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ANTICANCER GENE DELIVERY VEHICLES TO BONE-METASTASIZED PROSTATE
CANCERS

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14. ABSTRACT Adipose tissue derived MSCs (AT-MSCs) have tumor-homing potential and can be used as anti-cancer gene delivery vehicles to prostate cancer (PCa). Our investigations have elucidated the role of factors released by both tumor cells and tumor-associated stromal cells, in increasing tran-endothelial migration (TEM) and tumor-site specific homing of AT-MSCs. We have identified several cell-surface markers that enable this phenotype of AT-MSCs, and have demonstrated a crucial role of the SDF-1/CXCR4 axis. We have also shown that tumor-derived factors increase differentiation of AT-MSCs and rapidly augment several lineage restricted transcription factor, e.g. PPAR-gamma2 and RUNX2. Our goal is to demonstrate that AT-MSCs can deliver an inducible suicide gene (HSV-TK) to tumor foci which will locally increase ganciclovir (GCV) induced killing of the PCa cells. We have used enriched AT-MSCs stably/transiently expressing the CXCR4 and/or HSV-Tk genes, and have shown their in vitro ability to induce a 'bystander' killing effect in cocultured PCa cells. We have documented the tumor-homing ability of these AT-MSCs in tumor-xenografts in nude mice. We are in the process of investigating their enhanced tumor-homing property and their anti-tumor efficacy in vivo.					
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Introduction:

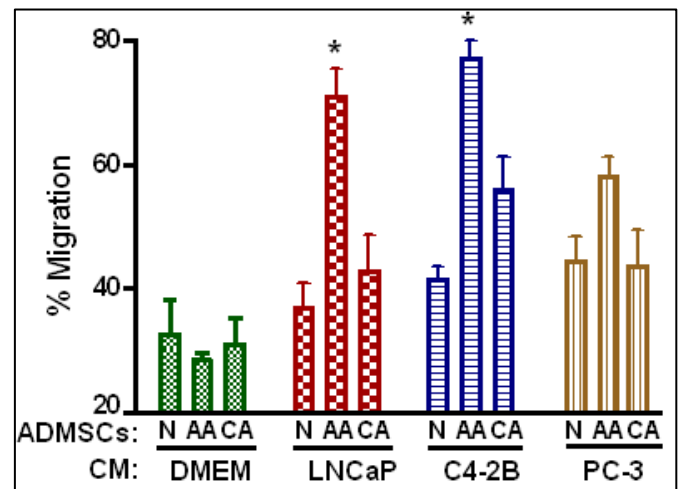
The uncanny ability of mesenchymal stem cells (MSCs) to traverse tissue barriers and seek out Prostate cancer (PCa) cells at metastatic sites, make them attractive candidates as vehicles for anti-cancer gene delivery to metastatic sites. Several recent publications have also demonstrated evidence of tumor-site specific localization of adipose tissue derived mesenchymal stem cells (AT-MSCs) [1-4]. Furthermore, because of their ease of availability from lipoaspirates, as well as their similar rates of propagation and differentiation as compared to bone marrow derived MSCs, the AT-MSCs hold significant promise in combined gene therapy and cellular therapy approaches against cancer. The first objective (*Task-1*) of our project is to investigate whether the tumor-homing potential of AT-MSCs can be enhanced, by either the *in vitro* enrichment of those AT-MSCs which possess higher tumor predilection, and/or their genetic manipulation in order to increase their metastatic site specific localization [5-7]. The second objective (*Task-2*) is to investigate whether the tumor-tropic AT-MSCs can be engineered to express a potent anti-cancer gene, e.g. the herpes simplex virus thymidine kinase (HSV-TK). The HSV-TK enzyme can phosphorylate ganciclovir (GCV), and thus activate its cytotoxic effects on tumors that recruit HSV-TK expressing AT-MSCs [4, 8]. The third objective (*Task-3*) is to identify specific signaling cues that are upregulated in AT-MSCs following their recruitment to the tumor foci. These signaling cues (transcription factor inductions) can be taken advantage of in order to augment the expression of HSV-TK, thus enabling localized GCV-induced cytotoxicity [9, 10]. Ultimately, our fourth and final objective (*Task-4*) is to demonstrate that our *in vitro* approach of enhanced AT-MSC localization and tumor-site specific HSV-TK expression is a successful anti-tumor strategy *in vivo*, and will be able to eliminate the PCa tumor xenografts in nude mice [1, 4, 12, 13]. Since the pro-drug activation strategy using HSV-TK/GCV can cause significant cytotoxicity in neighboring cancer cells via its ‘bystander effect’ and would also eliminate the genetically engineered AT-MSCs themselves due to a ‘suicide effect’, we believe that our combined cellular and gene-therapy approach will be effective in targeting the metastatic PCa tumor foci without manifesting the systemic side effects. Experiments carried out in the second year of funding have allowed us to generate crucial evidences towards the first three objectives, and we have optimized both AT-MSC migration and the cytotoxic gene delivery strategies (a Manuscript is *In Preparation* & a Patent has been filed; 61/516,671). These *in vitro* findings will be incorporated together to develop the best approach for tumor suppression at metastatic sites. Towards this last objective, we have initiated the *in vivo* studies in tumor-bearing mice, where preliminary studies showed tumor-site specific localization of green fluorescent protein (GFP) labeled AT-MSCs. Interestingly, similar to recent observations coinjection of AT-MSCs increased tumor growth *in vivo* [14,15]. This was evident when AT-MSCs obtained from PCa patients (both African Americans and Caucasians) were used, especially due to intracrine androgen production from these stem cells (Basis for a recently funded DoD grant #PC102056). However, we believe that the mitogenic effect of AT-MSCs will not be a drawback since the HSV-TK carrying AT-MSCs will act as a “Trojan horse” and should demonstrate a potent and localized cancer killing activity *in vivo*. In the following pages, we have listed our findings towards each of the specified ‘Tasks’. The crucial observations are provided in the ‘Key research accomplishments’ section.

Body:

Task-1. Identify factors that regulate AT-MSC migration towards PCa cells and develop strategies to increase their tumor-site specific homing.

During the first year, we had generated significant data to properly understand the factors which regulate tumor-homing potential of AT-MSCs, and several strategies were implemented to enhance the migration of AT-MSCs, e.g. tumor necrosis factor-alpha (TNF- α) stimulation and FACS sorting of highly migratory AT-MSCs, based on their surface marker phenotypes. In the second year, we have corroborated our findings on AT-MSC recruitment to the PCa foci *in vitro* and have observed statistically ($p < 0.05$ to < 0.001) significant differences in migration potentials between the AT-MSCs obtained from normal individuals (AT-MSC^N) and those obtained from PCa-patients, both Caucasians (AT-MSC^{CA}) and from African Americans (AT-MSC^{AA}). Our crucial findings from these studies formed the basis of a recently funded DoD grant (*Prostate Cancer Health Disparity Research Award: PC102056*) [Fig. 1].

