Distinct Effects of Adipose-derived Stem Cells and Adipocytes on Normal and Cancer Cell Hierarchy

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Abstract:

Adipose-derived stem cells (ASCs) have received considerable attention in oncology because of the known direct link between obesity and cancer as well as the use of ASCs in reconstructive surgery after tumor ablation. Previous studies have documented how cancer cells commandeer ASCs to support their survival by altering extracellular matrix (ECM) composition and stiffness, migration, and metastasis. This study focused on delineating the effects of ASCs and adipocytes on the self-renewal of stem/progenitor cells and hierarchy of breast epithelial cells. The immortalized breast epithelial cell line MCF10A, ductal carcinoma in situ (DCIS) cell lines MCF10DCIS.com and SUM225, and MCF10A overexpressing SRC oncogene were examined using a mammosphere assay and flow cytometry for the effects of ASCs on their self-renewal and stem-luminal progenitor-differentiated cell surface marker profiles. Interestingly, ASCs promoted the self-renewal of all cell types except SUM225. ASC co-culture or treatment with ASC conditioned media (CM) altered the number of $CD49f^{high}/EpCAM^{low}$ basal/stem-like and $CD49f^{medium}/EpCAM^{medium}$ luminal progenitor cells. Among multiple factors secreted by ASCs, IFNγ and HGF displayed unique actions on epithelial cell hierarchy. IFNγ increased stem/progenitor-like cells while simultaneously reducing the size of mammospheres, whereas HGF increased the size of mammospheres with an accompanying increase in luminal progenitor cells. ASCs expressed higher levels of HGF, whereas adipocytes expressed higher levels of IFNγ. Since luminal progenitor cells are believed to be prone for transformation, IFNγ and HGF expression status of ASCs may influence susceptibility for developing breast cancer as well as on outcomes of autologous fat transplantation on residual/dormant tumor cells.

Implications: This study suggests that the ratio of adipose-derived stem cells to

adipocytes influences cancer cell hierarchy, which may impact incidence and progression.

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Introduction:

Adipose stem cells (ASCs) are increasingly being used for aesthetic and reconstructive surgery in multiple surgical disciplines (1-3). Their main utility lies in the repair of soft tissue contour deficits either as a result of aging (aesthetic) or tumor removal, trauma, and congenital malformations (reconstructive). While their use in cosmetic surgery is less controversial, there are some lingering doubts about their safety when used at sites of tumor extirpation. Additionally, injection of fat into hormonally sensitive sites such as the breast could carry long-term consequences. Long-term follow up data in these patients are still not available to ascertain the safety of ASCs in these patients. Nonetheless, it is important to investigate the effects of ASCs and adipocytes on cancer cells because of the established link between obesity and cancer (4). A recent review in Cancer Research identified 37 articles that investigated the influence of ASCs on cancer progression and metastases (5). ASC-derived factors such as Vascular Endothelial Growth Factor, Interleukin 6 (IL-6), IL-8, Tumor Necrosis Factor Alpha (TNFα), Hepatocyte Growth Factor (HGF), Leptin, and Insulin-like growth factor (IGF) are suggested to promote angiogenesis, inflammation, cell proliferation, and hypoxia in the tumor microenvironment (5-7). Reciprocally, breast tumor cells promote transdifferentiation of ASCs into cancer-associated fibroblasts, which enhance tissue stiffness (8,9).

In contrast to ASCs, the role of adipocytes in cancer progression remains controversial. Many reports indicated pro-tumor growth effect including adipocyte-mediated induction of epithelial to mesenchymal transition (EMT) and resistance to ERBB2-targeted therapy (10,11). The adipokine Lipocalin2 secreted by adipocytes

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linked obesity to breast cancer progression (12). However, a recent study showed reduced proliferation of breast cancer cells that are in direct contact with neighboring adipocytes (13).

There are several gaps in our understanding of the role of ASCs and adipocytes on breast cancer. The first, do normal and tumor cells respond similarly to ASCs? Second, to what extent do ASCs alter stem-luminal progenitor-differentiated cell hierarchy in normal and cancer breast epithelial cells? Addressing this question is critical because most breast cancers are believed to originate from luminal progenitor cells (14,15). Third, do ASCs and adipocytes have distinct effects on normal and tumor cells and if so, can these differences be attributed to ASCs and adipocytes-enriched chemokines/cytokines?

To address the above questions, we utilized an in vitro system where we measured the effects of conditioned medium (CM) from ASCs and ASC derived adipocytes on stem-luminal progenitor-differentiated cell hierarchy under growth conditions that promote self-renewal as well as differentiation of stem cells. Spontaneously immortalized breast epithelial cell line MCF10A, MCF10A cells with enhanced EMT characteristics due to overexpression of SLUG (MCF10A-SLUG) (16), a MCF10A derivative that generates ductal carcinoma in situ (MCF10DCIS.com) (17) and MCF10A cells transformed by the SRC oncogene (MCF10A-SRC) were tested (18). We demonstrate that ASCs and adipocytes have distinct effects on the hierarchy of immortalized and transformed breast epithelial cells. HGF, secreted at higher levels by ASCs, and interferon gamma (IFNγ), secreted at higher levels by adipocytes, altered breast stem-luminal progenitor-differentiated cell profiles. We propose that the inter-individual differences in the ratio of ASCs to adipocytes in fat tissue or the ability of ASCs to

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differentiate into adipocytes could influence the rate of differentiation of breast epithelial

cells with ultimate impact on breast cancer incidence and progression.

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Materials and Methods:

Cell lines and mammosphere assay: MCF10A and MCF10DCIS.com cell lines were

purchased from ATCC. MCF10A cells overexpressing SLUG have been described (16).

