Adipocytes sustain pancreatic cancer progression through a non-canonical WNT paracrine network inducing ROR2 nuclear shuttling

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Running Title: Adipocytes accelerate pancreatic cancer progression

Keywords: pancreatic cancer, obesity, ROR2.

Financial Support: This work was supported by the Investigator Grant n°19111 to DM and n°18599 to GT through the Associazione Italiana per la Ricerca sul Cancro (AIRC), by the Basic Research Project 2015 through the University of Verona to DM, and by the Nastro Viola patients' association donations to DM.

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ABSTRACT

Background: Solid epidemiological evidences connect obesity with incidence, stage, and survival in pancreatic cancer. However, the underlying mechanistic basis linking adipocytes to pancreatic cancer progression remain largely elusive. We hypothesized that factors secreted by adipocytes could be responsible for epithelial-to-mesenchymal transition (EMT) induction and, in turn, a more aggressive phenotype in models of pancreatic preneoplastic lesions.

Methods: We studied the role of factors secreted by two adipogenic model systems from primary human Bone Marrow Stem Cells (hBMSC) in an in vitro experimental cell transformation model system of human pancreatic ductal epithelial (HPDE) cell stably expressing activated KRAS (HPDE/KRAS),

Results: We measured a significant induction of EMT and aggressiveness in HPDE and HPDE/KRAS cell lines when cultured with medium conditioned by fully differentiated adipocytes ($ADIPO^{CM}$) if compared with the same cells cultured with medium conditioned by hBMSC ($hBMSC^{CM}$) from two different healthy donors. Several genes coding for soluble modulators of the non-canonical WNT signaling pathway, including FRZB, SFRP2, RSPO1, WNT5A and 5B were significantly overexpressed in fully differentiated adipocytes than in their respective in hBMSC. $ADIPO^{CM}$ induced the overexpression and the nuclear translocation of the Frizzled family member Receptor tyrosine kinase-like orphan receptor (Ror) 2 in HPDE and HPDE/KRAS cells. Vantictumab, an anti-Frizzled

monoclonal antibody, reduced ROR2 nuclear translocation and in turn the EMT and aggressiveness in HPDE and HPDE/KRAS cells.

Conclusions: We demonstrated that adipocytes could induce EMT and aggressiveness in models of pancreatic preneoplastic lesions by orchestrating a complex paracrine signaling of soluble modulators of the non-canonical WNT signaling pathway that determine, in turn, the activation and nuclear translocation of ROR2. This signaling pathway could represent a novel target for pancreatic cancer chemoprevention. Most importantly, these factors could serve as novel biomarkers to select a risk population among obese subjects for screening and, thus, early diagnosis of pancreatic cancer.

INTRODUCTION

Cancer and obesity are the two major epidemics of the 21th century [1]. Pancreatic cancer is the fourth leading cause of cancer-related mortality among adults in the developed countries [2]. The poor prognosis for patients with pancreatic cancer could be mainly attributed to its aggressive course, the limited efficacy of available systemic treatments, and, in particular, to the invariable metastatic behavior demonstrated along the progression of the disease [3]. Thus, the identification of the earliest molecular events responsible for the metastatic dissemination of pancreatic cancer remains critical for early diagnosis, prevention, and treatment interventions [4]. Solid evidences supporting the model that metastasis is an early event in pancreatic carcinogenesis have been provided by using a genetically engineered murine model of pancreatic cancer in which the pancreatic epithelial cells could be tracked during tumor progression through the expression of YFP allele into the *KRas* plus *p53* or *p16* mutant background. In this model, even low-grade PanINs showed evidence of cells that have crossed the basement membrane, migrated from the glandular epithelium into the surrounding tissue and circulatory system, and seeded the liver prior to pancreatic cancer formation. This behavior was associated with an early epithelial- to-mesenchymal transition (EMT) genetic program in the premalignant lesions [5].

Several epidemiological studies demonstrated positive associations between the prevalence of obesity as judged by increased BMI, cancer incidence [6], poorer treatment outcome, worsened prognosis, and increased cancer-related mortality [7]. Pancreatic cancer obeys this rule, as several studies reported elevated risks of developing this tumor in obese individuals compared with individuals with a normal weight [8, 9]. In particular, a recent prospective cohort study demonstrated that higher prediagnostic BMI was associated with a significantly decreased survival among patients with pancreatic

cancer. This association was stronger for BMI measured a greater number of years before cancer diagnosis, suggesting that chronic exposure to the consequences of obesity may be important in affecting patient survival. More interestingly, this study prospectively demonstrated a statistically significant association between prediagnostic BMI and cancer stage, with more obese patients presenting with metastatic disease than patients with healthy weight [10].

Although the epidemiological evidence connecting obesity with cancer incidence is strong, the underlying mechanistic basis linking obesity per se to tumor-initiating events remains largely elusive [11]. Three main mechanistic connections have been proposed for this association, including the antiapoptotic effects of obesity-associated hyperinsulinemia, the enhanced aromatization of sex steroids in adipose tissue, and the elaboration of paracrine and endocrine factors, or adipokines, that promote either tumorigenesis or angiogenesis directly from adipocytes and stromal cells within fat pads [12]. However, a comprehensive analysis aimed to directly identify the actual adipokines responsible for the paracrine molecular networks linking obesity and cancer progression has not been performed before.

In this present study, we hypothesized that factors secreted by adipocytes could be responsible for EMT induction and, in turn, a more aggressive phenotype in models of pancreatic preneoplastic lesions, representing the molecular mechanisms linking obesity with pancreatic cancer progression.

METHODS

Cell lines and reagents

Human papillomavirus type 16 early gene 6 and 7-immortalized (HPDE) human pancreatic ductal epithelial non-tumorigenic cells stably expressing activated KRAS (HPDE/KRAS) have been previously described in [13]. The HPDE were routinely cultured in keratinocyte serum-free medium supplemented by epidermal growth factor and bovine pituitary extract (Life Technologies, Inc., Grand Island, NY). Cell lines used in this study were authenticated using DNA fingerprinting at the genomic core facility at Wayne State University (2009) and tested for mycoplasma contamination. Vantictumab (OMP-18R5) was purchased by OncoMed Pharmaceuticals, (Redwood City, California, USA) and used at 1μg/mL.