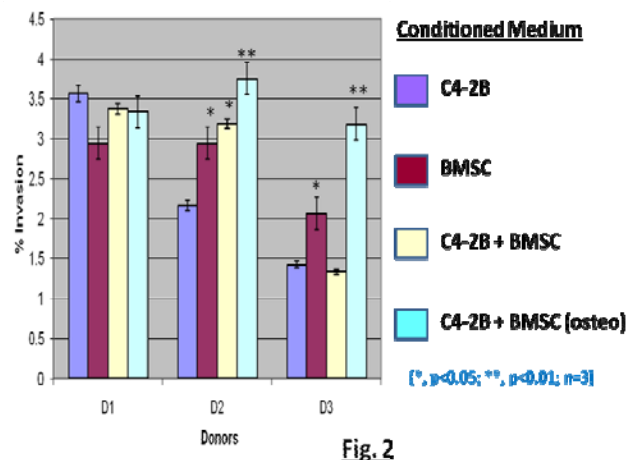
a. The African American patient-derived stem cells (AT-MSC^{AA}) showed the highest tropism towards bone metastatic PCa cells. To closely mimic the marrow microenvironments containing bone PCa cells, we developed *in vitro* coculture model for trans-endothelial migration (TEM) in a transwell culture (TWC) system. This model consisted of bone marrow endothelial cells (BMEC-1; 2×10^5 cells) grown on top of a matrigel-coated membrane insert (8 μ m pore size) in TWCs, and a confluent EC barrier was generated by Evans blue dye permeability to the bottom chamber. Calcein-stained AT-MSC^N, AT-MSC^{AA} and AT-MSC^{CA} cells (1×10^4 , $n=7$ per group) were then added to the upper chamber and their ability to migrate towards conditioned media (CM) from different PCa cells (LNCaP, C4-2B or PC-3) were monitored at 48 hrs post coculture. The percent migrations towards 10% fetal bovine serum (FBS) were used as a positive control (100%) and the comparative migration to either serum free DMEM medium or to PCa-CM were evaluated. As shown in Fig. 1 (right), the ADMSC^{AA} (African American) cells have higher (2-3 fold) intrinsic propensity to migrate towards PCa cell-derived factors (CM), as compared to ADMSC^{CA} (Caucasian American) or ADMSC^N (normal healthy subjects) cells ($p < 0.05$). However, even after 48 hrs these highly migratory populations showed that only a small subpopulation (about 3-5% of the total cells added) migrated towards LNCaP or C4-2B cell derived factors. Thus, suggesting that this small subpopulation of AT-MSCs may possess unique surface phenotype that enable their selective adhesion to BMECs and possess enhanced attraction properties due to higher chemokine receptor expression.



Earlier, we had documented that a small subset of AT-MSC^N cells migrated towards PCa-CM and their rate of migration could be enhanced by TNF- α stimulation of AT-MSCs. Here, we present compelling evidence showing that AT-MSCs from PCa-patients (AT-MSC^{AA} and AT-MSC^{CA}) have higher migration towards PCa cells, possibly due to their activated phenotype. Furthermore, we are presenting evidence that AT-MSC migration is much higher when conditioned medium from tumor cell and stromal cell cocultures (PCa-BMSC) were used (Fig.2, below).

b. AT-MSC migration occurs more rapidly towards PCa cells seeded within bone marrow stromal cultures.

In order to identify whether tumor-associated stroma also releases factors that enhance AT-MSC migration, we have carried out studies using CM from of PCa cells (C4-2B) alone, or with CM from cocultures with BMSCs (either control or osteogenically stimulated cells) (Fig.2). For these transmigration studies, AT-MSCs from three different donors were used, consisting of D1 (AT-MSC^{AA}), D2 (AT-MSC^{CA}) and D3 (AT-MSC^N) which had shown varying degrees of migration in our previous studies. The differences in percent migration, as compared to total number of cells added to the top chamber of the TWCs, are shown in the bar graphs. Although a small subpopulation migrated, we observed significant differences in the migratory phenotype of different AT-MSCs (D1, D2 & D3) towards CMs from either C4-2B or BMSCs alone, or towards



CM from C4-2B-BMSC cocultures. These observations clearly suggested a crucial role of factors secreted by the tumor cells as well as from the tumor-associated stromal cells, in dictating the tumor-site specific recruitment of AT-MSCs. Interestingly, the AT-MSC^{AA} cells showed high rate of migration to CM from C4-2B cells which was not significantly increased when CM from PCa-BMSC cocultures were used. However, both AT-MSC^{CA} and AT-MSC^N cells showed higher rates of migration towards CM from PCa-BMSC cocultures. Furthermore, the highest level of migration in these cells was clearly evident when CM from the osteogenically stimulated C4-2B + BMSC cocultures was used. These findings may elucidate why PCa cells primarily metastasizes to osteoblastic areas of the bone marrow [16].

c. Crucial role of SDF-1 α /CXCR4 chemokine axis in AT-MSC migration towards PCa-BMSC cocultures.

The enhanced migration to tumor-stroma may utilize attraction towards chemokine gradients [17-19]. Since osteoblasts secrete large quantities of the chemokine stromal derived factor-1 alpha (SDF-1 α) we first measured SDF-1 α levels in PCa-BMSC cocultures by using an SDF-1 α specific ELISA assay (**Fig.3A, below**). Flowcytometry analyses were also carried out to determine the level of CXCR4 expression in both unstimulated and TNF- α (10 U/ml) stimulated AT-MSC^N cells (**Fig. 3B, below**). Our investigations showed that supernatants from both C4-2B cells and BMSCs had minimal levels of SDF-1 α (30-70 pg/ml), however, the PCa-BMSC cocultures, especially the osteogenically stimulated cultures, showed significantly ($p < 0.01$) higher level of SDF-1 α production (100-200 pg/ml). In addition, flowcytometry analyses using a fluorescent (PE) conjugated antibody to CXCR4 suggested that a small subpopulation of the AT-MSCs (<5%) expressed CXCR4 (receptor for SDF-1 α) under unstimulated condition, however, a significant increase in surface CXCR4 expression 8-10 fold was observed in these AT-MSCs following their TNF- α (10 U/ml) stimulation. This clearly indicated the crucial role of SDF-1 α /CXCR4 chemokine axis in tumor-stroma specific homing and corroborated our hypothesis that AT-MSCs possessing an activated phenotype can migrate better towards osteoblastic areas of the bone.

