy in women with a worldwide incidence of more than half a million per year. Cervical cancer has a death toll of more than 265000 every year pointing to a less than 60% 5-year survival [1]. Pap smear and Human papillomavirus (HPV)-DNA screening programs, as well as the vaccination, reduce cervical cancer incidence and mortality in developed countries. However, the mortality in developing countries is still remarkably high [2]. The therapy modality (surgery combined with radiotherapy and/or chemotherapy) is depicted by the stage, size, location, and type of cervical cancer [3].

HPV infection is a major predisposing factor in the etiology of cervical cancer. The estimated worldwide prevalence of HPV among women is 11.7% and the incidence of HPV positivity in Turkey was found to be 3.5% (35517/1000000) according to the first results of the community-based HPV-DNA screening program [4,5]. The fundamental mechanisms of viral oncogenesis in mucosal cells have not been fully understood [6,7]. Previous reports showed that HPV E6 and E7 have several targets to modulate the cell cycle, apoptosis, cell polarity, and cytoskeletal organization [8,9]. Among several signaling cascades that impact cancer progression, activation of the Wnt/beta-catenin signaling in HPV-infected keratinocytes leads to overexpression of cyclin D1, c-myc, and c-jun leading to cell cycle control towards cancer progression [10-12]. Furthermore, HPV E6 directly targets several members of the Wnt/beta-catenin signaling pathway to modulate the cytoplasmic/nuclear level of beta-catenin [10,13]. Besides the Wnt/beta-catenin signaling, uncontrolled action of the notch pathway may also further enhance the proliferation of cancer cells [14]. Notch signaling promotes malignant conversion by increasing proliferation, epithelial-mesenchymal transition (EMT), angiogenesis, and blocking apoptosis via upregulating of myc or PKB/Akt pathway [15].

Mesenchymal stem cells (MSCs) are multipotent stromal cells that reside in a variety of somatic tissues such as the bone marrow, adipose tissue, umbilical cord, and peripheral blood [16]. Due to the diverse multipotency of these cells, comprehensive studies further aim to utilize MSCs for regenerative therapy [17]. MSCs are the sources of several secretory mediators that account for their anti-inflammatory properties as well as contributing to accelerated tissue regeneration [18,19]. Studies including us have reported secretion of soluble factors as well as several cytokines and chemokines including Vascular endothelial growth factor (VEGF), Insulin-like growth factor 1 (IGF1), C-X-C Motif Chemokine Ligand 12 (CXCL12), Interleukin 4 (IL4), IL10 that contribute to the therapeutic potential of MSCs [20-22]. It has been elucidated that the MSCs may impact the microenvironment through these secreted factors at the site of tumor growth as well as injury and repair. This modulation may be through either inhibitory or stimulatory aspects [23]. Thus, there is a need for the identification of the specific targets and signal pathways of tumor cells affected directly by MSCs or indirectly through their secreted factors. The aim of this study is to investigate the influence of MSCs' conditioned media (MSCs-CM) on Wnt/beta-catenin and Notch-1 signaling as well as the apoptosis in HeLa cells. This study revealed that the MSCs-CM induces beta-catenin and inhibits Notch-1 expression.

MATERIALS and METHODS

Cell culture experiments

Human bone marrow-derived MSCs were obtained and characterized as described our previous report in accordance with the Hacettepe University Local Ethical Committee (LUT12/134–16) [22]. Conditioned media of P2 and P3 MSCs were freshly collected and filtered (0.22 µm filters) before use. The effect of MSCs-CM on HeLa cell proliferation was analyzed with MTT assay. Cells were cultured in DMEM/Ham's F12 (DMEM/F12) medium supplemented with 2 mM L-glutamine, 10% fetal bovine serum and 100 IU/ml Penicillin-Streptomycin (Biochrom, Germany). Briefly, HeLa cells were seeded on to 96-well plates at a density of 4×10^4 cells/ml and incubated at 37°C and under 5% CO_2 in air. Following 24 hours of incubation, the medium was replaced with MSCs-CM while untreated cells served as controls. Cells were incubated for 24, 48, and 72 hours. MTT assay was performed for each incubation period using the standard procedures. Absorbance was measured at 570 nm using a microplate reader (EZ Read 400 Microplate Reader, Biochrom, UK). The cell morphology was visualized and documented using an Olympus IX70 inverted microscope equipped with an Olympus DP71 digital camera (Olympus, Japan). In order to visualize cell viability Acridine orange/Propidium iodide (AO/PI) staining was performed. Stained cells were visualized with fluorescence microscopy equipped with fluorescein (FITC) (520-560

nm) and rhodamine filters (510-560 nm) (Olympus IX70, Japan).