Isolation and adipogenic differentiation of hBMSCs

hBMSCs were isolated ad described in [14]. Briefly, human Bone Marrow Stromal cells were isolated from bone marrow aspirates of healthy donors (informed consent, approved by Ethical Committee of Azienda Ospedaliera Universitaria Integrata Verona; N. 1828, May 12, 2010- "Institution of cell and tissue collection for biomedical research in Onco-Hematology"). Bone marrow aspirates were cultured in 225-cm^2 flasks at 5×10^5 nucleated cells/cm^2 concentration in alpha-minimal essential medium (MEM), 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco). After 72 h, nonadherent cells were removed and the medium was replaced twice a week. Published protocol was followed to induce adipogenic differentiation of hBMSCs [15]. hBMSCs were cultured at a density of 5000~6000 cells/cm2. After reaching confluence, hBMSCs were cultured for one more week and induced in adipogenic medium containing 0.5 mM isobutyl-methylxanthine (Sigma-

Aldrich), 1 μM dexamethasone (Sigma-Aldrich), 10 μM insulin (Roche), 100 μM indomethacin (Sigma-Aldrich) for three days and maintained in medium with 10 μM insulin for one day. The treatment was repeated four times, after which the cells were washed three times with PBS and maintained in DMEM without FBS for four days. The conditional medium was collected, centrifuged. The cells were subjected to oil red O staining to detect cytoplasmic triglyceride.

Oil red O staining

Cells were washed three times with PBS and then fixed for 2 min with 3.7% formaldehyde. Oil red O (0.5% in isopropanol) was diluted with water (3:2) filtered through a 0.45 μm filter and incubated with the fixed cells for 1 h at room temperature. Cells were washed with water and the stained fat droplets in the cells were visualized by light microscopy and photographed.

Cell Proliferation Assay and Western blotting

On day 0, 1.0×10^3 cells/well were seeded in 96-well plates. At the indicated hours, sulforhodamine B (SRB) (Sigma, St. Louis, Missouri, USA) assay was used to obtain relative estimates of viable cell number as previously described in [16]. Western blotting analyses were carried out as described previously in [17]. Briefly, cell lines were washed twice with cold phosphate-buffered saline and lysed at 4°C into RIPA buffer (50 mM Tris–HCl , pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) plus protease inhibitor mix (Sigma-Aldrich). Each lysate was separated by SDS-PAGE and probed with antibodies against E-Cadherin, β-catenin (Abcam, Cambridge, UK), ROR2, Histone H3, GSK3α/β and p-GSK3β-(S9) (Cell Signaling Technology, Boston, MA), vimentin (Dako, Denmark), GAPDH and γ-tubulin,

(Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive proteins were detected using an enhanced-chemiluminescence reagent (ECL, Millipore, Billerica, MA) according to the manufacturer's instructions. Images were captured by LAS4000 Digital Image Scanning System (GE Healthcare, Little Chalfont, UK).

Reverse transcription-PCR and Real-Time PCR

RNA was isolated by Trizol reagent as manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription-PCR (RT-PCR) assay was performed accordingly with the High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) as previously described in [18]. The mRNA expression of CDH1, Vimentin, Leptin, ADIPOQ, WNT5A, WNT5B, FRZB, ZEB1, SFRP2, DKK1, DKK2 and DKK3 was quantified by using a SYBR green based real-time PCR analysis and the ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Each gene was tested in each cell line in four replicates and three independent experiments were performed. To quantify the relative changes in gene expression, the $2^{-\Delta\Delta CT}$ method was used and reactions were normalized to endogenous control gene β-actin expression levels.

Immunofluorescence

Immunofluorescence analysis were carried out as described previously in [19]. Briefly, HPDE cell lines were cultured for 24 hours on cover slips in a 24 multi-well plate. The cells were harvested and the medium completely replaced with $hBMSC^{CM}$, $ADIPO^{CM}$ with or without vantictumab. After 24 hours cells were fixed with 2.5% formalin and permeabilized with 0.1% Triton for 10 minutes at 4°C. The cells were then incubated with the primary antibody specific for ROR2 (Abcam, Cambridge, UK) or FZD for 1 hour at room temperature and with fluorophore (FITC) or AlexaFluor546 –conjugated as

secondary antibody (Invitrogen, Carlsbad, CA, USA). Nuclei were stained with To-Pro-3 (Invitrogen, Carlsbad, CA, USA). Cover slips were mounted with pro-long antifade mountant (Invitrogen, Carlsbad, CA, USA). The images were obtained with a confocal microscopy (LMS510, Zeiss, Oberkochen, Germany).

Wound healing and transwell invasion assay

Wound healing assay was performed as previously described in [20]. Briefly, HPDE in vitro cell transformation model systems were seeded at 70% of confluence in 100mm cell culture dishes. After 24 hours, medium was completely replaced with $hBMSC^{CM}$ or $ADIPO^{CM}$. After 48 hours a straight scratch was made using a pipette tip to simulate a wound. The cells were washed gently with cold phosphate-buffered saline (PBS1X) and rinsed with $hBMSC^{CM}$ or $ADIPO^{CM}$. Photographs of at least five different points were taken immediately and after 24 hours of culture. In vitro invasion assay were performed using 24- well transwell unit with polycarbonate filters (Corning Costar, Cambridge, USA). HPDE were suspended at density of $5x10^5$/ml in culture medium and then placed in the upper part of transwell. Meanwhile, conditional medium from hBMSC and adipocyte cells with or without vantictumab (1μg/mL) were added at the lower wells of the chambers.. Cells were incubated for 24 hours, fixed with ethanol and stained with 0,05% Crystal violet for 30 min. Cells in the upper chamber were removed with cotton swab. Cells that invaded through the Matrigel (MatrigelTM Basement Membrane Matrix, BD Biosciences, USA) to the underside of the filters were counted and picture taken under a Leica DMIL-Led microscope equipped with a Leica EC3 camera (Leica, Wetzlar, Germany). Three invasion chambers were used for treated and untreated group. The values obtained were calculated by averaging the total number of the cells from three filters. All experiments were performed in triplicates.