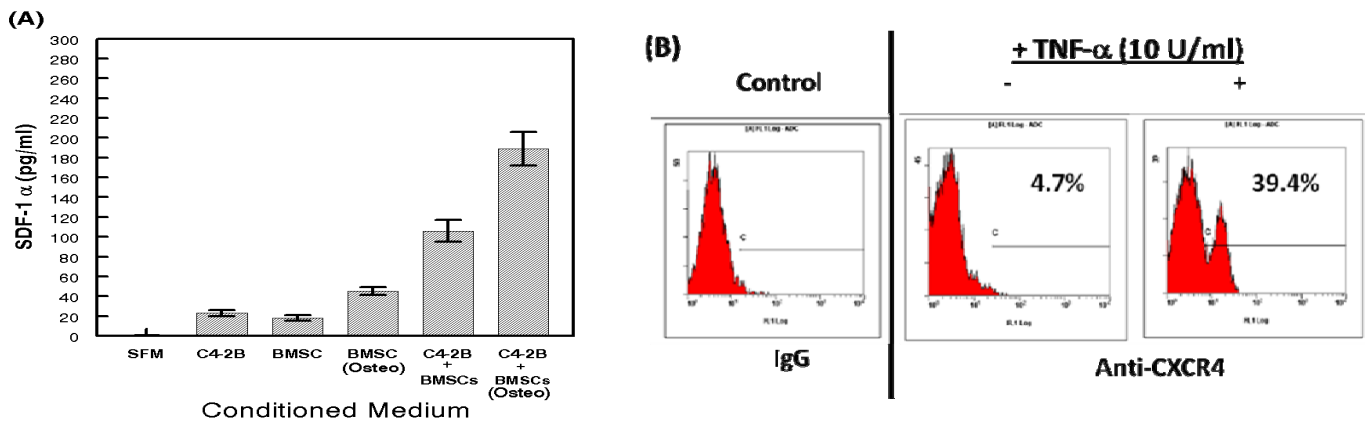
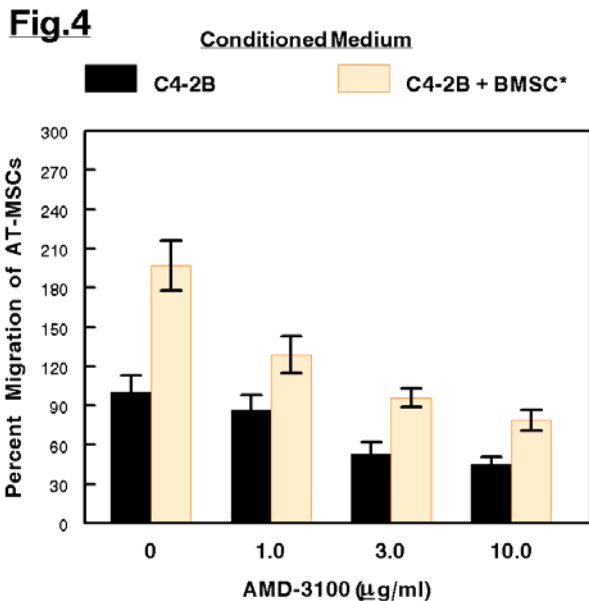
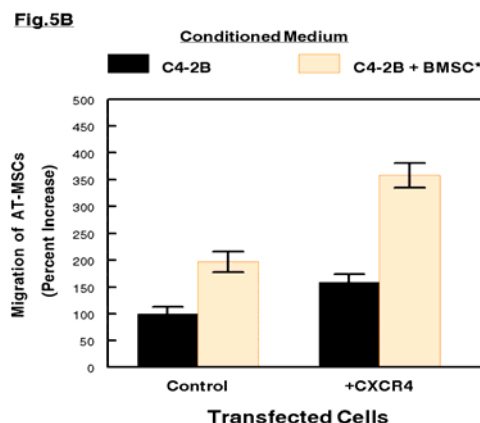
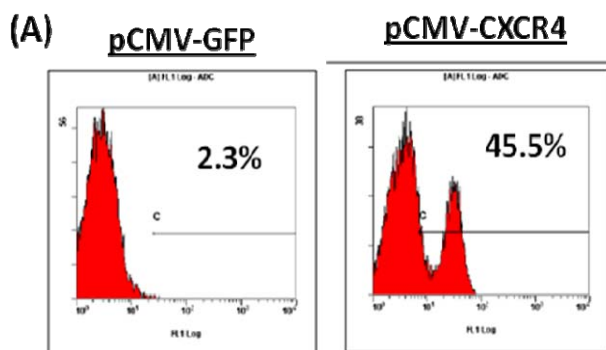


Fig.3



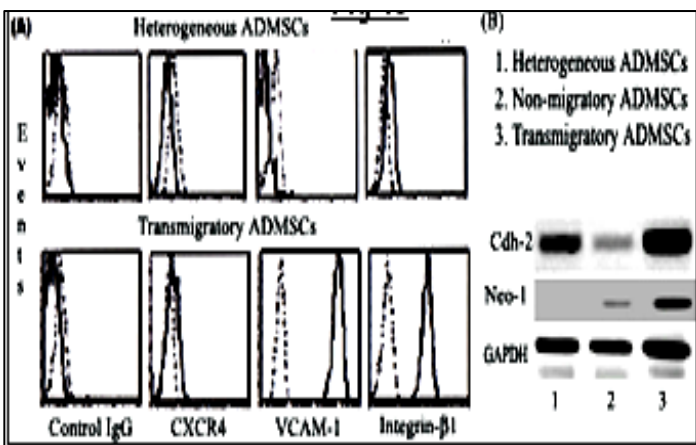
In order to determine whether the SDF-1 α /CXCR4 axis is indeed involved in the enhanced AT-MSC migration towards PCa-BMSC cocultures, we monitored the effects of a CXCR4 antagonist, AMD-3100 (**Fig.4**). We observed that pre-incubation of AT-MSCs with AMD-3100 (1-10 μ g/ml) significantly inhibited their migration towards PCa-BMSC conditioned medium.

Next, we hypothesized that since the SDF-1 α /CXCR4 axis is crucial in AT-MSC recruitment, then it may also be utilized to enhance AT-MSC homing to tumor microenvironments expressing SDF-1 α . In AT-MSCs transiently transfected with a CXCR4 expression plasmid (pCMV-CXCR4), we observed significantly ($p < 0.05$) increased surface levels of CXCR4 by flowcytometry analysis (**Fig.5A, next page**). In addition, we observed that AT-MSC migration towards conditioned medium from tumor-stroma cocultures could be enhanced by transient overexpression of CXCR4 in these cells (**Fig.5B, next page**).