Dr. Kevin Struhl (Harvard Medical School) provided MCF10A cells overexpressing SRC

oncogene linked to the ligand binding domain of estrogen receptor (MCF10A-SRC) and

these cells have been described previously (18). SUM225 cells were purchased from

Asterand (Detroit, MI) and maintained in media recommended by Asterand. Primary

ASCs were isolated from abdomen, hips, axilla and/or flank and cultured as previously

described (19). SUM225 and MCF10DCIS.com cells were purchased for this project

alone and were cultured for the first time for this study. MCF10A cells have been

authenticated within last two years using STR Systems for Cell line identification (DNA

Diagnosis Center, Fairfield, OH). Supplementary Table 1 (Table S1) provides details of

sites of fat tissue collection, age, and BMI of individuals from whom ASCs were

collected. All procedures of collecting human adipose tissue were approved by the

Indiana University School of Medicine Institutional Review Board. All samples were

from females. These ASCs have been characterized by flow cytometry for cell surface

markers and for osteogenesis and adipogenesis as described previously (19,20). ASCs

were subjected to adipocyte differentiation by culturing in adipogenic differentiation

media (21). Conditioned medium (CM) from confluent cultures was used. The

mammosphere assay has been described previously (22). Briefly, 5000 cells were plated

on ultra-low adherent six well plates in MammoCult media from Stemcell Technologies

(Vancouver, Canada). In co-culture experiments, equal number of ASCs and Green

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Fluorescent Protein (GFP)-labeled MCF10A or MCF10A-SRC cells was plated.

Mammospheres were imaged and/or subjected to flow cytometry 6-10 days after plating.

Cytokines/chemokines/growth factors, Antibodies and flow cytometry: Human cytokine

array panel A (R&D systems, Minneapolis, MN, catalogue #ARY005) was used to

measure secreted factors in CMs of ASCs and adipocytes. Assays were done as per

instructions from the manufacturer. HGF, IFNγ, CXCL1, SERPINE1, IL-17E, IL23, and

CD40L were purchased from R&D Systems or Peprotech, Inc (Rocky Hills, NJ).

Concentrations of cytokines/chemokines/growth factors used in mammosphere assays are

indicated in figure legends. Antibodies against CD49f, CD44, CD24 and EpCAM used

for flow cytometry have been described in our recent publication (23). Mammospheres

were collected, washed and exposed briefly to trypsin to obtain single cell suspensions.

Cells were stained with indicated antibodies and subjected to flow cytometry. The

percentage of cells in each quadrant or region is presented in the manuscript. For 2D

cultures, cells were treated with indicated cytokines/chemokines/growth factors for 48

hours, collected by trypsinization, and subjected to flow cytometry.

RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-

PCR): RNeasy kit from Qiagen (Redwood City, CA) was used to isolate total RNA. qRT-

PCR was performed as described previously and expression levels were normalized to

GAPDH (22). Primers used were: HGF Forward 5'-TGG GAA CCA GAT GCA AGT

AAG-3', Reverse 5'-AAT GAG TGG ATT TCC CGT GTA G-3'; IFNγ Forward 5'-CCC

ATG GGT TGT GTG TTT ATT T-3', Reverse 5'-AAA CCG GCA GTA ACT GGA

TAG-3' and GAPDH 5'-GGT GTG AAC CAT GAG AAG TAT GA-3', Reverse 5'-

GAG TCC TTC CAC GAT ACC AAA G-3'.

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Statistical analysis: All data presented are mean ±standard deviation from 2-6

experiments. In experiments with similar trends but with experimental variability,

experiments with least variability were selected for statistical analysis. Student *t* test was

used to measure the statistical significance between measurements (Graphpad.com).

Results:

ASCs enhance self-renewal of breast epithelial cells

The mammosphere assay is used routinely as a surrogate assay to measure self-renewal;

previous studies in neurosphere cultures have shown that the size of the spheres indicates

the extent of self-renewal (24,25). We used this assay to determine the effect of ASCs on

self-renewal of immortalized and transformed breast epithelial cells. Since breast

epithelial cells are marked with GFP, spheres formed by breast epithelial cells and ASCs

could be easily distinguished by fluorescent microscopy. MCF10A cells formed smaller

spheres and the size of the spheres were much larger when co-cultured with ASCs

(Figure 1A). ASCs from three different individuals gave similar results. ASCs did not

have consistent effects on number of spheres but the sphere size increased. For example,

ASC #4 increased the size of spheres from 73 x 40 micrometer to 111 x 80 micrometer.

Furthermore, ASCs increased the size of the MCF10A-SRC-derived mammospheres

(from 80 x 69 micrometer to 170 x 144 micrometer). We next determined whether CM

from ASCs similarly influenced the size of mammospheres. Indeed, CM treatment alone

was sufficient to increase the size of mammospheres (Figure 1B). Thus, transacting

soluble factors from ASCs are sufficient to enhance the self-renewal of immortalized and

transformed breast epithelial cells.

ASCs expand basal/luminal progenitor cell population of MCF10A cells

Breast epithelial cells with basal/stem-like, luminal progenitor, and non-

clonogenic/differentiated characteristics display CD49f+/EpCAM-, CD49f+/EpCAM+,

and CD49f-/EpCAM+ cell surface marker profile, respectively (26,27). We used these

two marker combinations to determine the effects of ASCs on phenotype of cells in the

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mammospheres. ASCs themselves do not stain for CD49f or EpCAM (data not shown).

Nonetheless, we examined the phenotype of all cells in the co-culture or cells gated for

GFP positivity. ASC co-culture increased the number of CD49f+/EpCAM+ MCF10A

cells (Figure 2A). Results of two independent co-culture experiments are shown in Figure

2B. ASC CM also increased the number of CD49f+/EpCAM+ cells (Figure 2A).

We recently reported that the EMT-associated gene SLUG increases MCF10A

stemness (16). Since EMT is linked to various aspects of cancer progression including

drug resistance (28,29), we then examined whether ASCs can further alter the phenotype

of MCF10A-SLUG cells. ASC CM increased the size of MCF10A-SLUG-derived

mammospheres with corresponding elevation in number of cells with luminal progenitor

phenotype (Figure 2C).

Transformed cells respond differently to ASC and adipocyte CMs

To determine whether ASCs and adipocytes have different effects on breast

epithelial cells, we obtained ASCs from three additional individuals and collected CMs

from undifferentiated and adipocyte-differentiated ASCs. CMs from ASCs were more

effective than CMs from adipocytes in increasing the size of MCF10A-derived

mammospheres (Figure 3A). ASC CM (all three) but not adipocyte CM (one out of three)

increased the number of CD49f+/EpCAM- cells in the mammospheres (Figure 3B and C).

Note that these three samples were obtained from individuals with higher BMI and ASCs

from these individuals increased stem rather than luminal progenitor cells (Compare

Figures 2 and 3). Thus, there may be inter-individual variation in the effect of ASCs on

stem/progenitor composition of breast epithelial cells.

