Abstract

**Background:** Wnt signaling pathways are taking a part in regulation of cell fate decisions in normal and cancerous cells. In some cancer types, a transition from canonical to non-canonical Wnt signaling pathways was identified, a phenomenon, that in return led to increase proliferation, invasiveness and metastasis.

**Methods:** In the current in vitro study we investigated the influence of MSCs, co-cultured in direct and indirect contact with OS cells, on the role of Wnt signaling pathways and tumor aggressiveness. Sub-populations were separated using Boyden chambers. Gene expression profiles were determined by qPCR.

**Results:** The results revealed that interactions with MSCs increased migration and invasion capacities along with OS proliferation. Moreover, canonical Wnt signaling activity was low in OS, and co-culture with MSC. However, MSCs did not trigger a switch between the canonical to the no-canonical Wnt pathways. In addition, a more aggressive OS sub-population tend to undergo a transition towards the non-canonical pathway. Moreover, this aggressive subtype presented cancer stem-cells like characteristic.

**Conclusions:** We submit that the progression in OS aggressiveness is attributed to a transition in Wnt signaling from canonical to non-canonical pathways, although MSCs are likely to take a part during the tumor progression, in the case of OS, they did not affect the Wnt switch. These complex tumor promoting interactions may be found in the natural and tumorigenic bone microenvironment. A better understanding of the molecular signaling mechanisms involved in the tumor development and metastasis may contribute to development of new cancer therapies.

Abbreviations

OS: Osteosarcoma; MSC: Mesenchymal Stem Cell; HSC: Hematopoietic Stem Cell; HCC: Hepatocellular Carcinoma; PBS: Phosphate buffered saline; OD: Optical density; TBST: Tween-20 in Tris buffered saline; cDNA: complementary DNA; TBP: TATA-binding protein; qPCR: quantitative real time Polymerase Chain Reaction; SD: Standard Deviation; SEM: Standard Errors of the Mean; RQ: Relative quantity

Introduction

Osteosarcoma (OS) is the most common primary malignancy of the bone, which typically affects long bones of the appendicular skeleton. OS commonly metastasize to the lungs and has poor prognosis in patients [1]. The bone microenvironment is composed of different cell types, including osteoblasts and osteoclasts, and their progenitor cells; mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs), respectively, which together orchestrate bone maintenance and remodeling [2,3]. OS cells secrete different factors, such as CXCL12 and VEGF, that attract and recruit MSCs and endothelial progenitor cells that further support tumor growth [4–7]. In OS microenvironment, MSCs may differentiate into tumor-associated fibroblasts or...
myofibroblasts and secrete various active molecules, including cytokines, chemokines and growth factors that promote cell migration, invasion and angiogenesis [3,8]. However, the exact role of MSCs in cancer development is still controversial [9]; while some researchers link tumor progression to MSCs, including OS [7,10-13], others ascribed tumor suppressive effects to MSCs [14-18]. The complex tumor microenvironment is influenced by the interplay between a variety of molecules for signaling and communication, including Wnt, that are excreted by the multiple cell types therein [19]. Wnt signaling pathways are evolutionarily conserved and control cell fate decisions, proliferation, differentiation, motility, cell polarity, apoptosis, survival and adhesion. The activation of Wnt signaling pathway is initiated by binding a Wnt ligand to Frizzled and co-receptors, which interact with Dishevelled and then activates the pathway [20]. In the canonical Wnt pathway β-catenin accumulates in the cytoplasm, is then translocated into the nucleus, and there participates in the transcription of the target genes c-Myc and Cyclin-D1, which induce cell growth and division [21,22]. In absence of canonical Wnt ligands, intracellular levels of β-catenin are kept low by its degradation [23]. The noncanonical pathways, or the β-catenin independent pathways, can be further divided into the planar cell polarity (PCP) and calcium-dependent pathways. These pathways regulate cytoskeletal remodeling, transcriptional regulation and migration. Noncanonical Wnt signaling expression may react with the β-catenin dependent pathway in a dynamic signaling complex and inhibit canonical TCF/β-catenin-dependent transcription [20]. Aberrant regulation of Wnt signaling may lead to neoplastic proliferation and tumor formation [20,24]. Upregulation of the canonical Wnt pathway resulted in OS tumor progression via reduced cell differentiation levels, increased cell invasion, migration, proliferation, and eventually metastasis [25-28]. Conversely, high expression levels and constant activation of non-canonical Wnt5a/b and Ror2 in OS were conferred to invasiveness and migration [29]. The contrary onset of Wnt signaling pathways during tumor formation and progression was ascribed to the cell differentiation level in hepatocellular carcinoma (HCC), thus well-differentiated cells relied on activation of canonical Wnt pathway, while the more invasive poorly-differentiated cells were associated with expression of noncanonical ligands and repression of canonical Wnt pathway. Therefore, Yuzugullu, et al. (2009) proposed that canonical and noncanonical Wnt pathways have convergent roles in HCC cancer, the canonical pathway contributes to tumor initiation and the noncanonical pathways cause tumor progression [30]. However, the dynamics of canonical and noncanonical Wnt signaling during OS progression, as well as the effects of MSCs on these processes, are not yet fully understood.