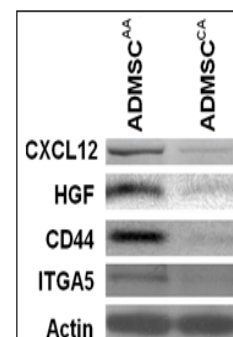
These observations clearly underscored the importance of SDF-1 α released by the PCa cells and from the tumor-associated BMSCs. Our findings also demonstrated that the tumor-homing properties of AT-MSCs, which may be largely dependent upon their expression of CXCR4, may be taken advantage of in future gene therapy strategies to increase tumor-site specific recruitment *in vivo*. (Manuscript *In Preparation* & Provisional Patent filed; # 61/516,671).

d. Tumor-tropic AT-MSCs possess different cell surface phenotypes. It is believed that in addition to the attractive properties of chemokine receptor expressing cells, the PCa cells expressing specific cell adhesion molecules (CAMs) and integrins can be preferentially recruited to the bone marrow endothelium which facilitates their homing properties.



Because of their crucial role in PCa cell adhesion to bone endothelium, we investigated the expression of VCAM-1 and Integrin- β 1 (in CD151 complexes) in total and selected AT-MSC^N populations (heterogeneous, transmigratory and non-migratory) (Fig. 6A, left). Flowcytometry studies clearly showed that, as compared to the control AT-MSCs, the transmigratory AT-MSCs showed augmented levels of both VCAM-1 and integrin- β 1, although CXCR4 expression was only slightly increased in these cells. In addition, as compared to either heterogeneous (control) or non-migratory population, the *in vitro* selected migratory AT-MSC^N cells showed higher mRNA expression of two other markers of cell adhesion and migration, e.g. Cdh2 and Neo1 genes, as measured by RT-PCR assays (Fig. 6B, left).

Since we had observed that a subpopulation of patient-derived AT-MSCs have higher tumor-homing potential, we wanted to identify specific surface markers that may enable these cells to migrate at a much faster rate as compared to the normal AT-MSCs. Preliminary studies towards the comparison of AT-MSC^{AA} and AT-MSC^{CA} cells by PCR-based array chip analysis (SABiosciences) had demonstrated selectively higher expression of the chemokine CXCL12 (a.k.a. SDF-1 α), Hepatocyte growth factor (HGF, a motility and morphogenic factor), CD44 (involved in cell-cell interactions, cell adhesion and migration) and ITGA5 (5 α -integrin) in the enriched AT-MSC^{AA} as opposed to ADMSC^{CA} (Fig.7, right). These results suggested that the patient-derived adipose stem cells appear to exhibit a more stem cell phenotype, and possesses a greater tendency for mobility. We aim to exploit these features for enrichment of AT-MSCs by FAC-Sorting and utilize these enriched tumor-tropic cells for targeted delivery.



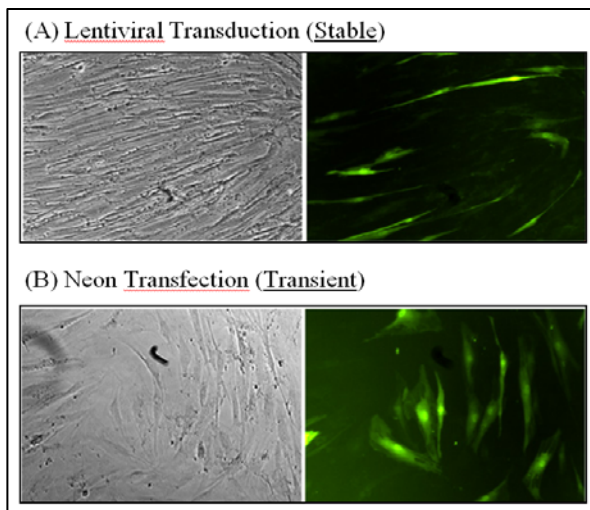
Indeed, during the first year, using the PCR-based array chip analysis system, we had identified several similar surface markers associated with the migratory phenotype of AT-MSCs and had hoped that these could be utilized for enriching cells with higher tumor-tropism. However, we have observed that although the sorting of migratory AT-MSCs was possible using a multi-color FACSorting system for high CDH1 (Cadherin-1) and CDH6 (Cadherin-6), the numbers obtained after sorting were too few to allow for large scale experimentations. Furthermore, we observed that upon long-term propagation, the sorted AT-MSCs reverted back to a more heterogeneous parental phenotype and lost their higher tumor-tropism. These observations suggested that the *ex vivo* sorting for tumor-tropic AT-MSCs for genetic engineering studies may not be the best approach that can be utilized for *in vivo* applications, since the migratory phenotype is transient. However, our current findings with the CXCR4 overexpressing cells has enabled a novel genetic engineering

strategy that may enable the availability of a larger and more stable population of AT-MSCs that possess enhanced tumor-tropism, and may be utilized alone or in combination with the sorting procedure to obtain cells with bone marrow tropism and specific predilection for the embedded tumor foci.

Task-2. Engineer AT-MSCs to express the HSV-TK enzyme within tumor microenvironments and monitor their ability to enhance GCV-induced 'bystander effect' on cocultured PCa cells.

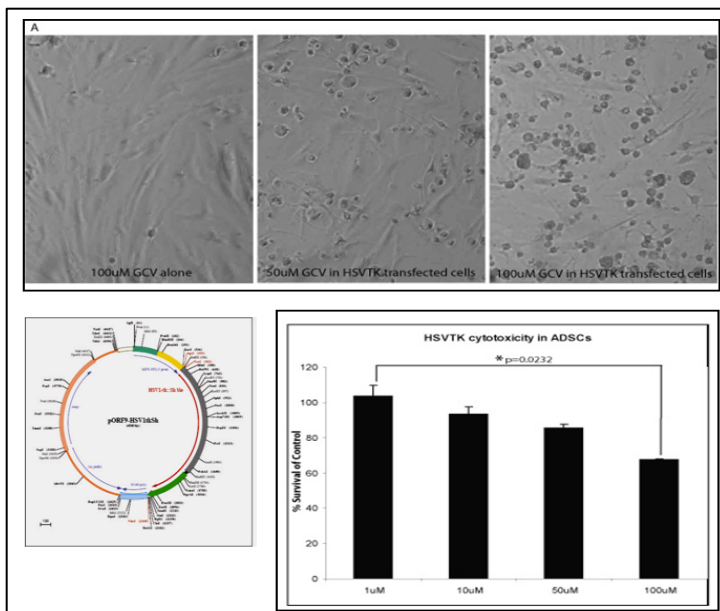
The second objective was to investigate whether the tumor-tropic AT-MSCs can be engineered to express the herpes simplex virus thymidine kinase (HSV-TK) gene which can locally activate the cytotoxic effects of ganciclovir (GCV) following their recruitment to tumor sites. During the first year, we had initiated our studies on lentivirus (LV) mediated gene delivery in AT-MSCs which enabled a high efficiency and stable expression of the transgene (green fluorescent protein; GFP). However, although our transduction data was promising, we observed that the LV-transduced AT-MSCs showed lower proliferative potential and had especially dampened differentiation capacities. This would be a significant deterrent to their utility in our future studies. Furthermore, LV integration in AT-MSCs may also have untoward long-term side effects and would be problematic towards their clinical use in patients. Hence, we envisioned that since AT-MSCs can localize to tumors within 2-3 days [1, 3, 4] where a transient expression of our prodrug therapy (HSV-TK/GCV) would be sufficient towards a potent anti-cancer effect, we looked for an alternative strategy for high efficiency gene delivery in these cells which would not affect their proliferative and differentiation capabilities.

a. A transient plasmid based transfection of AT-MSCs would be safe and efficacious in targeting of HSV-TK.