Gene Expression Microarray and Pathway Analysis

Differences in gene expression between cell lines were examined using Illumina Human 44k gene chips (Illumina, Milan, Italy). Briefly, synthesis of cDNA and biotinylated cRNA was performed using the IlluminaTotalPrep RNA Amplification Kit (Ambion), according to the manufacturer's protocol using 500ng of total RNA. Hybridization of cRNAs (750 ng) was carried out using Illumina Human 48k gene chips (Human HT-12 V4 BeadChip). Array washing was performed using Illumina High Temp Wash Buffer for 10' at 55°C, followed by staining using streptavidin-Cy3 dyes (Amersham Biosciences). Probe intensity data were obtained using the Illumina Genome Studio software (Genome Studio V2011.1). Raw data were Loess normalized with the Lumi R package and further processed with Excel software. Each microarray experiment was repeated twice. Differentially expressed transcripts were tested for network and functional interrelatedness using the IPA software program (Ingenuity Systems, Redwood, CA). Gene expression microarray data have been deposited in the GEO database with accession number (GSE59857).

Statistical Analysis

All results were expressed as the 95% confidence interval for at least three independent experiments performed in triplicate. All of the statistical analyses were performed using the GraphPad Prism software program (version 4.0c; GraphPad Software, La Jolla, CA).

RESULTS

Adipocytes secreted factors induce EMT and aggressiveness in *in vitro* models of pancreatic preneoplastic lesions

Initially we established two adipogenic models from human Bone Marrow Stem Cells (hBMSC) from healthy donors. hBMSC were grown in culture media able to induce hBMSC differentiation into mature and functionally active adipocytes, as confirmed by the formation of small cytoplasmic lipid droplets by light microscopy analysis and Oil Red O staining (Figure 1A). To further confirm adipocytes differentiation, we measured a significant increasing expression of ADIPOQ and Leptin genes in hBMSC, pre-adipocytes, and fully differentiated adipocytes (Figure 1B).

To test the hypothesis that factors secreted by adipocytes could be responsible for EMT and aggressiveness of pancreatic preneoplastic lesions, we used an in vitro experimental cell transformation models consisting in the stable expression of activated KRAS (HPDE/KRAS) in HPDE cell line. These cell lines were cultured with medium conditioned by hBMSC ($hBMSC^{CM}$), or by fully differentiated adipocytes ($ADIPO^{CM}$). We measured a significantly higher expression of the mesenchimal marker vimentin in HPDE and HPDE/KRAS cell lines when cultured with $ADIPO^{CM}$ if compared with the same cells cultured with $hBMSC^{CM}$ from two different healthy donors (Figure 1C and Supplementary Figure 1). We measured significantly higher proliferation rates in HPDE and HPDE/KRAS cells cultured with $ADIPO^{CM}$ compared with $hBMSC^{CM}$ (Figure 1D). Moreover, we measured also a significantly higher migration rate of these cell lines when co-cultured with adipocytes cells compared with hBMSC control cells at early time points when the differences in proliferation were negligible (Figure 1E). Interestingly, in co-cultured conditions this effect was more evident for HPDE/KRAS cells with respect to HPDE control cells (Supplementary Figure S2).

Adipocytes sustain pancreatic preneoplastic lesions aggressiveness through a WNT paracrine network

In order to identify the factors secreted by adipocytes that could be responsible for accelerating EMT in the pancreatic preneoplastic models, we compared gene expression profiles and narrowed our analysis to genes coding for proteins in the extracellular space that exhibited significantly higher expression in fully differentiated adipocytes than in their respective in hBMSC from two healthy donors. We found an overexpression of several genes coding for soluble modulators of the non-canonical WNT signaling pathway, including FRZB, SFRP2, RSPO1, WNT5A and 5B. Moreover, we measured the significant down-regulation of genes encoding for members of the dickkopf family proteins – DKK1, 3 – that are known to antagonize WNT canonical pathway (Figure 2A, and B). Consistently, we found a higher inhibitory phosphorylation of GSK3 in HPDE and HPDE/KRAS cells cultured with ADIPO^{CM} compared with hBMSC^{CM} (Figures 2C and D).

In order to demonstrate that these adipocyte-secreted WNT factors were indeed responsible for the EMT induced in HPDE cells, we used vantictumab, a monoclonal antibody directed against the extracellular portion of WNT receptors (FZDs). In this regard, we demonstrated a measurable reduction of the expression of vimentin, β–catenin and phospho-GSK3-β in HPDE and HPDE/KRAS cells when cultured with ADIPO^{CM} and treated with vantictumab if compared with the same cells cultured with ADIPO^{CM} and treated with a control mAb, at levels similar with those measured in the same cells cultured with hBMSC^{CM} (Figure 3A). Consistently, we measured a significant reduction in the proliferation (Figure 3B), migration (Figure 3C) and invasion rates (Figure 3D) of HPDE and HPDE/KRAS cells when cultured with ADIPO^{CM} and treated with vantictumab if compared with the same cells cultured with ADIPO^{CM} and treated

with mAb control. This effect was not observed in cells cultured with hBMSC^{CM} (Figures 3B, 3C and 3D).

Collectively, these results provide strong evidences supporting a model in which the induction of an EMT phenotype in pancreatic premalignant lesions by adipocytes involve the binding of WNT proteins to the FZDs receptors.

ROR2 nuclear shuttling mediates pancreatic preneoplastic lesions aggressiveness induced by adipocytes-derived WNT paracrine factors

Receptor tyrosine kinase-like orphan receptor (Ror) 2 is a Frizzled family protein which belongs to the 7 trans- membrane class of receptors. Ror2 interact with several of the Wnt ligands to activate a combination of noncanonical and canonical Wnt signaling activity. In particular, WNT5A induced the formation of a complex between ROR2 and Frizzled, resulting in Ser/Thr phosphorylation of Ror2 and the recruitment of Dvl, Axin, and GSK3B [21]. We measured a significantly higher expression of ROR2 in HPDE and HPDE/KRAS cell lines when cultured with ADIPO^{CM} if compared with the same cells cultured with hBMSC^{CM}. Immunofluorescence analysis showed co-localization of FZD receptors and ROR2 in these cells. Treatment with vantictumab was able to modulate ROR2 expression and to interfere with binding of FZD with ROR2, triggering to ROR2 cellular rearrangement (Figures 4A, and B).