Immunocytochemistry

HeLa cells incubated in 96-well plates were fixed with cold absolute methanol for 6 min and standard immunostaining was performed for each time-point (24h, 48h, 72h) [24]. Vectastain Elite Universal ABC kit (Vector Laboratories, USA) was employed for signal visualization. Monoclonal beta-catenin (1:100 dilutions, rabbit mAb, Cell Signaling Technology, USA, Cat#8480S, RRID: AB_11127855), monoclonal Notch-1 (1:100 dilutions, mouse mAb, Santa Cruz Biotechnology, Inc, Cat#sc-373944, RRID: AB_10917738) and monoclonal cleaved caspase 3 (1:400 dilutions, rabbit mAb, Cell Signaling Technology, USA, Cat#9664L, RRID: AB_2070042) antibodies were used.

Briefly, fixed HeLa cells were washed 2 times with Phosphate buffered saline (PBS)-Tween-20. PBS was used in all washing steps. To block nonspecific interactions, normal horse serum was used according to kit instructor. Then, specimens were blocked by using Avidin/Biotin blocking kit (Vector Laboratories, USA). After blocking steps, specimens incubated with diluted primary antibodies. The specimens were then incubated with the universal secondary antibody solution according to kit instructions. After the performing Vectastain reagent, specific chromogen signal was generated using 3,3-diaminobenzidine (Vector Laboratories, USA) and the samples were counterstained with Harris' hematoxylin (MERCK, Germany). The same protocol was applied to the negative controls where the primary antibody was omitted. Normal colon, placenta, and colorectal carcinoma tissues were used as positive controls for cleaved caspase 3, Notch-1 and beta-catenin respectively. Ten randomly selected fields at 20× magnification were captured and cells enumerated using IJ 1.46r version of ImageJ software (Fiji, https://imagej.nih.gov/ij/). For quantification of immunocytochemical staining; all positive and negative cells in view of beta-catenin, Notch-1 and cleaved caspase 3 were counted. The percentage of positive cells was calculated.

Statistics

The Statistical Package for the Social Sciences (SPSS Version 23.0 for Windows; SPSS, Chicago, IL) was used for statistical analysis. All values were expressed as mean±standard deviation of biological replicates. Shapiro-Wilks Normality test was performed to choose the statistical test for the comparison of the groups. Non-parametric Mann Whitney-U test was selected for comparing the treated and untreated groups. To elucidate the significance of time-course changes, Friedman's two-way analysis of variance by ranks was used. A *P*-value cut off of 0.05 was accepted as significant.

RESULTS

Cell viability and apoptosis

MTT cell viability results showed that through all incubation periods, there was no statistically significant difference between the MSCs-CM treated HeLa cells and the controls (p=0.774) (Figure 1). In parallel to MTT results, AO/PI stained cell number did not exhibit any variation over the incubation time. To observe the impact of MSCs-CM treatment of apoptotic cell death of HeLa cells, we investigated caspase 3 activity by using cleaved caspase 3 immunostaining. MSCs-CM treatment of HeLa did not reveal any significant change in cleaved caspase 3 immunoreactivity (p=0.312) (Figure 1, Table 1).

Beta-catenin and Notch-1 immunoreactivity

Beta-catenin immunoreactivity was upregulated following treatment with MSCs-CM from 24h to 48 and 72 hours; respectively (Figure 1). This increase was statistically significant (p<0.001) (Table 1). A time-course analysis also revealed a statistically significant increase (p<0.001) (Table 2).

Notch-1 immunoreactivity was assessed using an antibody against the cytoplasmic tail of Notch-1 receptor. MSCs-CM treatment showed statistically significant decrease in Notch-1 receptor expression (p<0.001) (Figure 1 and Table 1). All of the observation time-points displayed same decrease profile (p=0.273) (Figure 1 and Table 2).

Table 1. Comparison of immunocytochemical expressions of beta-catenin, Notch-1, and cleaved caspase 3 expression in treated and untreated HeLa cells.