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CD44+/CD24- cells are considered breast cancer stem cells and CD44 expression alone is linked to undifferentiated status of breast epithelial cells (27,30). We used these two markers to characterize the mammospheres for undifferentiated cells. ASC CM increased CD44+/CD24- cells compared with adipocyte CMs (Figure 3B and D). Because of experimental variability, representative data are shown in this figure. Thus, based on expression pattern of two sets of markers, we conclude that ASCs increase the number of either luminal progenitor or basal/stem-like cells when cells are grown under mammosphere growth conditions.

We next determined the influence of CM from ASCs and adipocytes on MCF10A cells grown under 2D culture. Unlike in mammosphere growth condition, neither ASC CM nor adipocyte CM influenced the phenotype of MCF10A cells (Figure 3E and F).

To determine whether transformed and non-transformed cells respond differently to ASCs and adipocytes, we next investigated the effects of CM from ASCs and adipocytes on MCF10A-SRC cells. In contrast to MCF10A cells, both ASCs and adipocyte CM increased the size of MCF10A-SRC derived mammospheres (Figure 4A). Flow cytometry of mammospheres revealed that CM from both ASCs and adipocytes increased the number of luminal progenitor cells (Figure 4B). Please note that ASC CM and adipocyte CM had minimum effects on CD44 and CD24 status under mammosphere growth condition (Figure 4B). However, under 2D condition, adipocyte CM but not ASC CM increased the number of CD44+/CD24- undifferentiated cancer stem-like cells while neither CM had an effect on CD49f/EpCAM status (Figure 4C and D). Thus, transformed status and culture conditions determine the influence of ASCs and adipocytes on breast epithelial cells.

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ASCs increase the number of luminal progenitors in ductal carcinoma in situ (DCIS) cells

Identifying features of DCIS that can predict the potential to re-emerge as invasive cancer has been a great challenge and is an unmet need (31). Clinically, increased BMI is associated with ER-positivity and radiographic abnormalities of DCIS (32). To determine whether ASCs influence the self-renewal and differentiation status of DCIS, we examined two cell lines that are routinely used to study DCIS. MCF10DCIS.com cells give rise to DCIS like lesions when implanted subcutaneously into nude mice (17). ASC but not adipocyte CM increased the size of mammospheres (Figure 5A). ASC CM increased the number of luminal progenitor cells (CD49f^{high}/EpCAM+) and cancer stem like undifferentiated (CD44+/CD24-) cells (Figure 5B and C). Adipocyte CM was ineffective in altering differentiation/stem status of these cells (Figure 5D).

The cell line SUM225 is also being used as a model for DCIS (33). Unlike all other cell types tested, SUM225 cells were unresponsive to ASC and adipocyte CM both with respect to mammosphere formation and changes in the marker profiles (Figure S1). SUM225 cells overexpress ERBB2 oncogene and it is possible that ASCs and ERBB2 activate overlapping signaling pathways to alter differentiation status of breast epithelial cells.

Individual variation in soluble factors secreted by ASCs

We performed cytokine array analysis of ASC CM (two patients) and adipocyte CM (one patient) to identify secreted factors that may alter differentiation of breast epithelial cells. Factors identified include CCL2, CD40L, CD54, CSF2, CXCL1,

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CXCL11, IFNγ, IL-6, IL-8, IL-16, IL17E, IL-23, and SERPINE1 (Figure S2). CD40L, CSF2, IFNγ, IL-23, and SERPINE1 were common between ASCs of two patients. Differentiation into adipocytes reduced the levels of these factors with the exception of IL-23.

HGF and IFNγ alter mammosphere size and/or differentiation status of breast epithelial cells

We systematically analyzed the effects of recombinant proteins corresponding to several ASC secreted factors for mammosphere formation and/or alteration in stem/progenitors in mammospheres. Only positive results are described below. We included HGF in this assay because previous studies have shown an important role of ASC-derived HGF on invasion of breast cancer cells (6). HGF and IFNγ consistently increased the size of mammospheres derived from MCF10DCIS.com cells (Figure 6A). However, in transformed MCF10A-SRC cells, HGF increased but IFNγ reduced the size of mammospheres (Figure S3A). None of the other factors had an effect on the size of mammospheres (data not shown). In MCF10A cells, HGF had no effect on the size of the mammospheres, whereas IFNγ reduced the size of mammospheres (Figure S4A).

We next determined the phenotypic effect of HGF and IFNγ on cells in the mammosphere. Similar to the effects of ASC CM, HGF altered CD49f^{low}/EpCAM+ and CD49f+/EpCAM+ ratio in favor of elevated CD49f+/EpCAM+ progenitor MCF10DCIS.com cells (Figure 6B and C). Similar effects were noted with IFNγ, although there was marked experimental variability. Note that neither HGF nor IFNγ altered CD44/CD24 staining pattern in MCF10DCIS.com-derived mammospheres,

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although overall CD44 staining intensity was higher in HGF-treated and lower in IFNγ-treated mammospheres.

HGF and IFNγ displayed distinct effects on the phenotype of MCF10A-SRC-derived mammospheres. While HGF increased CD49f+/EpCAM+ luminal progenitor cells, IFNγ increased the number of stem cells (Figure S3B). In fact, IFNγ increased the intensity of CD49f staining, whereas HGF increased EpCAM staining intensity (Figure S3C). In case of MCF10A cells, HGF increased the number of CD44+/CD24- cells similar to ASC CM albeit weakly (Figure S4B). IFNγ increased the number of CD49f+/EpCAM+ progenitor and CD49f+/EpCAM- stem/basal cells (Figure S4C and D). It appears that IFNγ blocked differentiation of stem/progenitor cells as mammospheres had no CD49f-/EpCAM+ cells. These results show that HGF and IFNγ distinctly alter the stem or progenitor cell composition of immortalized, DCIS, and invasive cancer cells. Since the effects of ASC CM and HGF/IFNγ overlapped partially, it appears that combined effects of multiple factors in ASC CM contribute to mammosphere expansion.

To determine whether there is any relationship between HGF and IFNγ expression in ASCs and adipocytes, we measured their mRNA levels by qRT-PCR. Differentiation of ASC to adipocytes caused significant drop in HGF levels with concomitant increase in IFNγ levels (Figure 6D).