By using a NeonTM transfection system from Invitrogen (a high-efficiency and low toxicity electroporation technique) we have first optimized a transient transfection protocol with 0.5 µg of a plasmid encoding the EGFP in AT-MSCs (~10 million cells). The following conditions were found to be optimal: Pulse voltage of 1400 volts, pulse width of 10 milliseconds and pulse number of 2-3. In **Fig.8, left**, we are presenting data comparing the efficiencies of the LV infection and NeonTM transfection protocols by using vectors expressing EGFP (reporter gene). In (A), transduction with VSVG pseudotyped LV was carried out, and in (B) transient transfection with the Neon system was carried out (**Fig.8, left**). The EGFP expression was checked after 5 days and photographed using a TS-100 nikon inverted microscope. Our data showed that optimized NeonTM transfection system show high efficiency of AT-MSC transfection. This enabled almost 80-90% transfection efficiency in AT-MSCs and showed less than 70% cytotoxicity. Furthermore, the

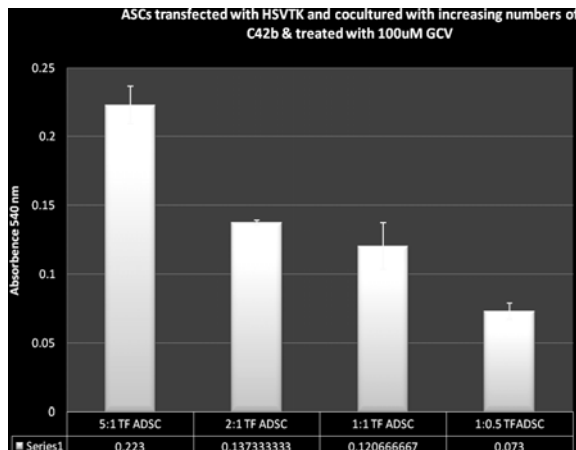
transiently transfected cells were competent for both proliferation and lineage specific differentiation. In addition, the EGFP expression was seen to be stable for at least 10-12 days. Hence, we modified our approach from a stable to a more transient gene expression strategy using the plasmid based gene therapy approach.



As had been documented in Fig.5, we had utilized the NeonTM transfection system to express CXCR4 (pCMV-CXCR4; Missouri cDNA resource center) in the AT-MSCs. This transient transfection system of CXCR4 overexpression was indeed able to increase the migration of AT-MSCs towards an SDF-1α gradient as well as towards PCa-BMSC conditioned medium.

Recently, we used the NeonTM transfection protocol to transfect AT-MSCs with a HSV-TK expression plasmid (pORF-HSVtk; Invivogen). The plasmid pORF-HSVtk constitutively expresses the transgene under the control of a eukaryotic elongation factor (EIF-2α) enhancer [plasmid map in **Fig.9, left**]. Approximately 24 hrs post transfection, cells were exposed to different concentration of ganciclovir [GCV (1-100 µM) for 5 days. Exposure to the higher concentrations of GCV (50 & 100 µM) was significantly more cytotoxic (p=0.0232) and induced > 45% death in the HSV-TK transfected cells only (**Fig.9**).

The light microscopy images show cell death and bar graphs showing percent survival following exposure to increasing concentrations of GCV. These results indicated that GCV treatment of cells transfected with HSVtk can be effective in *suicide gene therapy*.



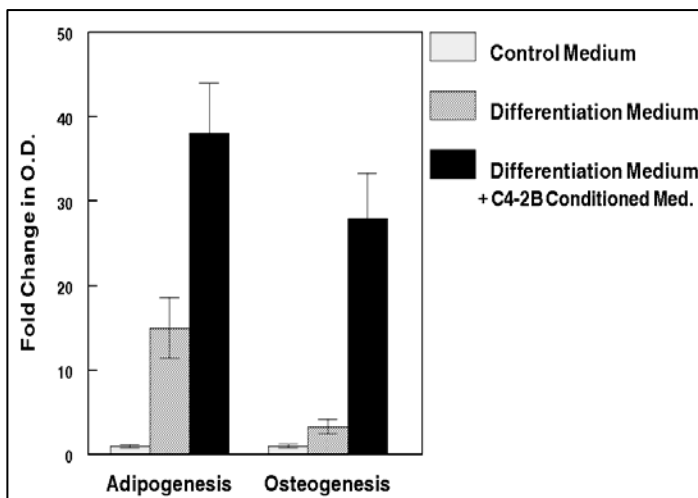
Next, we wanted to observe whether coculturing of the HSV-TK expressing AT-MSCs with untransfected PCa cells would be able to show a *bystander killing effect* (**Fig. 10, below**). The AT-MSCs expressing HSV-TK were cocultured at different dilutions with the C4-2B cells and percent cell viability in 5-days were measured by MTT assays. The ratios of C4-2B cells were varied with a constant number of transfected AT-MSCs. Higher rate of killing was clearly evident when cells were plated in 1:1 and 1: 0.5 ratios of PCa cells to AT-MSCs. As much as a 70-90% elimination of the PCa (C4-2B) cells was observed in this coculture experiments, thus clearly demonstrating a bystander killing effect. Hence, we have established that the *NeonTM* transfection system can be used to efficiently express both CXCR4 and HSV-TK in AT-MSCs. We are in the process of finalizing a Manuscript which will be submitted soon.

Interestingly, the GCV-induced cytotoxicity was far greater in the PCa cells (Fig. 10) as compared to the suicide killing effect observed in the AT-MSCs (Fig.9], further corroborating that the residual (surviving) AT-MSCs may have potential long-term anti-tumor effect following multiple GCV exposures *in vivo*. However, we also envision that even low level of AT-MSC migration to systemic sites and the expression of HSV-TK at these nonspecific areas would have significant non-target toxicity from GCV, which will be unacceptable in a clinical setting. Hence, signaling cues will need to be in place in order to regulate the HSV-TK expression only following tumor-site specific recruitment of the engineered AT-MSCs, which formed the basis of our Task-3.