Proteins	Treatment	Mean of Percentages Mean ± SD	P-value
Beta-catenin	Untreated Treated	5.61 ± 9.24 9.24 ± 4.63	<0.001*
Notch-1	Untreated Treated	5.57 ± 3.30 2.73 ± 2.28	<0.001*
Cleaved caspase 3	Untreated Treated	2.84 ± 4.23 3.80 ± 7.30	0.312+

*: P<0.001, +: P>0.05

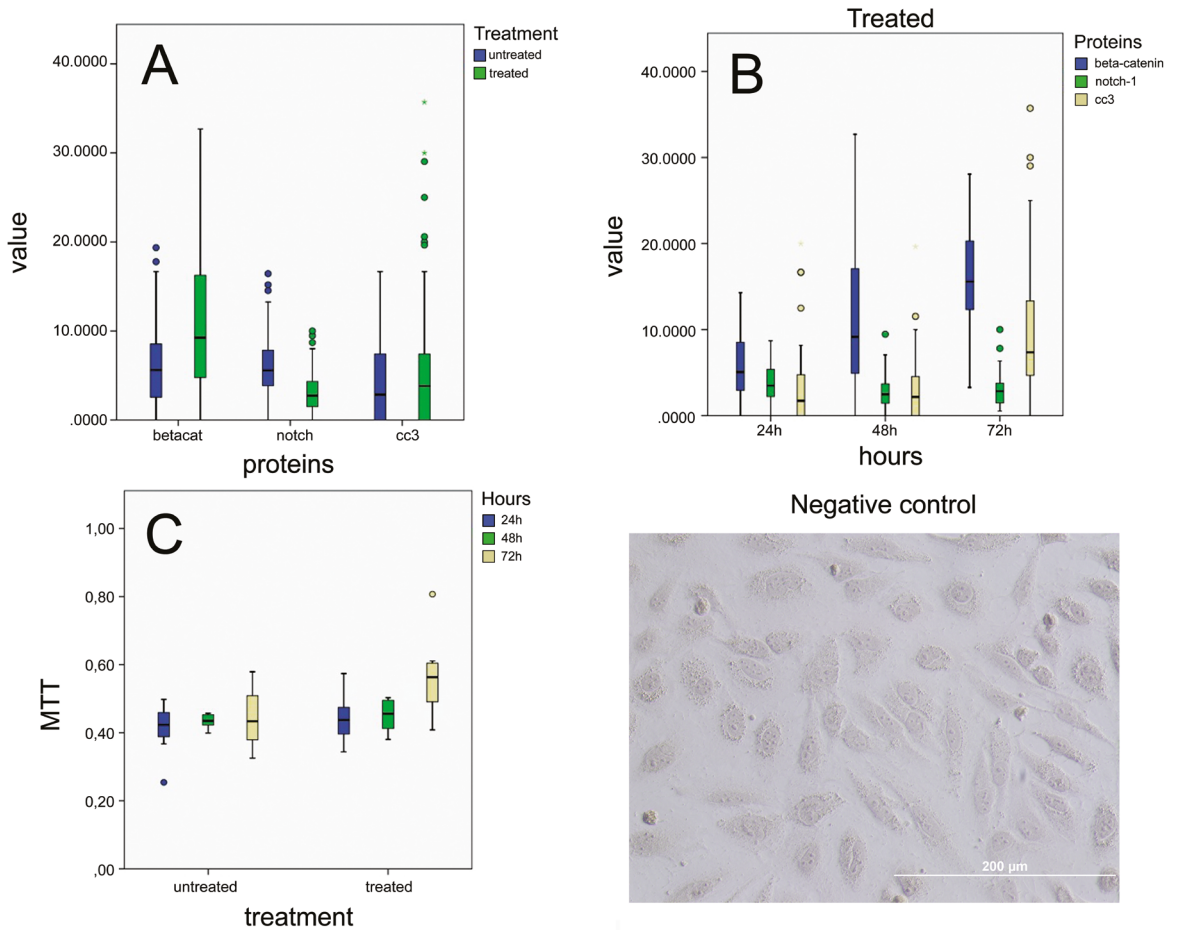

Figure 1. A) Box plot chart of beta-catenin, Notch-1, and cleaved caspase 3 depict the mean of percentages (value: percentages of positively stained cells) in treated and untreated HeLa cells. MSCs-CM treatment shows statistically significant increase in beta-catenin (p<0.001) and decrease in Notch-1 receptor (p<0.001), however the expression of cleaved caspase 3 (CC3) is equivalent in treated and untreated group (p=0.312), B) Box plot chart of beta-catenin, Notch-1, and cleaved caspase 3 expression in treated HeLa cells depends on time, C) MTT (Absorbance) cell viability results show that through all incubation periods, there was no statistically significant difference between the MSCs-CM treated HeLa cells and the controls D) Negative control was performed without primary antibodies (Scale bar: 200 µm, magnification ×20)