Since recent evidences have been provided for the nuclear translocation of the other member of the ROR family, ROR1 [22], we performed a bioinformatics analysis on the potential nuclear translocation of ROR2. Based on NLS-mapper software (http://nls-mapper.iab.keio.ac.jp/), we identified several nuclear translocation sequences (NLS) that have good potential for ROR2 nuclear localization (Table 1). In this regard, we performed a Western blotting and immunofluorescence analyses of ROR2 in the different cellular compartments, and we demonstrated that ADIPO^{CM} induced not only a

ROR2 overexpression but also the nuclear translocation of its 50 KDa short isoform. Treatment with vantictumab was able to modulate ROR2 expression and to interfere with its nuclear localization (Figures 5A, and B).

These results suggest that the WNT paracrine factors secreted by adipocytes could sustain cell proliferation and invasion in pancreatic premalignant lesions by inducing the nuclear shuttling of ROR2.

DISCUSSION

In this study, we demonstrated that adipocytes could induce EMT and aggressiveness in models of pancreatic preneoplastic lesions by orchestrating a complex paracrine signaling of soluble modulators of the non-canonical WNT signaling pathway that determine, in turn, the activation and nuclear translocation of ROR2.

The most compelling preclinical evidences about the link between obesity and pancreatic cancer carcinogenesis indicated that a high-fat, high-calorie diet could lead to obesity and accelerates early pancreatic neoplasia in the conditional K-Ras G12D mouse model [23]. More recently, a complex cross-talk between adipocytes, tumor-associated neutrophils, and pancreatic stellate cells has been described to promote desmoplasia, accelerated tumor growth and impaired delivery/efficacy of chemotherapeutics, with IL1β secreted by all these cells playing a major role in this cooperation [24].

Our study contributes to this field by providing evidence, through an inductive approach, of a role for several modulators of the non-canonical WNT signaling as the paracrine network between adipocytes and EMT induction in preneoplastic pancreatic lesions. The anti- WNT receptors monoclonal antibody vantictumab was able to modulate these effects. Interestingly, EMT was established as one of the pharmacodynamic biomarker in surrogate tissues and tumor tissues from serial biopsies in the first-in-human Phase 1a study for vantictumab in patients with advanced solid tumors [25].

Current papers reported that depending on receptor context, WNT proteins have the potential to both activate the non-canonical pathway [26] as well as to antagonize the canonical pathway by promoting the activation of tyrosine kinase-like orphan receptors (ROR)-1 and -2 [27]. In the case of canonical WNT signaling, ROR2 expression is lost, supporting its role as a gatekeeper of the canonical pathway. Alternatively, in cancers driven by non-canonical WNT signaling, ROR2 expression is increased and is thought to play a critical role in driving tumorigenesis [28].

ROR2 is a tyrosine kinase receptor with a well-established role in the activation of non-canonical WNT signaling pathways. Recent evidences showed that Ror2 was highly expressed in an increasingly long list of cancers, including pancreatic cancer. In the majority of these cancer types, ROR2 has been identified as a potential high value target for therapeutic development [21] [28].

Previous studies indicated that ROR2 specifically interacts only with non-canonical WNT5A. This activation is partially due to the ability of WNT5A to interact with ROR2 and FZD and form a tripartite complex Ror2–Wnt5a–Fzd [29] [26].

The current study revealed that adipocytes secreted factors could enhance the aggressiveness of pancreatic preneoplastic lesions through a mechanism that require the interaction of ROR2-WNT-FZD

The inhibition of WNT receptors FZDs by using the monoclonal antibody vantictumab, reduced ROR2- FZD interaction and could moderate the proliferation, migration and invasion rate of pancreatic preneoplastic lesions induced by adipocytes secreted factors. A recent paper showed a domain of the ROR1 cytoplasmic region that mediated the translocation event through Ran GTPase involvement [22]. Since ROR1 and ROR2 shared protein sequences with high identity matrix we hypothesize a similar behavior for ROR2. A bioinformatics analysis based of NLS domain recognizes multiple ROR2 domains that

play a critical role in nuclear accumulation. Confocal immunofluorescence and cell compartment analyses corroborate this evidence showing a well-defined nuclear translocation of ROR2 in premalignant cells when cultured with adipocytes^{CM}. Vantictumab treatment was able to reduce ROR2 protein expression and its nuclear translocation.

Most importantly, to our knowledge this is the first study that demonstrate ROR2 as potential factors involved in PC initiation and progression and could be of help in establishing novel therapeutic approaches by targeting the molecular signaling pathways responsive to adipocytes-mediated stimuli.

Moreover, the identification of these factors could facilitate the development of novel therapeutic strategies for the targeting of ROR2 in an obese high risk pancreatic cancer population.

Acknowledgment: Part of the work was performed at the Laboratorio Universitario di Ricerca Medica (LURM) Research Center, University of Verona. We thank Dr. Marzia Di Chio at the Department of Diagnostic and Public Health, University of Verona for technical execution of immunofluorescence analyses.

Conflicts of interest: The authors declare no conflicts of interest.