Task-3. Utilization of lineage specific factors to activate the HSV-TK transgene in tumor-tropic AT-MSCs in order to localize the cytotoxic effects of GCV.

a. Tumor-derived factors can increase both osteogenic and adipogenic differentiation of AT-MSCs.

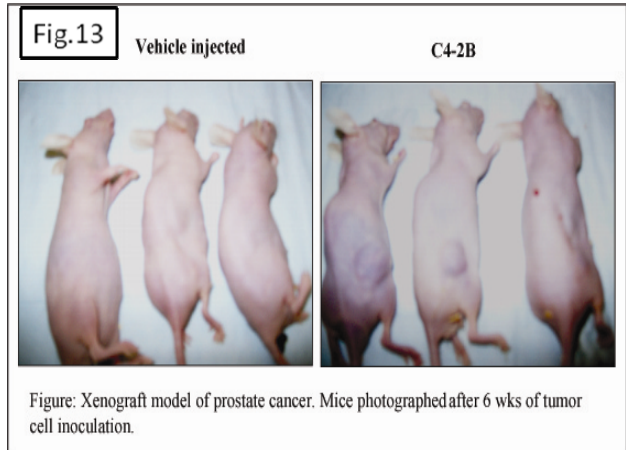
Previously, we had determined the effect of tumor-derived factors (C4-2B CM) on AT-MSC differentiation towards either adipogenic or osteogenic lineages. For adipogenic differentiation, cells were incubated in DMEM medium (0.5 μ M 1-methyl-3 isobutylxanthine, 1 μ M dexamethasone, 10 μ g/ml insulin and 100 μ M indomethacin) for 3 weeks. To visualize the extent of lipid droplets, cells were fixed with 4% formalin and stained with Oil red-O dye (Fig. 9A). For osteogenic differentiation, cells were incubated in medium containing 100 nM dexamethasone, 10 mM β -glycerophosphate and 0.05 mM L-ascorbic acid-2-phosphate for 3 weeks. Mineralization of the extracellular matrix was visualized by staining with Alizarin red dye (Fig. 9A). The extent of differentiation was measured colorimetrically by organic phase extraction (isopropanol) of dye and measuring optical density (OD). Our findings showed that tumor-derived factors can increase both osteogenic and adipogenic differentiation of AT-MSCs (**Fig.11, left**). Recently, we also measured osteogenic and adipogenic commitment in AT-MSCs by monitoring both mRNA levels of Osteonectin (OCN) or lipoprotein lipase (LPL), respectively. Our data clearly showed a more rapid increase in these differentiation markers in AT-MSCs exposed to tumor-derived factors, as compared to control AT-MSCs (not shown)



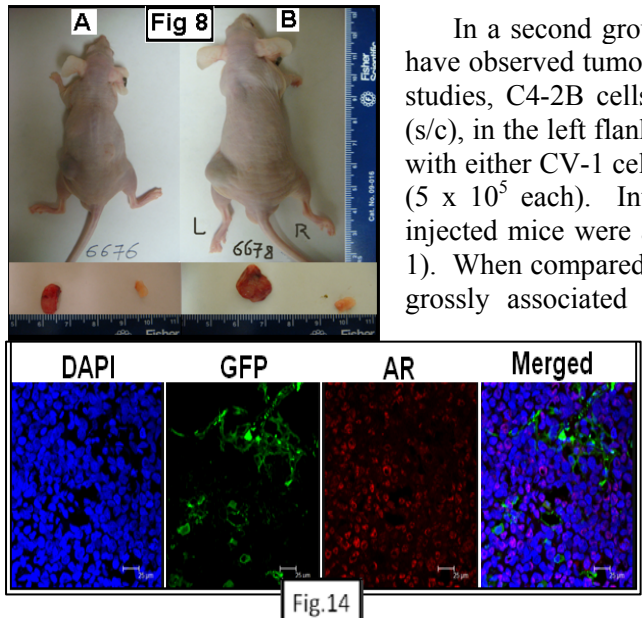
Since our ultimate goal would be to utilize the lineage specific transcription factors in regulating HSV-TK expression from these cells, during the second year, we have monitored the temporal expression of two lineage specific transcription factor genes, PPAR- γ (adipoenic) and RUNX2 (osteogenic) by RT-PCR []. We are presenting preliminary data obtained from one AT-MSC line (Donor-3) where the mRNA levels for GAPDH, PPAR- γ and RUNX2 were measured every day following differentiation induction, for 7-8 days [**Fig. 12, next page**]. Following differentiation induction, the AT-MSCs were exposed to either serum free medium (-) or conditioned medium from C4-2B cells (+). The most significant changes in control and tumor-exposed AT-MSCs were observed between day-2 and day-5 (48-120 hrs) post differentiation

induction, and results from these time points are included in the figure [Fig. 12 left]. In this representative AT-MSC line, the lineage specific transcription factors were rapidly increased within 48 hrs post exposure to either adipogenic or osteogenic media. Similar to the enhanced differentiation observed in Fig. 11, the increase in both PPAR- γ and RUNX2 was found to be much higher in cells coexposed to the PCa-CM, as well. The induction of PPAR- γ (adipoenic) was more robust and more consistent, whereas expression of RUNX2 was weaker and its induction was seen in both adipogenic or osteogenic cells, especially at the later time point. Hence, we are in the process of measuring the nuclear protein levels of these two transcription factors, following with we plan to clone the RUNX2 or PPAR- γ binding site upstream of the HSV-TK gene. From this data it became evident that the PPAR- γ response element may be used to effectively increase HSV-TK gene expression in tumor-recruited AT-MSCs. By using this vector, we propose that AT-MSCs recruited to tumors will enhance their HSV-TK expression and GCV cytotoxicity *in vivo*.

Task-4. Demonstrate the feasibility of our approach of enhanced AT-MSC localization and tumor-site specific HSV-TK expression to eliminate the PCa tumor cells in vivo.



We have initiated the *in vivo* studies to develop PCa xenografts in immunocompromised mice (an IACUC approved protocol). In brief, in a 6-week old nu/nu mice ($n=5$ /experiment), C4-2B cells (2×10^6) were mixed with Matrigel and injected subcutaneously (s/c), alone or along with CV-1 cells (control) in the right flank. Tumor growth on each flank of the same mice was measured for 6 weeks, at weekly intervals. Measureable tumor growth was clearly observed within 3-4 weeks in at least 80% of the animals injected with the C4-2B cells. However, no such tumors were visible even at 6 weeks when the CV-1 cells were used [Fig. 13, left]. These C4-2B derived tumors were palpable, however, tumors generated with two other PCa cell lines, PC-3 and LNCaP, showed lesser efficiency of tumor engraftment and showed smaller and softer tumor mass even at 6 weeks. Hence, our preliminary studies showed that subcutaneous (s.c.) injection of C4-2B cells ($2-5 \times 10^6$ cells) develop visible tumors within 4-weeks and this xenograft model can be used to monitor AT-MSC colocalization and then GCV-mediated anti-tumor effects.