**Results**

**MSCs increased tumorigenic potential of OS**

In order to investigate the effect of MSCs on metastatic and tumorigenic properties of OS, we assessed the migration, proliferation and invasion potential. Migration capacity of OS was evaluated during direct and indirect interactions with MSCs, using a wound healing assay on direct co-cultures of OS and MSCs and a transwell system for indirect co-cultures. The examination of the scratch assay images and the subsequent calculation of the migration rates revealed that cells grown in co-culture showed higher motility in comparison with the other two homogenous culture groups (Figure 1A-B). In addition, 76.2±11 % of the cells in the wound frame were determined as red–labeled OS cells (Figure 1C, n=5), which implies that mainly OS cells were responsible for the rapid wound closure. Furthermore, a proliferation rate comparison of all cultures presented an intermediate level for the coculture group, which was exceeded by the proliferation of OS cells alone (Figure 1D). Therefore, the increased migration of the co-culture group was probably not due to increased OS proliferation, but was rather induced by cell–cell interactions. For further investigation of the MSCs–effect on OS cells migration, we established indirect co-cultures using the transwell system. The results revealed that in the presence of MSCs, two times more OS cells crossed the membrane towards the MSCs, in comparison with the control, thus elevating OS motility (Figure 2A). Moreover, the proliferation rate of OS cells was enhanced by the vicinity of MSCs by about 33% (Figure 2B), further indicating an increase in OS aggressiveness after both, direct and indirect contacts with MSCs.

OS cells invasiveness examination was simulated in vitro by a Matrigel matrix, which resembles the basement membrane. Within the assay, invasive cells may enzymatically degrade the Matrigel by proteases secretion, as they invade towards the chemoattractant [33]. The Matrigel–drop assay displayed that twice the amount of OS cells invaded the Matrigel when MSCs were embedded inside the drop (Figure 3A-C). In addition, OS cells invaded for greater distances in presence of MSCs, reaching 700 μm distance from the drop margins with a positively skewed invasion profile, relative to a gradual decrease till 300 μm in the absence of MSCs (Figure 3D). It appeared that OS cells were highly attracted by MSCs, since OS cells entered further into the Matrigel towards the MSCs, and vice versa even some MSCs exited the Matrigel directing towards the OS cells. Noteworthy, the strong OS–MSCs interactions resulted in the phenomena of cell–cell fusion and fusion of extracellular vesical released by MSCs, which could be observed occasionally (Supplementary Figure S1).