DISCUSSION

MSCs-CM shows promising opportunities for the therapy of various diseases including cancer, inflammation-induced tissue damage, and neurodegenerative diseases [25,26]. It has been shown that factors secreted by MSCs-CM influence cell proliferation, differentiation, immunoregulation, as well as regeneration [22,23,25-27].

MSCs are known to differentiate into cancer-associated cells in the vicinity of tumor site and are known to facilitate tumor growth and invasion. Thus, investigating MSC-CM on the behavior of cancer cells is of particular interest [27,28]. Here, we aimed to elucidate the impact of MSCs-CM on beta-catenin and notch signaling for the first time on HeLa cells.

Previous studies addressing the content of MSCs-CM showed that it contained several soluble factors including chemokines, cytokines, and growth factors that may be responsible for the modulatory role of MSCs in inflammation and cell fate determination [29,30]. The impact of MSCs-CM is cell and context-dependent [31]. Here, we did not observe any significant change in HeLa cell proliferation. Likewise, MSCs-CM treatment did not exhibit any change in caspase 3 activation, confirming the cell viability results. Previous reports showed that MSCs could induce apoptosis in activated T-cells and breast cancer cells [32,33]. On the contrary, MSCs inhibited the apoptosis of chondrocytes and neuronal cells [25,34]. Our results suggest that MSCs-CM had no significant effect on the proliferation or apoptosis of HeLa cells.

Table 2. Comparison of immunocytochemical expressions of beta-catenin, Notch-1, and cleaved caspase 3 expression in treated HeLa cells depends on time.

Proteins	Hours	Mean of Percentages Mean ± SD	P-value
Beta-catenin	24 h 48 h 72 h	5.07 ± 3.87 9.16 ± 8.06 15.08 ± 6.34	<0.001*
Notch-1	24 h 48 h 72 h	3.48 ± 2.38 2.48 ± 2.27 2.83 ± 2.17	0.273+
Cleaved caspase 3	24 h 48 h 72 h	1.72 ± 5.60 2.17 ± 4.37 7.36 ± 9.08	<0.001*