In a second group of mice, we injected GFP-labeled AT-MSCs at a distal site and have observed tumor-site specific localization of these cells [Fig. 14, below]. For these studies, C4-2B cells (2×10^6) were mixed with Matrigel and injected subcutaneously (s/c), in the left flank. At four weeks post inoculation, tumor-bearing mice were injected with either CV-1 cells (A) or with GFP-transduced AT-MSC^{AA} cells (B) at the left flank (5×10^5 each). Interestingly, at 6-weeks, we observed that the tumors in AT-MSC injected mice were almost two times larger than those injected with control cells (CV-1). When compared to C4-2B cells alone, the tumors generated in coinjected mice were grossly associated with increased neovasculatures, suggesting that AT-MSCs may induce angiogenesis which can contribute to the increased PCa tumor mass. In panel (C), data from immunohistochemical (IHC) analysis of tumor sections with antibodies to androgen receptor (AR) and immuofluorescence microscopy (IFM) for GFP-labeled cells, are shown. DAPI stained nuclei are shown in blue. The engraftment of GFP-transduced AT-MSCs (green) in C4-2B xenografts expressing AR (red) is clearly evident in the merged image which demonstrated tumor-site specific colocalization of AT-MSCs.

Similar to a number of recent publications [14, 15] inoculation of AT-MSCs increased PCa tumor volume (compare A & B). Although this may contradict the utility of using AT-MSCs as anti-cancer gene delivery vehicles, we believe that our approach to locally activate the cytotoxic effects of GCV should be able to suppress the mitogenic effects of these cells. The combination of a ‘suicide gene’ and its ‘bystander effect’ should ultimately be able to eliminate the PCa tumors at metastatic sites *in vivo*.

We are currently monitoring whether the CXCR4 expressing AT-MSCs have a higher rate of tumor-site specific localization and whether the PPAR γ regulated HSV-TK expression by the tumor-recruited AT-MSCs can induce GCV cytotoxicity in s.c. tumors. If we observe promising data with our gene delivery vehicles in the s.c. tumor model, we propose to carry out similar studies with intraosseous PCa tumors in the later part of the third year, which will underscore the validity of our hypothesis and demonstrate the clinical utility of our approach.

Key Research Accomplishments:

- *In vitro* studies comparing the migration capacity of AT-MSCs from both normal individuals (AT-MSC^N) and from PCa-patients (AT-MSC^{AA} and AT-MSC^{CA}) showed differential migratory ability towards PCa tumors, implicating the therapeutic potential of using autologous (patient derived) AT-MSCs as highly efficient anti-cancer gene delivery vehicles.
- AT-MSCs stimulation (TNF- α or IL-1 β) increased their endothelial adhesion and tumor-site migratory phenotype, suggesting that patient derived AT-MSCs may have a more activated phenotype and surface markers expressed on these cells enable their higher tumor predilection.
- AT-MSC migration occurred more rapidly towards Prostate cancer (PCa) cells seeded within bone marrow stromal (BMSC) cultures, suggesting the role of stroma-derived factors and conditioned medium from PCa-BMSC cocultures were seen to produce high quantities of SDF-1 α .
- Stimulation of AT-MSCs enhanced the surface expression of CXCR4 and other migration associated genes, e.g. integrins, CAMs and MMPs, which were constitutively higher in the patient-derived AT-MSCs.
- Pre-incubation of AT-MSCs with the CXCR4 antagonist, AMD-3100 inhibited migration potential, suggesting the crucial role of SDF-1 α /CXCR4 chemokine axis in AT-MSC recruitment.
- Although lentivirus transduction was efficient in AT-MSCs, the LV-transduced cells showed significantly lower proliferative potential and differentiation capacities.
- A transient transfection system (NeonTM) was optimized to express CXCR4 or HSV-TK genes, and showed no significant effect on their proliferative potential and differentiation capacities.
- As compared to untransfected AT-MSCs, AT-MSCs overexpressing CXCR4 showed higher *in vitro* migration towards both tumor-derived factors, as well as towards tumor-associated stroma.
- AT-MSCs expressing HSV-TK demonstrated very potent GCV-induced cytotoxicity in cocultured PCa cells, and *bystander killing* of PCa cells was dependent upon both GCV dose and AT-MSC number.
- Coexposure to tumor derived factors (C4-2B CM) enhanced both adipogenic and osteogenic differentiation and rapidly increased the expression of PPAR γ 2 and Runx2 expression in tumor-recruited AT-MSCs.
- Systemically injected AT-MSCs (GFP-labeled) were able to localize to the tumor xenografts.

Reportable Outcomes:

Meeting Abstracts

1. Debasis Mondal, Geetika Chakravarty, Yijun Yang, Marxa L. Figueiredo and Asim B. Abdel-Mageed. *Optimizing the use of adipose tissue-derived mesenchymal stem cells (AT-MSCs) as highly effective anti-cancer gene delivery vehicles.* **2011.** IMPACT Conference, Orlando, FL.
2. Robert Davis, Geetika Chakravarty & Debasis Mondal. *Enhancement of ganciclovir cytotoxicity with adipose stem cell driven prodrug therapy for prostate cancer.* **2011.** Tulane Health Science Research Day, New Orleans, La.

Patents

Provisional Patent filed (# 61/516,671).

Enhancement of Ganciclovir Cytotoxicity with Genetically Engineered Adipose Stem Cell Driven Prodrug Therapy for Cancer.

Grants

Recommended for funding:

DoD; Qualified Collaborator Award: PC102056 (*Prostate Cancer Health Disparity Research Award*).

Stem-Cell Based Therapeutic Targeting of Metastatic Prostate Cancer Residual Androgens in African Americans

Submitted

NIH (NCI); U01: PAR-09-161

Estrogen-ERbeta Axis in Health Disparity of Prostate Cancer

Conclusions:

We have confirmed our previous findings that a small subpopulation of AT-MSCs has high tumor-homing potential. We have shown that several *in vitro* strategies can be implemented to enhance the percentage of AT-MSCs that migrate to PCa tumors and towards tumor-stroma. We have identified the SDF-1 α /CXCR4 chemokine axis as a main determinant of the invasive phenotype of AT-MSCs and overexpression of CXCR4 increased their tumor-predilection. We have optimized a highly efficient transfection protocol which showed that both migration and anti-cancer therapy using AT-MSCs can be increased by transient gene delivery of CXCR4 and HSV-TK, respectively. We have identified certain signaling cues, rapidly activated in tumor-recruited AT-MSCs, which may be utilized to increase tumor-site specific HSV-TK expression and GCV-induced *bystander effect*. We have generated human PCa tumors (C4-2B cells) in immunocompromised (nu/nu) animals and have demonstrated the ability of fluorescent labeled (GFP) AT-MSCs to seek out the tumor foci *in vivo*. Ultimately, we envision that with the use of autologous AT-MSCs, constitutively expressing CXCR4 and conditionally expressing the HSV-TK, our anti-cancer gene delivery strategy would show GCV-induced killing of PCa cells at metastatic sites.