Noticing that MSCs affect OS in a paracrine manner, we decided to evaluate gene transcription of tumorigenicity and migration related genes, including the chemokines CCL5 and CCL2, chemokine receptor CXCR4, which binds CXCL12, and S100A4 on an mRNA level. The expression levels of CCL5 increased gradually over the time at all experimental groups, and on days 7 and 10 the expression in co-culture of OS and MSCs was much greater in comparison with the homogenous cultures of each type (Figure 4.A). The expression of CCL5 was about 2.5–fold higher in comparison with OS and about 4–fold higher relative to the MSCs homogenous cultures on day 7 and 10. However, CCL2 expression was almost not detectable in MSCs and was the highest on day 7 in the co-culture compared with the homogenous cultures (Figure 4B). CCL2 expression increased gradually in OS cultures, while in co-cultures an 8–fold increase in CCL2 expression was observed from day
5 to 7 followed by a 4-fold decrease from day 7 to 10. The expression of S100A4 was enhanced in co-cultures on day 10 by 2.6- and 6-fold relative to OS and MSCs homogenous culture respectively, while the lowest S100A4 levels were found in MSCs (Figure 4C). No significant differences were found in CXCR4 expression between the cultures at day 5 and 7 (Figure 4D). A subsequent decline appeared on day 10 in all cultures, which was less expressed in the coculture. Overall, MSCs triggered the proliferation, migration and invasion capacities of OS and upregulated the expression of metastasis and invasion related genes, thus contributing to an elevation of metastatic and tumorigenic potential of OS.

**Wnt signaling activity in co-culture of OS and MSCs**

The gene expression profile of key molecules that participate in Wnt signaling were determined using qPCR, to investigate the contribution of Wnt signaling to OS aggressiveness in the co-culture. mRNA expression of β-catenin, a key player in canonical Wnt signaling, did not show significant differences, neither between cell cultures nor between the culture duration (Figure 5A). During the activation of the canonical pathway, β-catenin accumulates in the cytoplasm, and is then translocated into the nucleus. Therefore, β-catenin levels in the cytoplasmic and nuclear fraction were examined by western blotting showing the highest protein levels for both fractions in MSCs, while low levels were determined in OS alone and in co-cultures (Figure 5B-C, Figure 2S), meaning the activity of canonical Wnt signaling is kept low in the presence of OS cells. The expression of the protooncogene c-Myc, a transcription target gene of Wnt signaling downstream of β-catenin,
increased in OS and co-culture from day 5 to 7, followed by a downregulation at day 10, while in MSCs cultures a gradual decrease was observed (Figure 5D). The expression of Wnt inhibitor factor 1 (WIF1) was downregulated in co-culture on day 7 by 2- and 6- folds, respectively, relative to OS and MSCs homogenous cultures, and 4- fold relative to MSCs on day 10 (Figure 5E), which may promote Wnt signaling activation. In order to elucidate the contribution of specific Wnt signaling pathways to the elevated aggressiveness of OS, gene expression of two ligands, Wnt4 and Wnt5a, widely considered as non-canonical Wnt pathways was assessed. Wnt4 is induced by canonical Wnt signaling and promotes cancer cell proliferation, while Wnt5a is a non-canonical Wnt signaling mediator that is implicated in cell migration and invasion.

Figure 3: OS invasion in Matrigel drops after 3 days of incubation. (A) In presence of DiO-labeled MSCs (green) and (B) in absence of MSCs. The dashed lines mark the Matrigel margins. (C) Quantification of invasive OS cells per Matrigel drop (Error bars represent SD, n=3). (D) Invasion distance profile from Matrigel margins. Error bars represent SEM, n=7, ** p<0.01.

Figure 4: Expression of migratory and tumorigenic related genes in OS and MSCs co-culture and homogenous cultures on day 5, 7 and 10. (A) Relative quantity (RQ) of CCL5 (B) CCL2 (C) S100A4 and (D) CXCR4 gene expression levels. Error bars represent SD, n=3, ** p<0.01, *** p<0.001, **** p<0.0001.