HGF/IFNγ mRNA ratio in the breast tumor microenvironment predicts outcome in patients with ER- or PR- breast cancers

Since adipocyte differentiation altered the ratio between HGF and IFNγ, we next determined whether the ratio between these two factors within the breast cancer environment has any prognostic value using a tool we developed recently (34).

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HGF/IFNγ ratio can be a surrogate for ASCs and adipocytes composition in the tumor

microenvironment. cBioportal analysis showed a tendency of mutual exclusivity in their

expression in breast cancer (data not shown). In the TCGA dataset, elevated HGF/IFNγ

ratio was associated with poor overall survival among patients with Estrogen Receptor

(ER)-negative and Progesterone Receptor (PR)-negative but not ER-positive and PR-

positive breast cancers (Figure S5). Since ER-negative and PR-negative breast cancers

are associated with poor outcome, ASC/adipocyte ratio may influence outcome in

patients with these highly aggressive forms of breast cancer.

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Discussion:

Multiple mechanisms have been proposed to link adiposity with cancer incidence and/or outcome (4). Several pro-tumorigenic and anti-tumorigenic factors have been identified in both ASCs and adipocytes. For example, adipocyte secreted leptin and adiponectin have opposing biological effects in the normal breast, which impacts breast cancer susceptibility (35). Xenograft models have shown that the type of adipocytes, particularly beige/brown adipocytes, influence breast tumor development and progression (36). The majority of mechanistic studies on the effects of adiposity on breast cancer have focused on leptin signaling, including leptin-STAT3-G9a axis, leptin-induced EMT, and angiogenesis (37-39). Several other soluble factors from ASCs have been implicated in this process including VEGF, IL-8, IGF, and HGF (5). A recent "meta PubMed" search identified 37 studies that have linked ASCs to cancer progression with the majority indicating unfavorable effects (5). Concurrently, breast cancer-derived factors themselves have been shown to alter plasticity and differentiation capacity of ASCs and promote their differentiation into cancer-associated fibroblasts (8,40).

Adiposity has not only been linked to cancer incidence but also to the molecular subtypes of breast cancer (4). For example, in premenopausal women, adiposity is associated with ~10% risk reduction in less aggressive ER+/PR+ breast cancers but ~80% increase in risk of aggressive ER-/PR- and triple negative breast cancers. In post-menopausal women, adiposity increases risk for both ER+/PR+ and ER-/PR- breast cancers. While multiple studies have independently analyzed the effects of ASCs and adipocytes, there has not been a comprehensive study comparing the effects of ASCs and adipocytes on "progression series" of breast epithelial cells, particularly in the context of

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epithelial cell hierarchy. Our study has addressed this issue by measuring the effects of ASCs and their differentiated counterparts on immortalized breast epithelial cells, non-invasive cancer cells representing DCIS, and invasive breast cancer cells. While ASCs were more effective than adipocytes in altering stem/progenitor cell composition of immortalized and non-invasive cancer cells, adipocytes were as effective as ASCs in altering progenitor/stem cell composition of invasive cancer cells. Please note that we did not observe a consistent increase in the number of mammospheres by any treatment suggesting that ASCs do not cause dedifferentiation of differentiated cells to stem/progenitor cells. Instead, ASCs or adipocytes have an effect on the self-renewal or expansion of existing pool of stem/progenitor cells. We also observed a lack of effect of ASC or adipocytes on a DCIS cell line with ERBB2 amplification suggesting that certain cancer-associated genomic events activate signaling cascades that overlap with adiposity-driven signals. Additional studies with multiple models are needed to identify the cancer subtypes that are not impacted by adiposity or ASC/adipocyte ratio.

The differential effect of ASC and adipocyte CM on non-invasive and invasive breast cancer cells is intriguing. We found ASC CM containing more soluble factors than adipocyte CM with very few being common to both. It is interesting that both ASC and adipocyte CM secrete IL-23, a cytokine that suppresses immune response during tumor initiation, growth, and metastasis (41,42). Therefore, ASCs and adipocytes may play a similar role in blocking host immune response to tumor. Additionally, adiposity may have an impact on response to recently developed immune checkpoint therapy for cancer (43).

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While the role of ASC-derived HGF on breast cancer cells has been studied (6), its influence on breast epithelial cell differentiation is unknown. Our studies indicate the ability of HGF to expand luminal progenitor cell population. Luminal progenitor cells in breast epithelial cell hierarchy are believed to be the most susceptible cell population for transformation and ASC composition in the breast may determine the proportion of cancer susceptible subpopulations of breast epithelial cells (14). Although HGF-mediated signaling events that promote expansion of luminal progenitor cells are yet to be elucidated, HGF-cMET axis has been shown to activate β-Catenin, which is a critical transcription factor in breast stem/progenitor cells (6,44).

We noticed unique effects of IFNγ on breast epithelial and cancer cells. Although we did not observe a uniform effect of IFNγ on the size of mammospheres, it promoted the accumulation of stem-like or progenitor cells and possibly blocked differentiation of MCF10A cells. It is unclear at present whether IFNγ selectively kills differentiated cells, promotes dedifferentiation of differentiated cells, or blocks differentiation at progenitor cell state. Nonetheless, the observed effect of IFNγ is significant because its expression is induced upon differentiation of ASCs to adipocytes (Figure 6) and its higher expression compared to HGF is associated with favorable outcome in ER- and PR- breast cancer patients (Figure S5). It is possible that IFNγ promotes dormancy of tumor cells by inhibiting their cell cycle progression and maintaining them in G0/G1 stem-like state. IFNγ has previously been shown to inhibit cell cycle progression of mammary epithelial cells by inducing the expression of p27kip1 (45). A recent study has shown stem cell-like properties of p27kip1-positive breast epithelial cells (46). Collectively, our studies suggest that ASC/adipocyte composition in the breast microenvironment determines

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HGF/IFNγ ratio, which ultimately has an impact on differentiation status of normal or cancerous breast epithelial cells. While this manuscript was being prepared for submission, Picon-Ruiz et al showed that immature and mature adipocytes promote cytokine production and drive SRC/SOX2/miR302b-mediated stem cell signaling in breast cancer cells (47). However, the study did not investigate adipocyte-derived factors involved in promoting cytokine production by cancer cells. It is possible that HGF/IFNγ levels might determine to extent to which ASCs/adipocytes influence feed-forward cytokine production loop in the breast environment.