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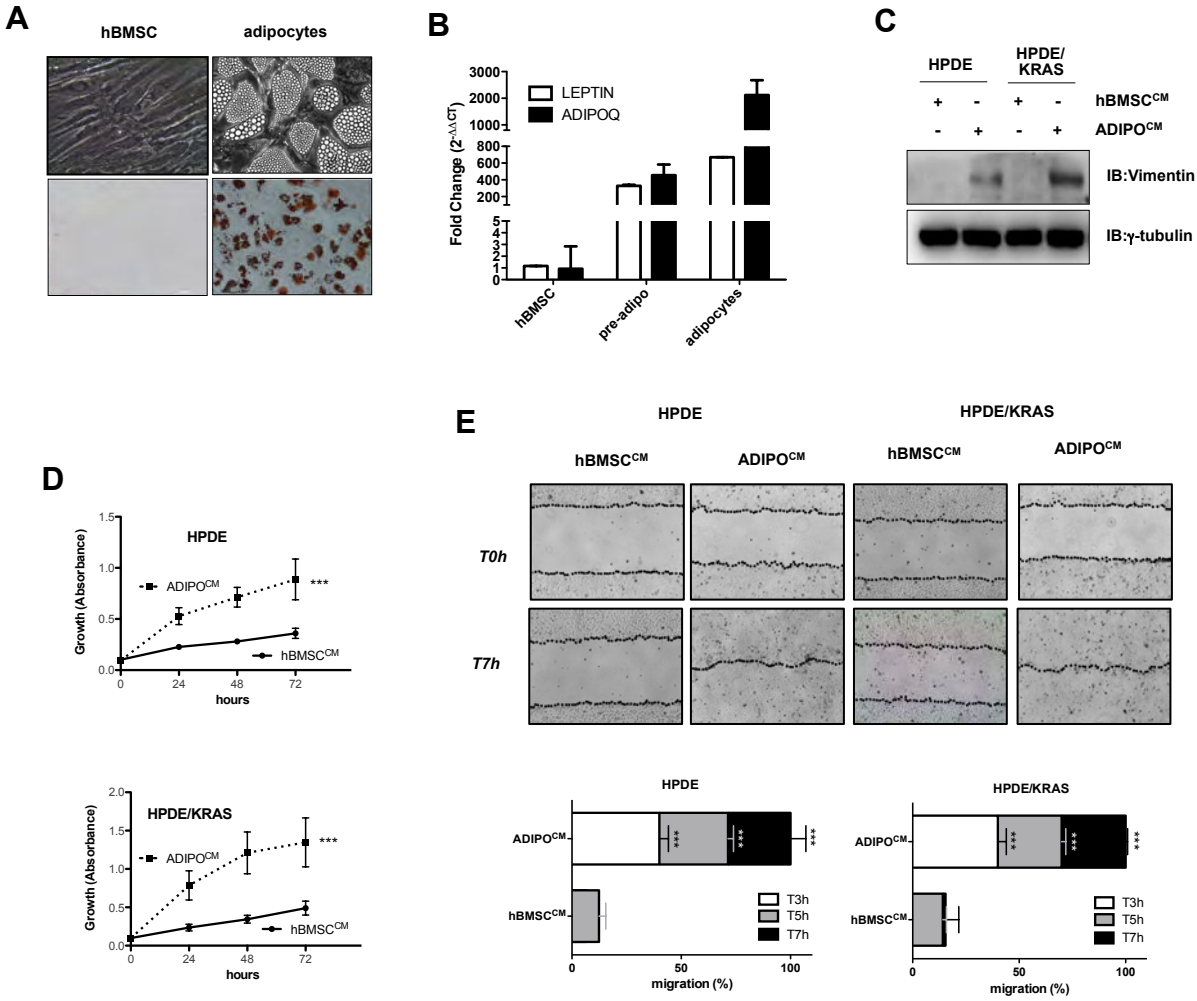
Figure 1


A
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LEPTIN
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IB:Vimentin
IB:γ-tubulin
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Growth (Absorbance)
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HPDE
HPDE/KRAS
hBMSC^CM
ADIPO^CM
T0h
T7h
ADIPO^CM
hBMSC^CM
T3h
T5h
T7h
migration (%)

Figure S1

A

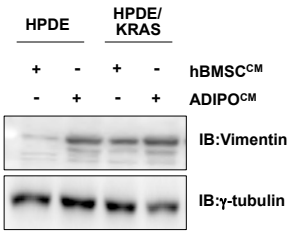
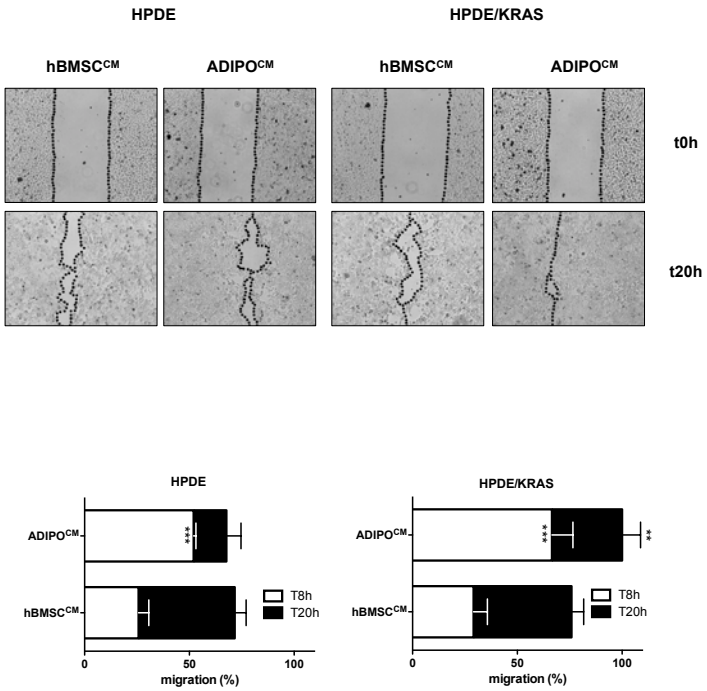