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canonical, and two widely considered canonical ligands, Wnt3 and Wnt3a [20–22] were examined. The expression of Wnt5a increased over time in the cocultures, while it remained almost undetectable in MSCs cultures (Figure 5F). Wnt4 expression did not change over time in the homogenous cultures, but a 70% increment was identified in co-culture on day 7 (Figure 5G). The expression of the canonical Wnt3a and Wnt3 declined in all cultures showing the lowest values on day 10 (Figure 5H–I). Therefore, the results imply that the interactions between MSCs and OS trigger the downregulation of the canonical Wnt ligands expressions and upregulation of the noncanonical Wnt ligands production. This imply that it may be followed by changes in downstream effectors. Interestingly, the transcription levels of most genes in the co-culture group represent average values
of the homogenous groups after short term culturing on day 5, while after longer culture periods, such as 7 and 10 days, different trends could be observed; meaning prolonged co-culture durations (longer than 5 days) are required for cell-cell interactions that affect the transcriptome.

**Wnt signaling effect on metastasis potential of OS**

In order to relate Wnt signaling activity of OS during the processes of metastasis, Wnt related gene expression profile of the high metastatic K7M2 OS was compared with a less metastatic and less aggressive K12 OS cell line, as an earlier developmental stage of OS. We found that the expression of β-catenin and c-Myc were upregulated in K7M2, and the expression of WIF1 was downregulated (Figure 6A–C). Nonetheless, the transcription level of the noncanonical Wnt5a was 4.5-fold higher in K7M2 as compared with K12, while the expression of Wnt4 was lower in the more metastatic cell line (Figure 6D). Concomitantly, the expression of canonical Wnt3a was lower in K7M2, and Wnt3 expression remained unchanged.

**Stem-cell-like sub-populations of OS at different microenvironments**

Given that a tumor is a cellular heterogenic entity, it may be comprised of cell subpopulations including cells with stem-cell-like characteristics, such as improved migration ability. Migrated OS cells were separated from the non-migrated OS cells in presence of MSCs or OS cells culture using the transwell system. The expression of CCL5, CCL2 and S100A4 was upregulated in the migrated OS population as compared with non-migrated cells (Figure 7A–C). Interestingly, migrated cells relative to non-migrated cells in the presence of OS showed a 13-fold, 3-fold and 12-fold rise in CCL5, CCL2 and S100A4 expression, respectively. In the presence of MSCs a 3-fold increase was noticed only for CCL5 expression in the migrated OS cells; CCL2 and S100A4 expression did not differ significantly. Furthermore, comparing the two migrated groups gene expression of CCL5, CCL2 and S100A4 was always about 3-fold higher in the presence of OS than MSCs. In contrast, the CXCR4 expression was downregulated in migrated OS cells regardless the presence of OS or MSC (Figure 7D), which may be due to the cells mobility. In order to elucidate the option that the migrated OS sub-population present cancer stem-cells like characters, we examined gene transcription of two cancer stem-cell markers; Nestin and Oct4 [34]. Nestin expression was upregulated in migrated cells, showing significant differences in presence of OS (Figure 7E). However, the expression of Oct4 was significantly downregulated in the migrated cells fraction, and values were about 350- and 200-fold less when exposed to OS and MSCs, respectively (Figure 7F). Moreover, in the non-migrated OS sub-populations the expression of Oct4 was 40% lower in presence of MSCs than in the presence of OS (Figure 7F). Thus, our results indicate that migrated cells possess greater migratory and tumorigenic potential and show gene expression that may point to the separation of a cancer stem-cell-like subpopulation.