Translation of these findings into clinical practice requires further study. While removal of the tumor is obviously important, reconstructive options to improve quality of life gain in meaning as long-term survival increases. One of the new options is the transplantation of adipose tissue augmented with or without ASCs into the breast extirpation site. This current study is the first to show that the ratio of adipocytes and ASCs may be a critical influencer of breast cancer cell hierarchy, with some of the control over this decision in the hands of the reconstructive surgeon. However, individual variations in the ability of ASCs to differentiate to adipocytes, the levels of secreted HGF and IFNγ, as well as the genomic aberration status and/or molecular subtypes of any residual tumor cells may ultimately determine the safety of ASC/adipocyte transplantation. Characterization of ASCs and tumors on an individual basis may help to establish safety levels and provide rational guidelines for safer decisions.

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Figure Legends:

Figure 1: ASCs increased the size of mammospheres: A) Effects of ASC co-culture on the size of mammospheres derived from MCF10A and MCF10A-SRC cells. Both cell lines express GFP and GFP-positive spheres were visualized under fluorescent microscope. ASCs themselves formed spheres, which were not fluorescent (bottom panel). B) ASC CM increased the size of mammospheres. CM from ASC #4 was used in this assay.

Figure 2: ASCs increased the number of MCF10A and MCF10A-SLUG cells with luminal progenitor features. A) CD49f/EpCAM staining pattern of GFP expressing MCF10A cells with or without ASC #4 coculture or CM. Under both conditions, ASCs increased CD49f+/EpCAM+ cells (with or without gating for GFP+ cells). B) Results of two independent experiments are shown. C) ASC CM increased the size of spheres and the luminal progenitor pool of MCF10A-SLUG cells. Image 10X in all figures. Results from three independent experiments are shown (right side).

Figure 3: ASCs and adipocytes displayed distinct effects on MCF10A cells: A) Effects of ASC and adipocyte CMs on MCF10A-derived mammospheres. Although MCF10A cells do not efficiently form the mammospheres, spheres formed in the presence of ASC CMs were larger in size. B) ASC CM increased the number of CD44+/CD24- and CD49f+/EpCAM- stem cells. Adipocyte CM was less efficient in inducing these changes. C) ASC CM was more efficient than adipocyte CM in increasing CD49f+/EpCAM- cells in mammospheres. D) ASC CM was more efficient than adipocyte CM in increasing the

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number of CD44+/CD24- cells in mammospheres. E) ASC and adipocyte CMs did not

alter CD49f/EpCAM profile of MCF10A cells under 2D culture condition. Cells were

maintained in DMEM/F12 with 10% FBS for five hours without any supplements,

switched to serum-free media for 48 hours and then treated with CM for 48 hours. F)

ASC and adipocyte CMs did not alter the CD44/CD24 profile of MCF10A cells under 2D

culture condition.

Figure 4: CM from both ASC and adipocytes increased the size of MCF10A-SRC-

derived mammospheres. A) Phase contrast images of mammospheres under three

conditions (10X). B) CD49f/EpCAM and CD44/CD24 profiles of MCF10A-SRC-derived

mammospheres under ASC or adipocyte CM-treated conditions. Both ASC and adipocyte

CMs increased CD49f+/EpCAM+ cells. ASC and adipocyte CMs did not affect CD44

and CD24 status, as these spheres were predominantly CD44+/CD24-. Results using CM

from ASC/adipocytes of patient #1 and 2 are shown. C) ASC and adipocyte CMs did not

alter CD49f/EpCAM status of MCF10A-SRC cells grown under 2D condition. D)

Adipocyte CM but not ASC CM increased CD44+/CD24- MCF10A-SRC cells grown

under 2D condition.

Figure 5: CM from ASCs but not adipocytes increased the size of MCF10DCIS.com-

derived mammospheres. A) Phase contrast images of mammospheres under three

conditions (10X). B) CD44/CD24 and CD49f/EpCAM staining pattern of cells from

mammospheres. C) ASC CM increased CD49f+/EpCAM+ and CD44+/CD24- cells in

patient-specific manner. Results of two or more experiments are shown. *p<0.05 Control

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versus ASC CM-treated condition. D) Adipocyte CM did not alter CD49f/EpCAM staining pattern of cells from mammospheres.