References

- 1: Cavarretta IT, Altanerova V, Matuskova M, Kucerova L, Culig Z, Altaner C. *Adipose tissue-derived mesenchymal stem cells expressing prodrug-converting enzyme inhibit human prostate tumor growth*. Mol Ther. **2010** Jan;18(1):223-31. PMID: 19844197.
- 2: Sun B, Roh KH, Park JR, Lee SR, Park SB, Jung JW, Kang SK, Lee YS, Kang KS. *Therapeutic potential of mesenchymal stromal cells in a mouse breast cancer metastasis model*. Cytotherapy. **2009**;11(3):289-98, 1 p following 298. PubMed PMID:19308770.
- 3: Corallini F, Secchiero P, Beltrami AP, Cesselli D, Puppato E, Ferrari R, Beltrami CA, Zauli G. *TNF- α modulates the migratory response of mesenchymal stem cells to TRAIL*. Cell Mol Life Sci. **2010**;67(8):1307-14. PMID: 20063037.
- 4: Matuskova M, Hlubinova K, Pastorakova A, Hunakova L, Altanerova V, Altaner C, Kucerova L. *HSV-tk expressing mesenchymal stem cells exert bystander effect on human glioblastoma cells*. Cancer Lett. **2010** Apr 1;290(1):58-67. PMID: 19765892.
- 5: Zhao BC, Zhao B, Han JG, Ma HC, Wang ZJ. *Adipose-derived stem cells promote gastric cancer cell growth, migration and invasion through SDF-1/CXCR4 axis*. Hepatogastroenterology. **2010** Nov-Dec;57(104):1382-9. PubMed PMID: 21443090.
- 6: Ryu CH, Park SA, Kim SM, Lim JY, Jeong CH, Jun JA, Oh JH, Park SH, Oh WI, Jeun SS. *Migration of human umbilical cord blood mesenchymal stem cells mediated by stromal cell-derived factor-1/CXCR4 axis via Akt, ERK, and p38 signal transduction pathways*. Biochem Biophys Res Commun. **2010** Jul 16;398(1):105-10. PMID: 20558135.
- 7: Park SA, Ryu CH, Kim SM, Lim JY, Park SI, Jeong CH, Jun JA, Oh JH, Park SH, Oh W, Jeun SS. *CXCR4-transfected human umbilical cord blood-derived mesenchymal stem cells exhibit enhanced migratory capacity toward gliomas*. Int J Oncol. **2011** Jan;38(1):97-103. PubMed PMID: 21109930.
- 8: Dong D, Dubeau L, Bading J, Nguyen K, Luna M, Yu H, Gazit-Bornstein G, Gordon EM, Gomer C, Hall FL, Gambhir SS, Lee AS. *Spontaneous and controllable activation of suicide gene expression driven by the stress-inducible grp78 promoter resulting in eradication of sizable human tumors*. Hum Gene Ther. **2004** PMID: 15212714.
- 9: Giusta MS, Andrade H, Santos AV, Castanheira P, Lamana L, Pimenta AM, Goes AM. *Proteomic analysis of human mesenchymal stromal cells derived from adipose tissue undergoing osteoblast differentiation*. Cytotherapy. **2010** Mar 15. [Epub ahead of print] PubMed PMID: 20230220.
- 10: DeLany JP, Floyd ZE, Zvonic S, Smith A, Gravois A, Reiniers E, Wu X, Kilroy G, Lefevre M, Gimble JM. *Proteomic analysis of primary cultures of human adipose-derived stem cells: modulation by Adipogenesis*. Mol Cell Proteomics. **2005** Jun;4(6):731-40. PMID: 15753122.
- 11: Jotzu C, Alt E, Welte G, Li J, Hennessy BT, Devarajan E, Krishnappa S, Pinilla S, Droll L, Song YH. *Adipose tissue derived stem cells differentiate into carcinoma-associated fibroblast-like cells under the influence of tumor derived factors*. Cell Oncol (Dordr). **2011** Feb;34(1):55-67. PMID:21327615.
- 12: Fang B, Li Y, Song Y, Li N, Cao Y, Wei X, Lin Q, Zhao RC. *Human adipose tissue-derived adult stem cells can lead to multiorgan engraftment*. Transplant Proc. **2010** Jun;42(5):1849-56. PMID: 20620536.
- 13: Josiah DT, Zhu D, Dreher F, Olson J, McFadden G, Caldas H. *Adipose-derived stem cells as therapeutic delivery vehicles of an oncolytic virus for glioblastoma*. Mol Ther. **2010** Feb;18(2):377-85. PMID: 19904233.

- 14: Prantl L, Muehlberg F, Navone NM, Song YH, Vykoukal J, Logothetis CJ, Alt EU. *Adipose tissue-derived stem cells promote prostate tumor growth*. Prostate. **2010** Nov 1;70(15):1709-15. PMID: 20564322.
- 15: Jeon ES, Lee IH, Heo SC, Shin SH, Choi YJ, Park JH, Park do Y, Kim JH. *Mesenchymal stem cells stimulate angiogenesis in a murine xenograft model of A549 human adenocarcinoma through an LPA1 receptor-dependent mechanism*. Biochim Biophys Acta. **2010** Nov;1801(11):1205-13. PMID: 20708100.
- 16: Ibrahim T, Flamini E, Mercatali L, Sacanna E, Serra P, Amadori D. *Pathogenesis of osteoblastic bone metastases from prostate cancer*. Cancer. **2010** Mar 15;116(6):1406-18. PMID: 20108337.
- 17: Sun YX, Schneider A, Jung Y, Wang J, Dai J, Wang J, Cook K, Osman NI, Koh-Paige AJ, Shim H, Pienta KJ, Keller ET, McCauley LK, Taichman RS. *Skeletal localization and neutralization of the SDF-(CXCL12) /CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites in vivo*. J Bone Miner Res. **2005** Feb;20(2):318-29. PMID: 15647826.
- 18: Wang J, Loberg R, Taichman RS. *The pivotal role of CXCL12 (SDF-1)/CXCR4 axis in bone metastasis*. Cancer Metastasis Rev. **2006** Dec;25(4):573-87. PMID: 17165132.
- 19: Christopher MJ, Liu F, Hilton MJ, Long F, Link DC. *Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization*. Blood. **2009** Aug 13;114(7):1331-9. PMID: 19141863.