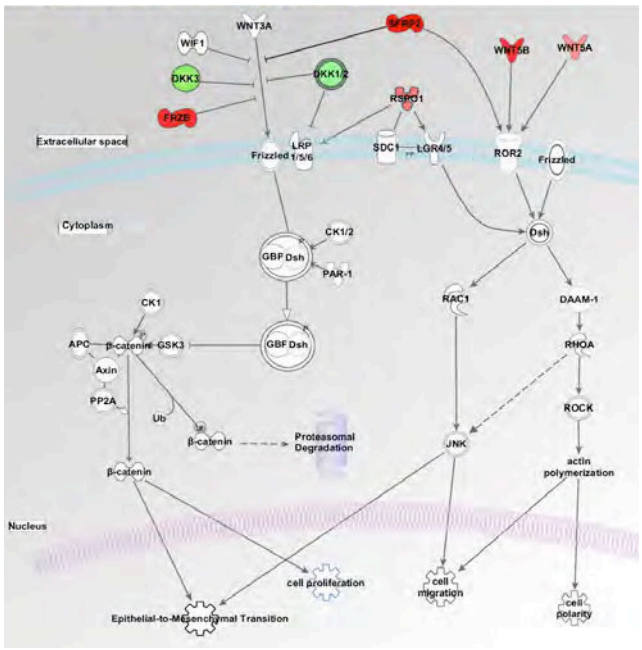
Figure S2



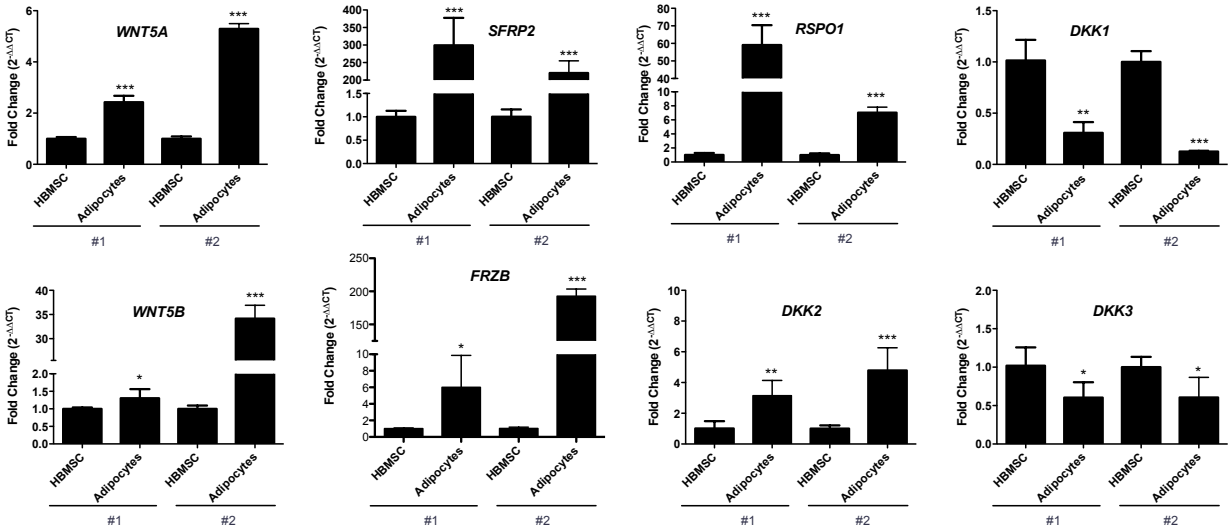
Co-culture: Adypocite-conditioned medium induces cell migration

Figure 2

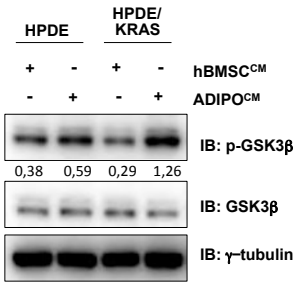
A



B



C



D

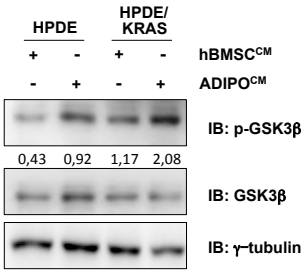


Figure 3

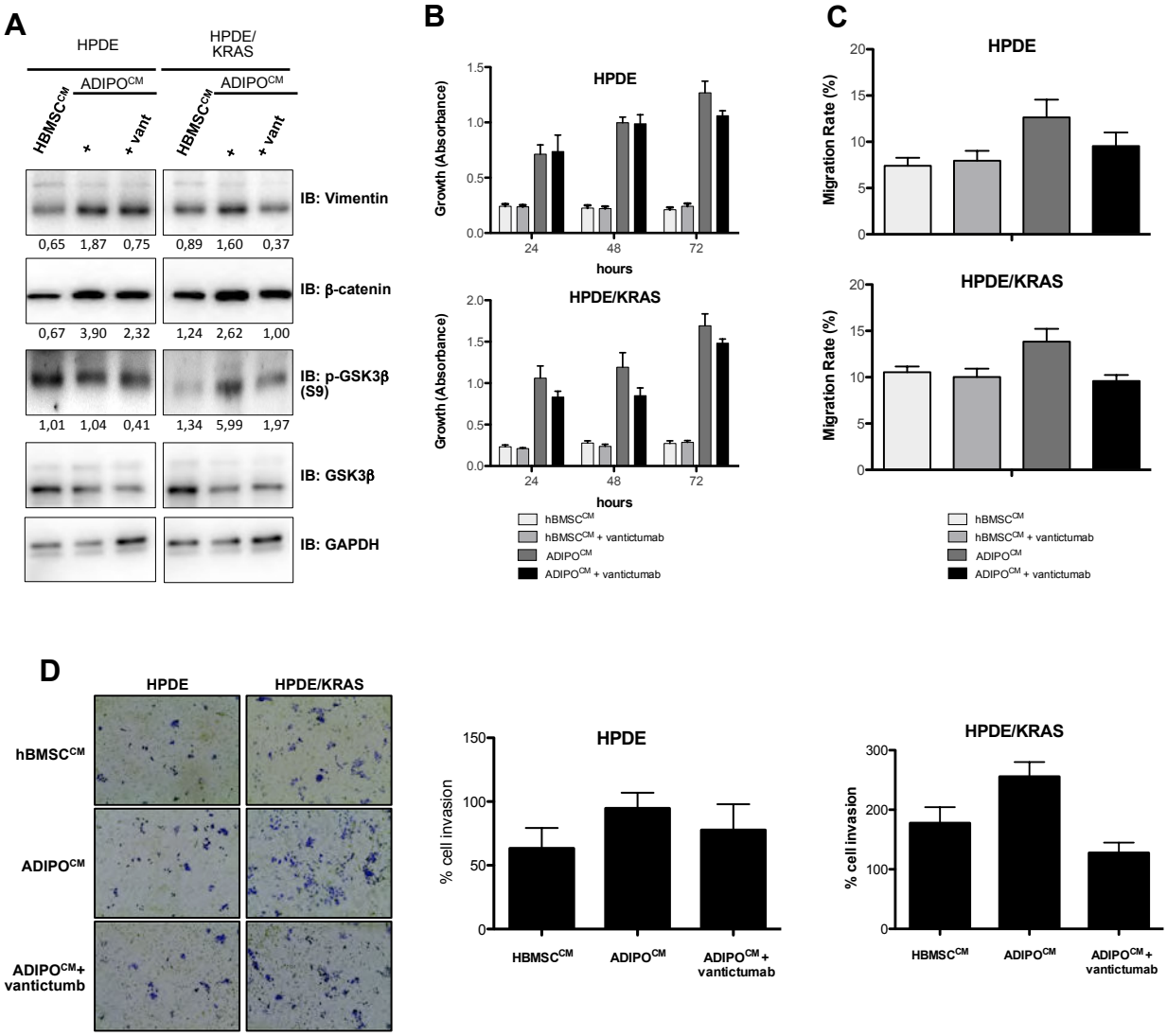
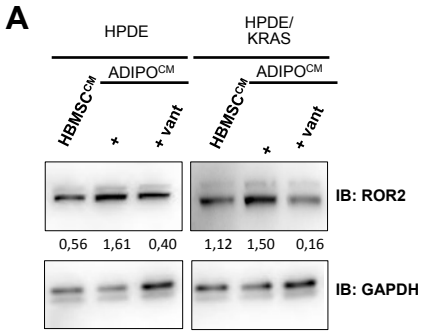