*: P<0.001, +: P>0.05

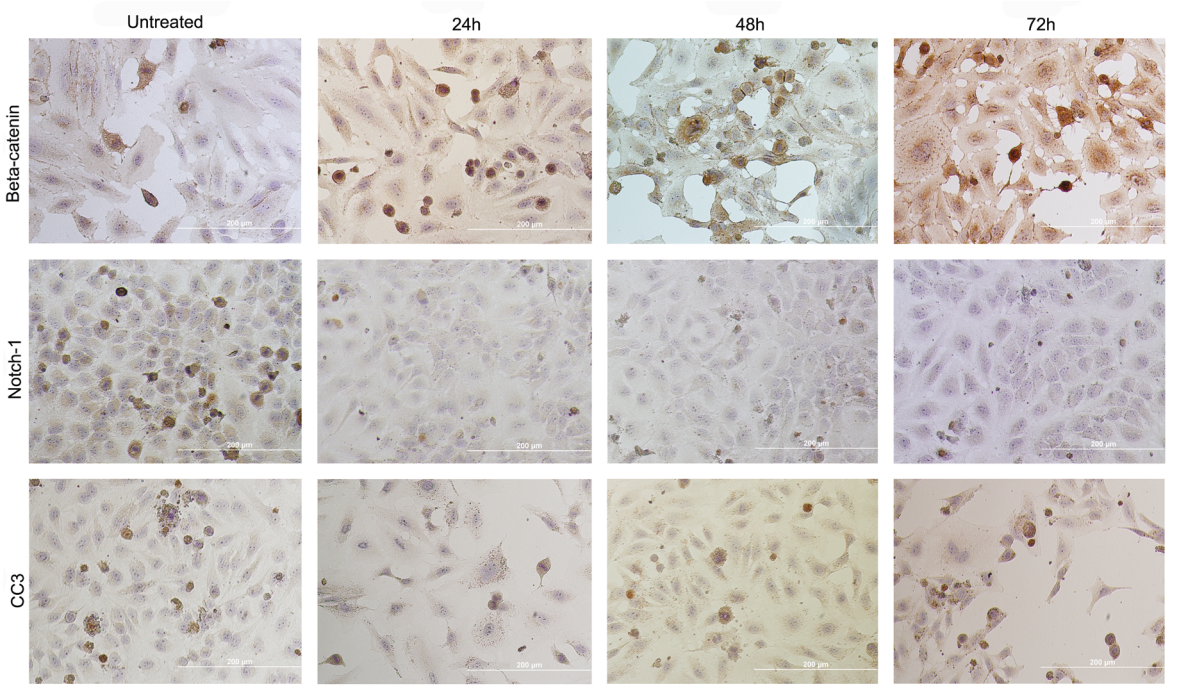

Figure 2. This figure shows the representative immunocytochemical staining results of beta-catenin, Notch-1, and cleaved caspase 3 (CC3) depends on MSCs-CM treatment and time. Expression of beta-catenin is upregulated following treatment with MSCs-CM from 24 hours to 48 and 72 hours, however, Notch-1 was downregulated. CC3 was not changed as a result of MSCs-CM treatment, but the expression of this enzyme was increased following treatment with MSCs-CM as a time-dependent manner (Scale bar: 200 µm, magnification ×20).

Wnt/beta-catenin and Notch-1 signaling pathways regulate cell proliferation, differentiation, and apoptosis. Thus, these pathways must be tightly regulated [35-38]. Aberrant activation of both these pathways is critical for tumor formation and cancer progression in colorectal, cervical, and breast cancers [10,11,35-41]. Here, we demonstrated that MSCs-CM treatment induced Wnt/beta-catenin signaling pathway, but inhibited Notch-1 signaling in HeLa cells. Furthermore, beta-catenin staining was observed both in the cytoplasm as well as the nucleus. This pinpoints that fact that Wnt/beta-catenin signaling was active in HeLa cells. Consistent with our results, previous studies showed that the cytoplasmic and/or nuclear stabilization of beta catenin correlates with the transcription of downstream in the Wnt/beta-catenin signaling pathway genes [42,43].

HPV infection is known to be an initiating factor for the formation of cervical cancer and uncontrolled activation of the Wnt/beta-catenin signaling pathway suggested as the second hit [10,11,44,45]. Here, we use HPV type 18 positive HeLa cells and consistent with previous reports, show strong beta-catenin staining in both cytoplasm and nucleus, confirming the activity of Wnt/beta-catenin signaling in HeLa cells [46] (Figure 2). Genes downstream of the Wnt/beta-catenin signaling pathway such as cyclin D1, c-myc, and c-jun were previously shown to be over-expressed in HPV infected epithelial cells [45].

Downregulation of Wnt/beta-catenin signaling is expected to inhibit tumor cell growth. Etheridge, et al. reported that MSCs secrete Dickopff-1 (Dkk-1) protein for the negative regulation of Wnt/beta-catenin signaling [47]. On the contrary, we observed beta-catenin to be significantly increased following MSC-CM treatment. Previous reports identified Wnt 2, 3a, 4, 5a, 11, and 16 expressions in MSCs [47,48]. Among these, especially Wnt3a has been shown to stimulate the Wnt/beta-catenin signaling pathway strongly. Thus, we suggest the presence of the Wnt activators in the MSCs-CM to stimulate the Wnt/beta-catenin signaling in HeLa cells.

Notch-1 signaling promotes malignant transformation of cells by upregulating c-myc and activating PI3K/Akt signaling pathway as well as stimulating epithelial-mesenchymal transition (EMT) and angiogenesis [15,49,50]. A possible link has been suggested between enhanced

Notch signaling and cervical cancer. Notch-1 is extensively positive in cervical cancer tissues and Notch expression is correlated with an invasive phenotype in high-grade precursor cells [39,51]. Notch-1 silencing induces growth inhibition of HeLa cells in vitro [52]. Consistently, Notch-1 was found to be positive in HeLa cells in this study. Here we observed that MSCs-CM treatment inhibit the Notch-1 signaling in HeLa cells (Figure 2). To the best of our knowledge, this is the first study to provide direct evidence for the modulatory activity of MSCs-CM on the Notch-1 signaling. It has been postulated that Notch-1 signal is active in MSCs and any modulation should be attributed to other related molecules in conditioned media [53,54]. Further descriptive analyses to investigate such regulatory factors need to be pursued. This may be regarded as a future goal as well as a current weakness of this study.