We further examined Wnt activity in the migrated and non-migrated sub-populations. The expression of β-catenin was 15-fold upregulated in migrated cells in the presence of OS, and only 1.7-fold in the presence of MSCs. However,
in the non-migrated cell fraction β-catenin expression was 3-fold higher after exposure to MSCs as compared with OS (Figure 8A). Nonetheless, c-Myc transcription was reduced in the migrated cells by 25- and 43-times in OS and MSCs vicinity, respectively (Figure 8B). Due to exposure to MSCs the expression of β-catenin and c-Myc were 3- and 1.5-times lower, respectively, between the migrated cells groups, which imply on reduced canonical activity. Moreover, WIF1 expression was downregulated by 3-fold in migrated cells, regardless the present cell type, which point to even higher Wnt activity (Figure 8C). The transcription of the determined Wnt ligands was downregulated in the migrated cells relative to the non-migrated cells, except Wnt5a that did not change its expression in the presence of OS (Figure 8D). However, between the nonmigrated groups; the expression of Wnt5a was 3.6-fold higher in the presence of MSCs as compared with OS, while Wnt3a expression was significantly lower and Wnt4 and Wnt3 expressions were of similar levels.

Discussion

The present results showed that MSCs were able to amplify OS proliferation and migration when grown in co-culture, during both, direct and indirect contacts (Figure 1, 2). Proliferation rates of the direct OS and MSCs co-cultures, which started with equal cell amounts, were found to be in an intermediate range. (Figure 1D). However, accelerated OS proliferation rates were found in the indirect co-culture (Figure 2B), which is in agreement with similar results reported, that bone marrow derived MSCs promoted in vitro OS cell proliferation and invasion [35]. In addition, MSCs proliferation was probably reduced concurrently resulting from MSCs differentiation, as was monitored in vivo in tumor-bearing mice by bioluminescence imaging [36]. Furthermore, indirect contacts, as demonstrated by the Matrigel drop experiments, showed that OS cells and MSCs attract each other (Figure 3). This mutual attraction between OS and MSCs was previously identified [7,59] who showed that the paracrine...
effect of CXCL12-CXCR4 and CCL5 was the reason for this convergence. In addition, we observed spontaneous fusions of MSCs and OS cells that may result in advance aggressiveness of the co-cultured cells. This is in agreement with recent reports indicating that cell fusion of MSCs with cancer cells led to an enhanced ability to metastasize in different cancer types [35-37].

We found an upregulated expression of the chemokines CCL5 and CCL2 and the receptor CXCR4 after prolonged culture periods (7 or 10 days) in OS and MSCS co-cultures as compared with the homogenous cultures, supporting the notion that cancer aggressiveness is accelerated by acquiring the necessary molecular tools over time, a process that in our study, was triggered by direct contact with MSCs. In addition, high levels of S100A4 may indicated for a higher tumorigenicity and metastasis potential [38-40].

We found that general transcription levels of β-catenin were comparable between all cell cultures; a fact that was not reflected by nuclear and cytoplasmatic β-catenin protein levels, which were low in both, OS homogenous culture and co-culture after 10 days of culturing, further corroborating the observations that MSCs increased the tumor promoting properties of OS. We found that γ-irradiation levels of β-catenin were comparable between all cell cultures; a fact that was not reflected by nuclear and cytoplasmatic β-catenin protein levels, which were low in both, OS homogenous culture and co-culture (Figure 5A-C). In addition, the mRNA expression levels of β-catenin target gene c-Myc were low at day 10, consistent with nuclear β-catenin levels in co-culture and OS cultures (Figure 5D). Therefore, the canonical Wnt/β-catenin pathway probably did not contribute to the enhancement of OS aggressiveness caused by MSCs and it may occur via secretion of array of chemokine and cytokine and signaling ques that are known to interact with cancer cells. Those results are consistent with previous studies on OS, which indicated the absence of β-catenin in the nucleus and pointed to an inactivation of the canonical Wnt pathway [41-46]. However, opposite trends of β-catenin and c-Myc expression were identified in MSCs cultures in comparison with OS and co-cultures, which may indicate that β-catenin was involved in transcription of other target genes besides c-Myc.