A
HPDE
HPDE/ KRAS
ADIPO^CM
HBMSC^CM
+
+ vant
IB: Vimentin
0,65 1,87 0,75
0,89 1,60 0,37
IB: β-catenin
0,67 3,90 2,32
1,24 2,62 1,00
IB: p-GSK3β (S9)
1,01 1,04 0,41
1,34 5,99 1,97
IB: GSK3β
IB: GAPDH
B
HPDE
Growth (Absorbance)
hours
HPDE/KRAS
hBMSC^CM
hBMSC^CM + vantictumab
ADIPO^CM
ADIPO^CM + vantictumab
C
HPDE
Migration Rate (%)
HPDE/KRAS
D
HPDE
HPDE/KRAS
hBMSC^CM
ADIPO^CM
ADIPO^CM+ vantictumb
% cell invasion
HBMSC^CM
ADIPO^CM
ADIPO^CM+ vantictumab

Figure 4


A
HPDE
HPDE/
KRAS
ADIPO^{CM}
ADIPO^{CM}
HBMSC^{CM}
+
+ vant
HBMSC^{CM}
+
+ vant
IB: ROR2
0,56
1,61
0,40
1,12
1,50
0,16
IB: GAPDH

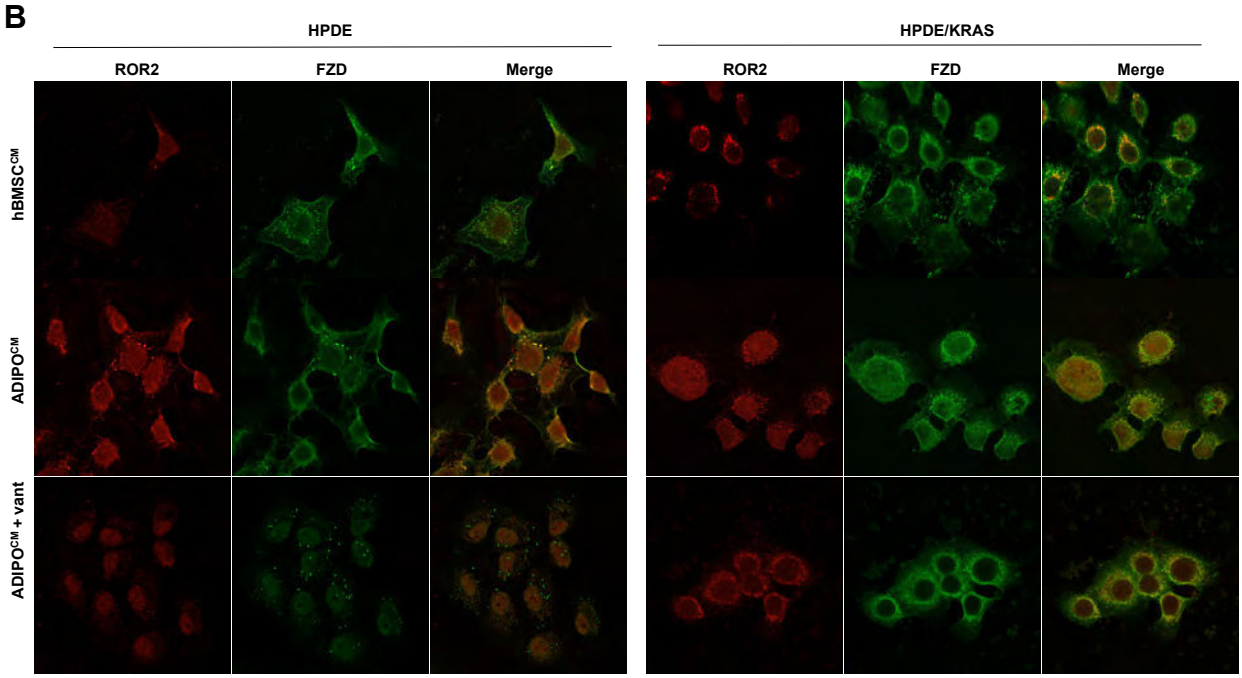
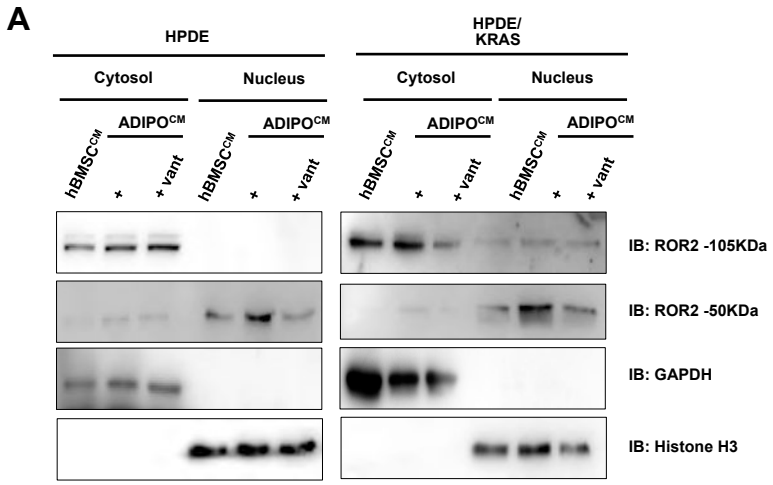