Cross-Talk between Wnt/beta-catenin and Notch-1 signaling in cancer progression has been previously reported [39,55,56]. Activation of Notch-1 signaling pathway may cause loss of membranous and cytoplasmic beta-catenin [55,56]. In this study, as a result of MSCs-CM treatment, we observed an increase in beta-catenin and a decrease in Notch-1 consistent with these results. This suggests that inhibition of Notch-1 signaling may be an important therapeutic target for cervical cancer (Figure 3).

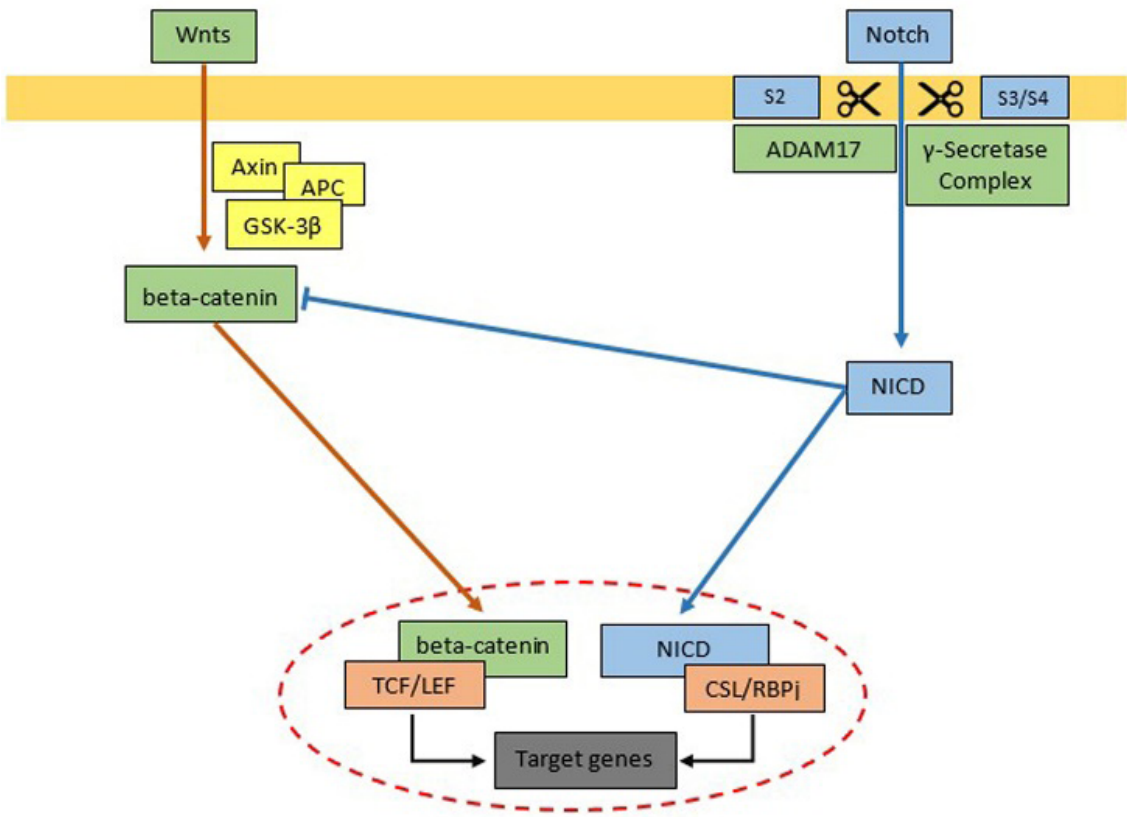

Figure 3. This figure indicated the cross talk between Wnt/beta-catenin signaling and Notch-1 pathway. As seen in figure, when Notch-1 signaling becomes activated, the level of cytoplasmic beta-catenin decreases. Figure was modified from [55].

In conclusion, our results demonstrate that MSCs-CM does not exert a cytotoxic nor growth promoting effect on HeLa cells. However, treatment with MSCs-CM enhanced the accumulation of beta-catenin and decreased Notch-1 in HeLa cells. Further descriptive analyses may further identify these regulatory factors and may provide promising opportunities for cancer therapy.

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