Downregulation of WIF1 expression, a Wnt inhibitor, led to increased tumor growth and lung metastasis in OS, moreover, WIF1 was also found to be a marker of late stage of normal osteoblast maturation, correlating with the OS differentiation state [22,47,48]. Here, we showed low WIF1 expression in OS and co-cultures after prolonged time periods, permitting increased Wnt activity, therefore causing reduced osteogenic differentiation and increased tumorigenicty of OS. The noncanonical Wnt5a was previously found to promote migration, invasiveness, metastasis and tumorigenicty in OS. Moreover, Wnt5a expression correlates with the severity of malignancy, since it is highly expressed in OS and constitutively activates the noncanonical Wnt pathway [29,49-51]. In addition, Wnt5a inhibits Wnt3a-induced canonical Wnt signaling in a dose-dependent manner by downregulating β-catenin transcription activity, rather than affecting β-catenin levels [52]. Even if recombinant Wnt3a protein was added canonical Wnt activity was not restored [43].

Our results showed high Wnt5a mRNA levels in OS and co-cultures, alongside with elevated gene expression of the noncanonical Wnt4 in migrated (Mig+) and non-migrated (Mig-) OS cells. Error bars represent SD, n=3, * p<0.05, **** p<0.0001. (RQ – Relative quantity).
This might be explained by the fact, that on one hand, cancer cells may recruit MSCs by secreting CCL5 and CCL2 and on the other hand MSCs as well secrete those cytokines, which increase metastatic and tumorigenic potential [7,11,53,54]. In a similar way S100A4 may react, which could be secreted from MSCs as it was reported for hepatocellular carcinoma [55]. In our case, MSCs directly interacted with OS, therefore migrated OS cells in the presence of MSCs had no necessity to express high levels of cytokines required for cell recruitment. Conversely, CXCR4 expression was downregulated in migrated OS subpopulation (Figure 7D), which may indicate for low retention to its native bone–marrow microenvironment and high mobilization into the blood circulation as described in different cell models [56–58]. However, the exposure of the sub-population to either OS or MSCs resulted in a similar CXCR4 expression pattern, as previously found in OS [59]. Investigating the migrated OS sub-populations for Wnt signaling activity, we found β-catenin upregulation in migrated OS, which was less expressed when exposed to MSCs (Figure 8A). However, expressions of c-Myc and WIF1 were downregulated (Figure 8B–C), which may point to a reduction of β-catenin transcriptional activity alongside with increased Wnt activity. In disagreement to our results, the proto-oncogene c-Myc has been reported as an essential factor in acquisition of migratory and invasive properties in OS [60–62]. Conversely, low cMyc levels were found in breast cancer and immortalized epithelial cells to enable migration [63,64], as was found in our migrated sub-population of OS. The reduced expression of Wnt ligands in the migrated cells (Figure 8D) suggests that during the migration process, OS cells relay probably on ligands expressed by other cells in their microenvironment in a paracrine manner. Relating increased mobility to stemness in migrated OS sub-population, we found that Nestin was upregulated, while Oct4 was downregulated. The contradictory low Oct4 expression was probably due to increased migratory and invasive properties, similarly to those gained by silencing Oct4 in a breast cancer cell line with low motility [65,66]. In conclusion, we suggest that the gain in OS aggressiveness can be related to a transition between canonical to noncanonical Wnt signaling, which is not apparently intensified in the presence of MSCs via these mechanisms. These tumor promoting interactions may take place in the bone and may further enhance the development of a tumorigenic microenvironment following invasion of metastatic cells. Due to the growing potential in using MSCs in various combination in cell therapy, their role during cancer development should be considered. A better understanding of the complex molecular signaling mechanisms involved in the tumor development and metastasis may contribute to development of new cancer therapies.