Figure 6: HGF and IFNγ had distinct effects on MCF10DCIS.com mammospheres. A) Effects of recombinant HGF (50 ng/ml) and IFNγ (50 ng/ml) on MCF10DCIS.com-derived mammospheres. Mammospheres were treated for 8-10 days with addition of new media ± recombinant proteins every three days. B) CD44/CD24 and CD49f/EpCAM staining pattern of mammospheres with and without HGF and IFNγ treatment. C) Both HGF and IFNγ increased the number of CD49f+/EpCAM+ cells. *$p<0.007$, control versus HGF or IFNγ, **$p<0.05$ HGF versus IFNγ. D) ASC to adipocyte differentiation caused reduction in HGF but an increase in IFNγ. All differences are statistically significant.
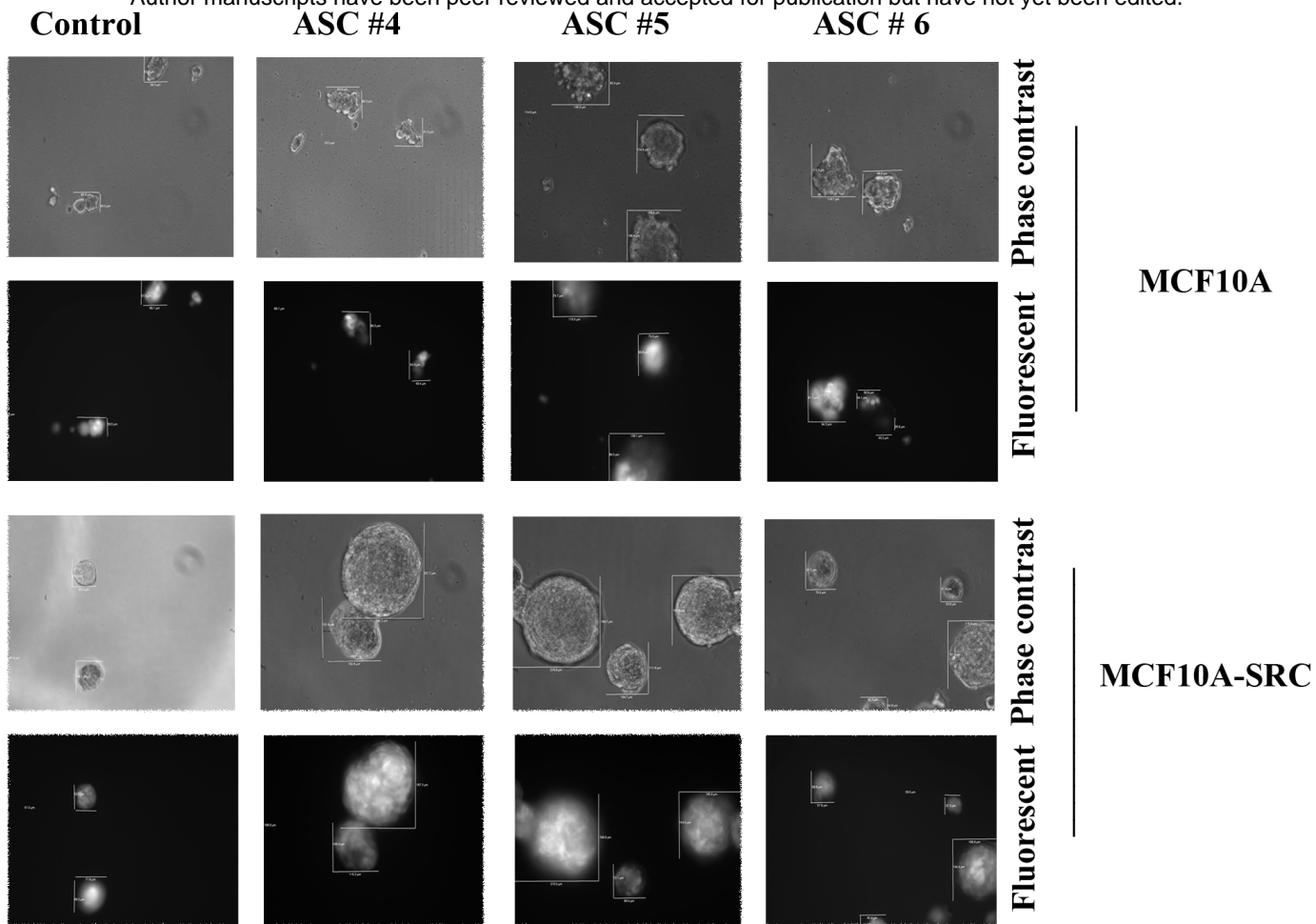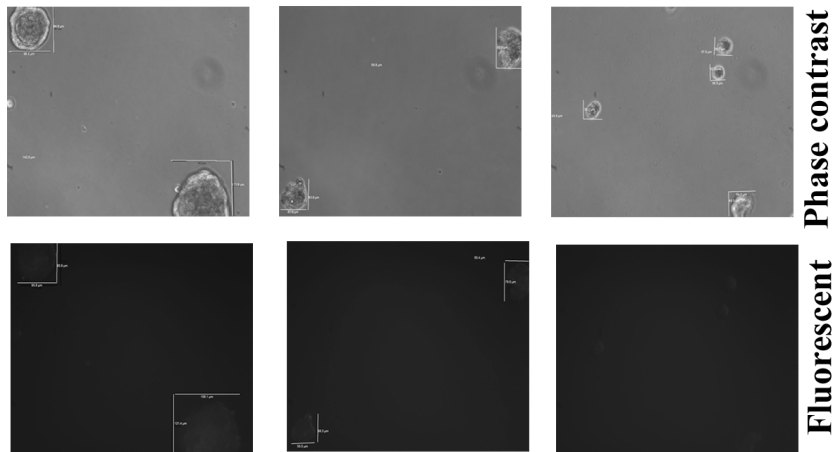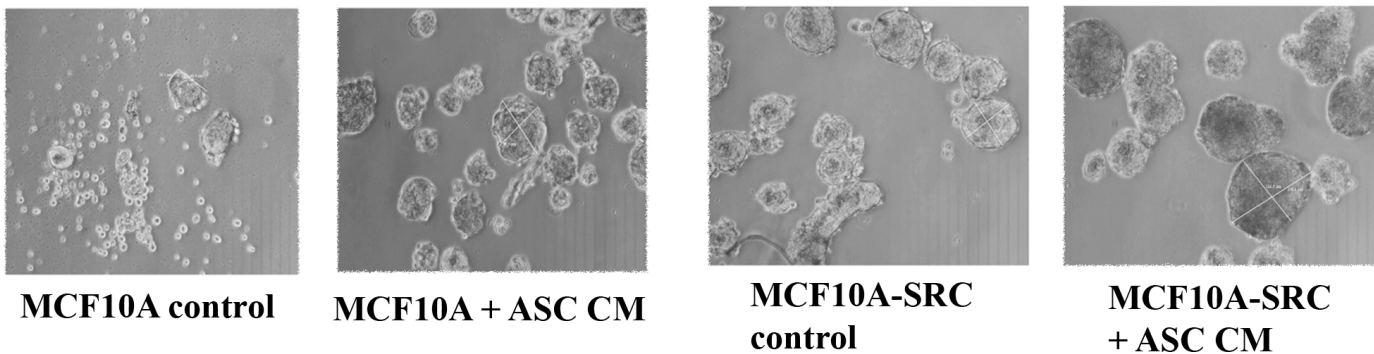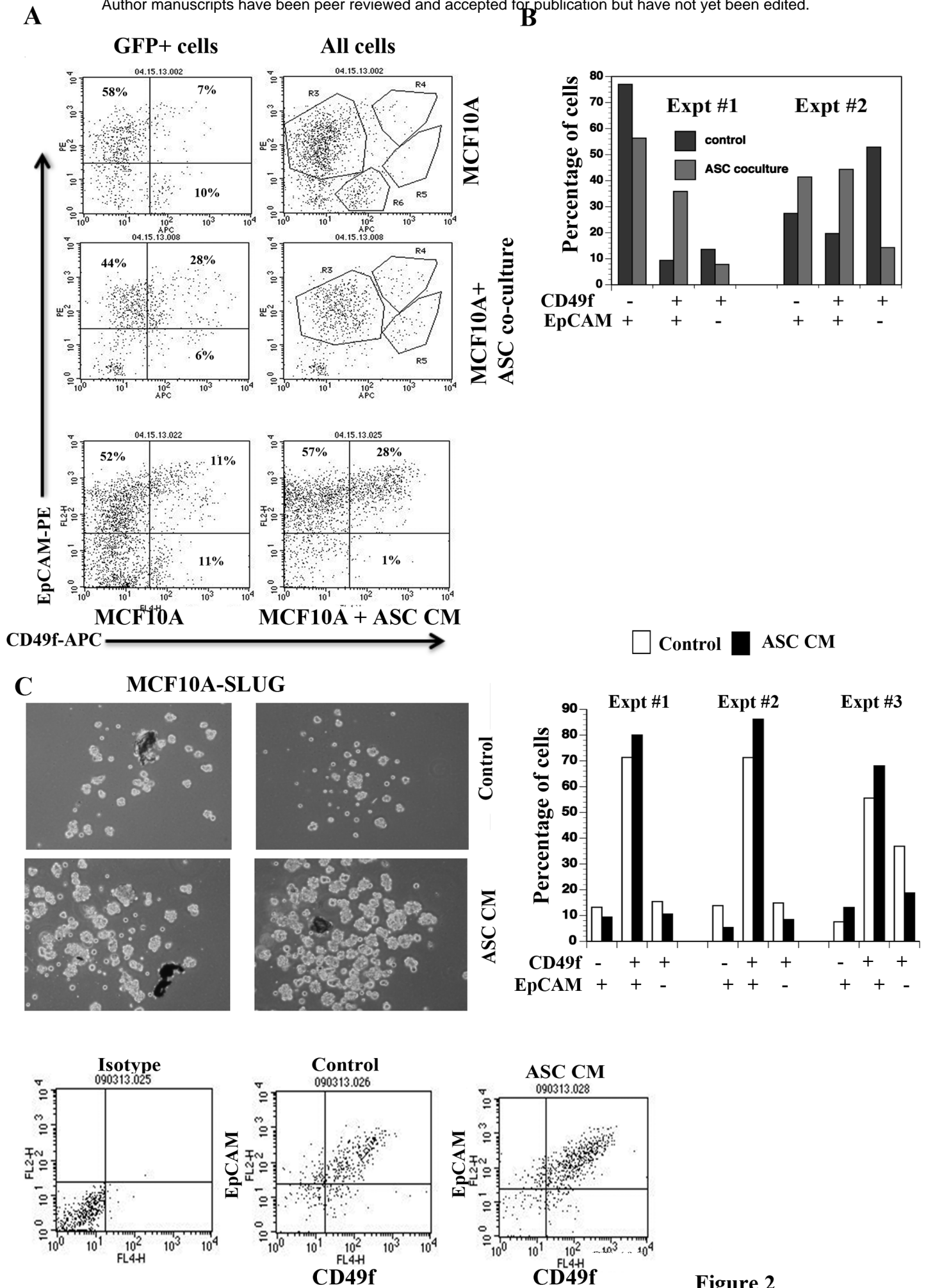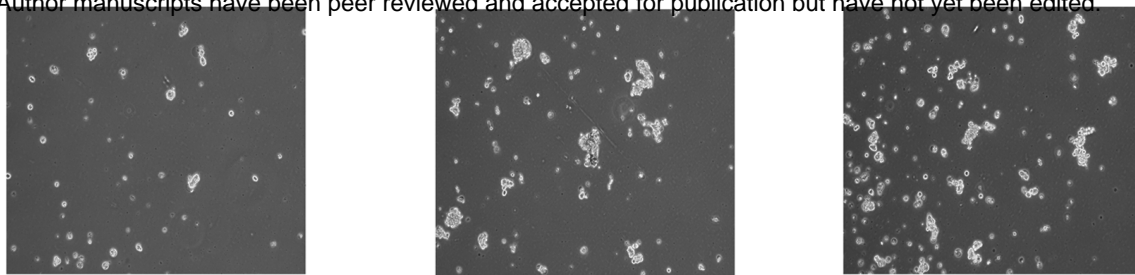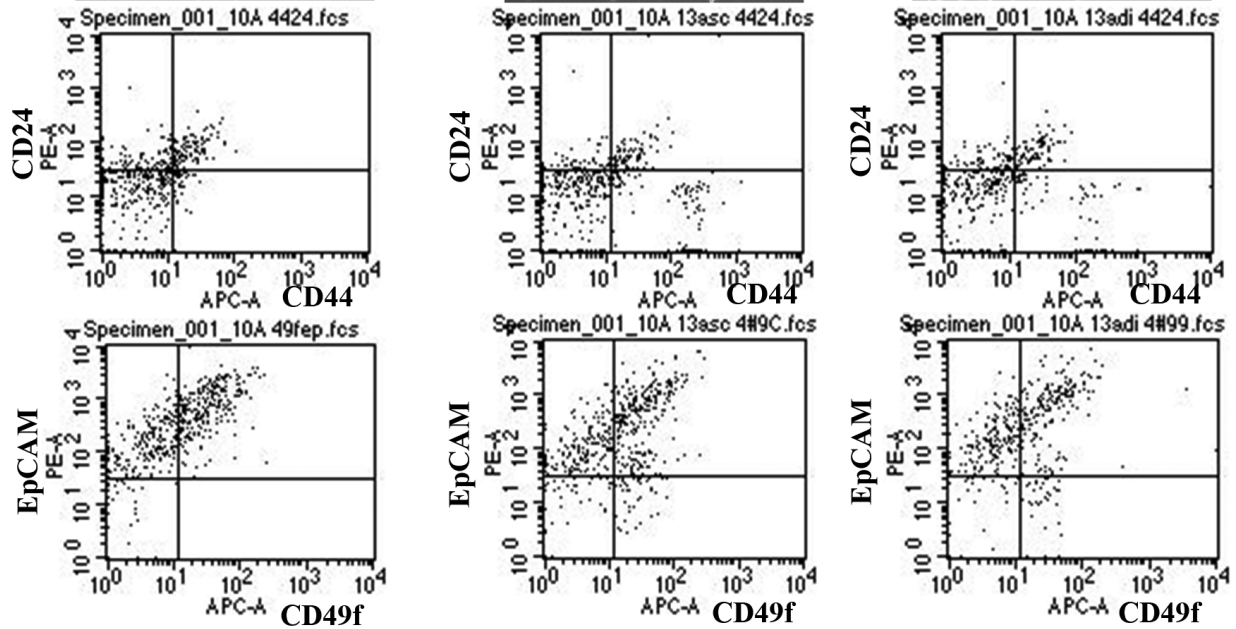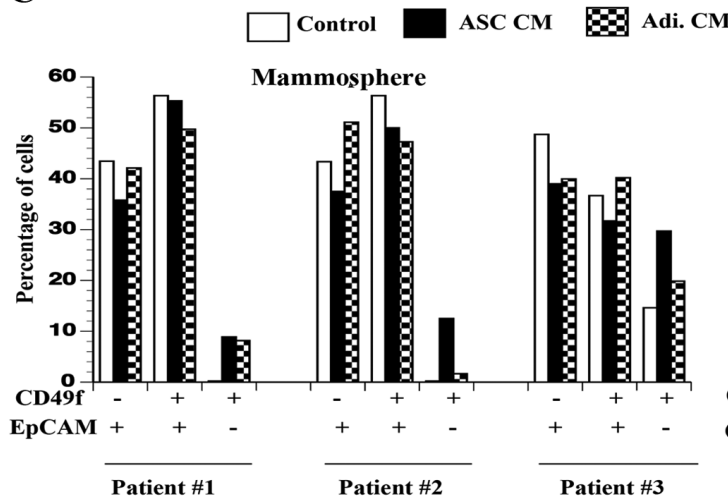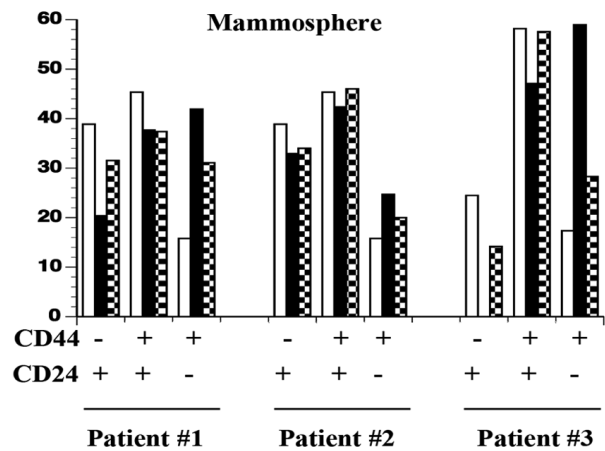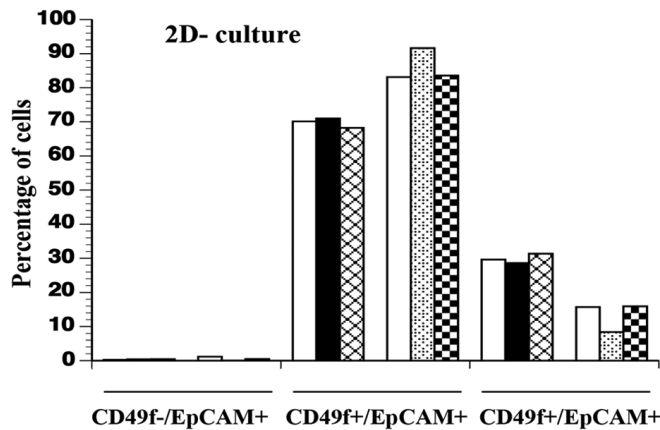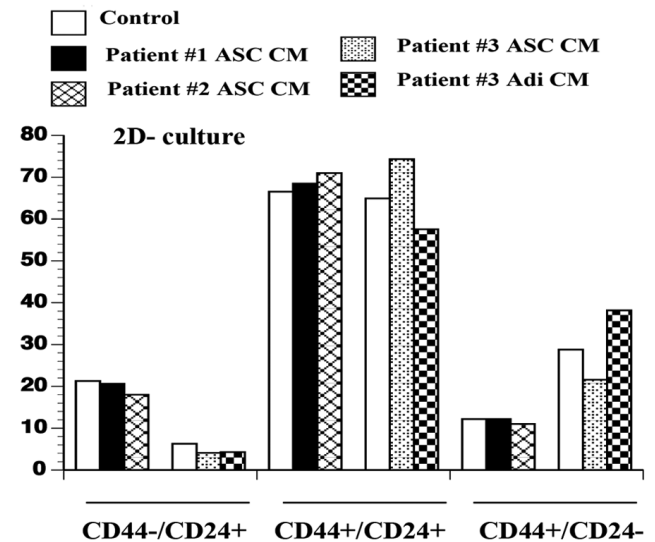
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Figure 1