B
HPDE
HPDE/KRAS
ROR2
FZD
Merge
ROR2
FZD
Merge
hBMSC^{CM}
ADIPO^{CM}
ADIPO^{CM} + vant

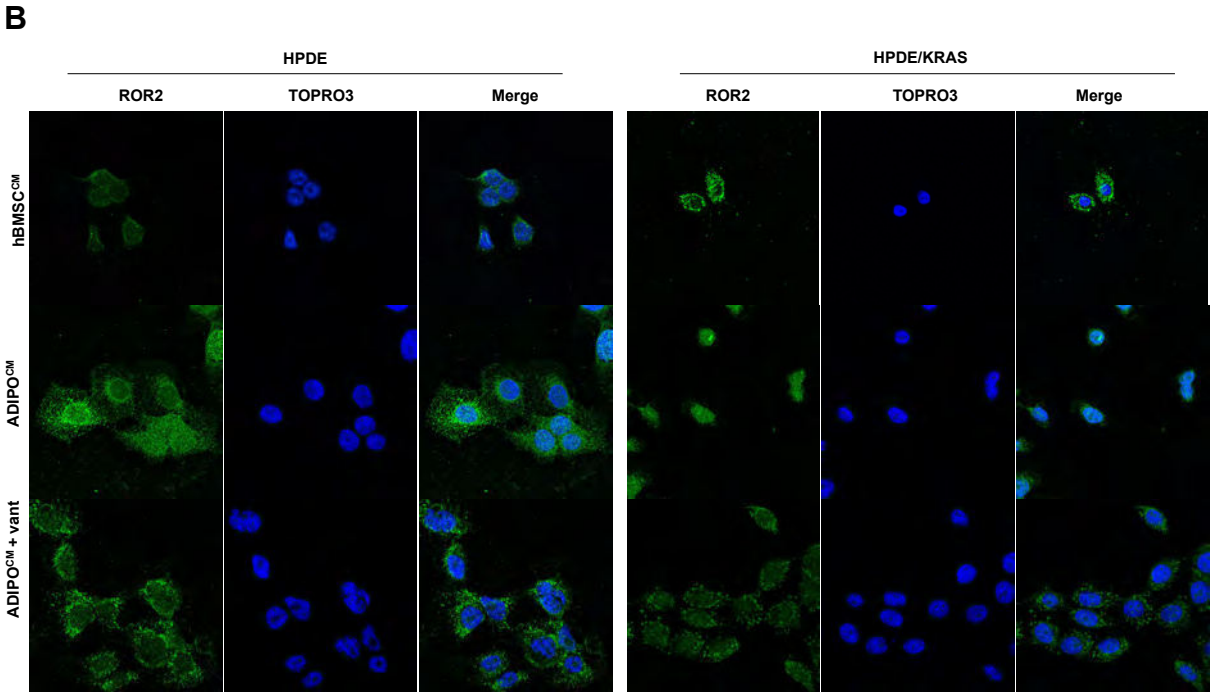
Table 1. Summary of the score for the nuclear-localization of ROR2 by NLS-mapper software.

Predicted Bipartite NLS			
Start position	Sequence	AA lenght	Score
242	**RSRTPKPRELCRDECEVLESDLCRQEYTIAR**	31	4
438	**TPQRRQLMASPSQDMEMPLINQHKQAKLK**	29	5.9
438	**TPQRRQLMASPSQDMEMPLINQHKQAKLKEI**	31	4.3
461	**KQAKLKEISLSAVRFMEELGEDRFGKVYKG**	30	7
465	**LKEISLSAVRFMEELGEDRFGKVYKG**	26	4.9
481	**EDRFGKVYKGHLFGPAPGEQTQAVAIKTLKDK**	32	4.4

Nuclear localizaion of ROR2 . Higher scores indicate stronger NLS activities:
Score of **8, 9**, or **10** is **exclusively** localized to the **nucleus**;
Score of **7** or **8 partially** localized to the **nucleus**,
Score of **3, 4,** or **5** localized to **both** the **nucleus and the cytoplasm**;
Score of **1** or **2** localized to the **cytoplasm**;

Figure 5


A
HPDE
HPDE/
KRAS
Cytosol
Nucleus
Cytosol
Nucleus
ADIPO^{CM}
ADIPO^{CM}
ADIPO^{CM}
ADIPO^{CM}
hBMSC^{CM}
+
+ vant
hBMSC^{CM}
+
+ vant
hBMSC^{CM}
+
+ vant
hBMSC^{CM}
+
+ vant
IB: ROR2 -105KDa
IB: ROR2 -50KDa
IB: GAPDH
IB: Histone H3


B
HPDE
HPDE/KRAS
ROR2
TOPRO3
Merge
ROR2
TOPRO3
Merge
hBMSC^{CM}
ADIPO^{CM}
ADIPO^{CM} + vant