Materials and methods

Cell cultures

The following cell lines were used for the experiments: murine MSCs (MSCs, ATCC/CRL 12424) at passages 4–5, murine OS K7M2 wt (ATCC/CRL 2836) previously transfected with mCherry as described [31], and murine OS K12. Cells were treated as described by I Levinger, et al. [67] Co-culture experiments were established by seeding K7M2 and MSCs at a 1:1 ratio, 5x10^3 cells per well, and in addition each cell type was seeded in homogeneous cultures. All the experiments were carried out using K7M2 cell line, the investigation of OS at different metastatic potentials was performed by the comparison of K7M2 cultures with the less aggressive OS cell line K12 [32].

Wound healing assay

Cultures were incubated for 7 days until full confluency was established. A single linear wound was made using p200 pipette tip. The cultures were photographed at the starting point (0 h) and after 24 hours, using a light inverted phase–contrast microscope (Eclipse–Ti, Nikon) connected to LED-based excitation system (CoolLED pE, Life Sciences & Analytical, UK), and fitted with a digital camera (DS–QiMc, Nikon). Cells migration rate was calculated based on the differences of cell coverage between the wound edges and was quantified and the percentage of red–labeled OS cells within the co-culture was assessed using CellProfiler software.

Cell proliferation assay

The cell proliferation levels of the co-culture experimental groups were quantified using XTT cell proliferation kit (Biology Industries, Beit Haemek, Israel) [67]. The proliferation rate was examined on days 1, 3, 4 and 6 post seeding, and the results were normalized to day 1.

Cell-labeling

Transient labeling of MSCs was established using Vybrant® DiO cell –labeling solution (green fluorescence dye, Invitrogen™, USA). Adherent cell cultures were washed with PBS followed by 20 min incubation at 37 °C with 5 µL DiO solution in 1 ml DMEM. Thereafter, the staining solution was removed, and the cells were washed three times with PBS to remove access color. Finally, the cells were detached by trypsinization and used for further experiments.

Invasion assay

The invasion levels of OS cells in presence of MSCs were assessed by the following experiment. DiO–labeled MSCs (2x10^4) were embedded in 50 μL drops of growth factor reduced Matrigel™ (BD biosciences), and 10x10^3 OS cells were seeded around the Matrigel drops. A group without MSCs inside the Matrigel drop served as control. Following 3 days of incubation, OS cells that entered the Matrigel drop were counted and photographed. The invasion profile of OS cells was constructed using NIS–Elements Basic Research (Nikon) and ImageJ (US National Institutes of Health) softwares.

Transwell migration assay

Indirect co–cultures of OS cells and MSCs were established using a transwell assay. OS cells (5x10^3) were seeded in the upper compartment of a Boyden chambers separated by a PET membrane of 8 µm pore size (ThinCerts™, Greiner Bio–One), and 5x10^3 MSCs or OS cells were seeded on the bottom of the culture well. After 7 days of incubation, cell migration and proliferation of OS cells were estimated, as well as the gene expression of the OS subpopulations.
Establishment of OS subpopulations

Based on the migratory capacities of the cells we have used Boyden chambers apparatuses, in order to obtain different subpopulations. The cells were seeded as described above and we collected the cells from the two facets of the mesh. The cells were detached using trypsin and counted. The first group, those that did not penetrate the membrane, (non-migrated) and the second, those cells that crossed the membrane towards the bottom area of the membrane, (migrated). The migrated cells were cultured for additional 4 days RNA prior extraction.

Western blotting

Cells were cultured for 7 days and proteins were extracted using ProteoJet™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas life sciences, Inc.). Total protein concentration was determined by Bradford assay (Bio-Rad laboratories, Inc.) using BSA as a standard. Samples containing equal concentrations of 25 μg proteins in 35 μL were denatured at 100 °C for 5 min in Laemmli sample buffer (Bio–Rad). The proteins were separated on a 10% SDS–polyacrylamide gel by electrophoresis (SDS–PAGE, PowerPac™ basic, Bio–Rad), and electroblotted onto nitrocellulose membranes (Trans–blot transfer medium, Bio–Rad). Even amounts of total proteins loaded on the gel was verified using Ponceau S (0.1% Ponceau in 5% acetic acid, Amresco®). The membranes were blocked with 5% (v/v) non–fat dry milk (in 0.1% (v/v) Tween-20 in PBS). The blotted protein bands were visualized by using ECL (ImageQuant LAS 4000, Life Sciences). The molecular size of β-catenin compared to the protein standards (Precision plus protein Dual Xtra Standards, Bio-Rad), and the second, those cells that crossed the membrane towards the bottom area of the membrane, (migrated). The migrated cells were cultured for additional 4 days RNA prior extraction.

Quantitative real-time Polymerase Chain Reaction (qPCR) analyses

Total mRNA was extracted from cultured cells using SV Total RNA Isolation System kit (Promega Inc.). RNA concentration and purity were determined using spectrophotometrically using NanoDrop™ (2000c, Thermo scientific, Inc.). Complementary DNA (cDNA) was synthesized using qScriptTM cDNA Synthesis kit (Quanta Biosciences™). mRNA expression was determined by qRT–PCR with the SYBR green PCR master mix (Bio–Rad, Los Angeles, CA, USA), for all genes except Oct4 and Nastin, primers with the sequences listed in Table 1. Gene transcription quantification of Oct4 and Nestin was performed using TaqMan® Fast Advanced Master Mix (Applied Biosystems) [67].

Table 1: Primer sequences for quantitative real time polymerase chain reaction (qPCR) analysis of gene expression. (designed and provided by Sigma-Aldrich, Rehovot, Israel).

| Gene   | Direction | Primer sequence (5'-3') |
|--------|-----------|------------------------|
| β-catenin | Forward   | TGGCTGCGAGGATCTCACA    |
|         | Reverse   | TGGGAGAATAAAGGACTGGCA   |
| CCL2   | Forward   | TGATCTCCCTGTAGCTCTCCA  |
|         | Reverse   | CATCCACGTTGTTGCTCA     |
| CCL5   | Forward   | CCTCTCTGCAGCTCTCCTC    |
|         | Reverse   | CAAGTGGCCTACTGGTGCGAG  |
| CXCR4  | Forward   | GAGTCTGCTAGGCAGAAGTGG  |
|         | Reverse   | CAAGAGGAGGAGTGGACAGTCTG |
| c-Myc  | Forward   | TGGCGCTGGAGAATGTCGCC   |
|         | Reverse   | TGTAATTGTGTCACACCTCAC  |
| S100A4 | Forward   | AGCTCTACGGCCTCTTCCCT   |
|         | Reverse   | CCCCTAATCCGTCTGGACACAC |
| TBP    | Forward   | CTGGGAGGCTGCGACTTTAAAGCAG |
|         | Reverse   | GGATGTCAGTCCACTCAGTGG  |
| WiFi   | Forward   | CTTTGGCTGGAAACAGTGGTCTC |
|         | Reverse   | GTGCTTAAGGGATGGTGGCC   |
| Wnt3   | Forward   | AAATTGTGTCGCGTCCGCC   |
|         | Reverse   | TCACACCTCTGGTCAAGT    |
| Wnt3a  | Forward   | ATCTGCTGCTGGTTCCTGTTG |
|         | Reverse   | GGCCAGTAGTCCTCAGTGG   |
| Wnt4   | Forward   | GAAGGTTGACGACAAAAAGGA |
|         | Reverse   | CTGCAAAAGGCCCACACCTG |
| Wnt5a  | Forward   | GAGACACATCACGTAGGGTCTACC |
|         | Reverse   | GTTGACCTGACACAGCTTGC |

Data analysis

All experiments consisted of at least three independent biological repeats, and the results were expressed as specified, using means ± standard deviation (SD) or standard errors of the mean (SEM). Two–tailed unpaired t–test with Welch’s correction, one–way or two–way ANOVA with Tukey’s multiple comparisons were performed as required using GraphPad software, and statistical significance was determined for each experiment.

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