A

GFP+ cells

All cells

MCF10A

MCF10A + ASC co-culture

EpCAM-PE

MCF10A

MCF10A + ASC CM

CD49f-APC

B

Percentage of cells

Expt #1   Expt #2

control
ASC coculture

CD49f − + +   − + +
EpCAM + + −   + + −

Control   ASC CM

C

MCF10A-SLUG

Control

ASC CM

Percentage of cells

Expt #1   Expt #2   Expt #3

CD49f − + +   − + +   − + +
EpCAM + + −   + + −   + + −

Isotype   Control   ASC CM

EpCAM

CD49f   CD49f

Figure 2

Figure 3

Figure 4

Figure 5

A

B

C

D

Figure 6

AACR American Association
for Cancer Research
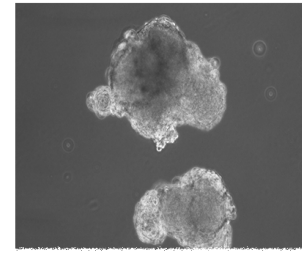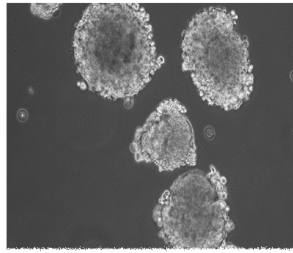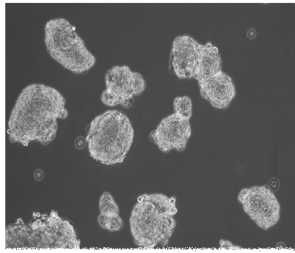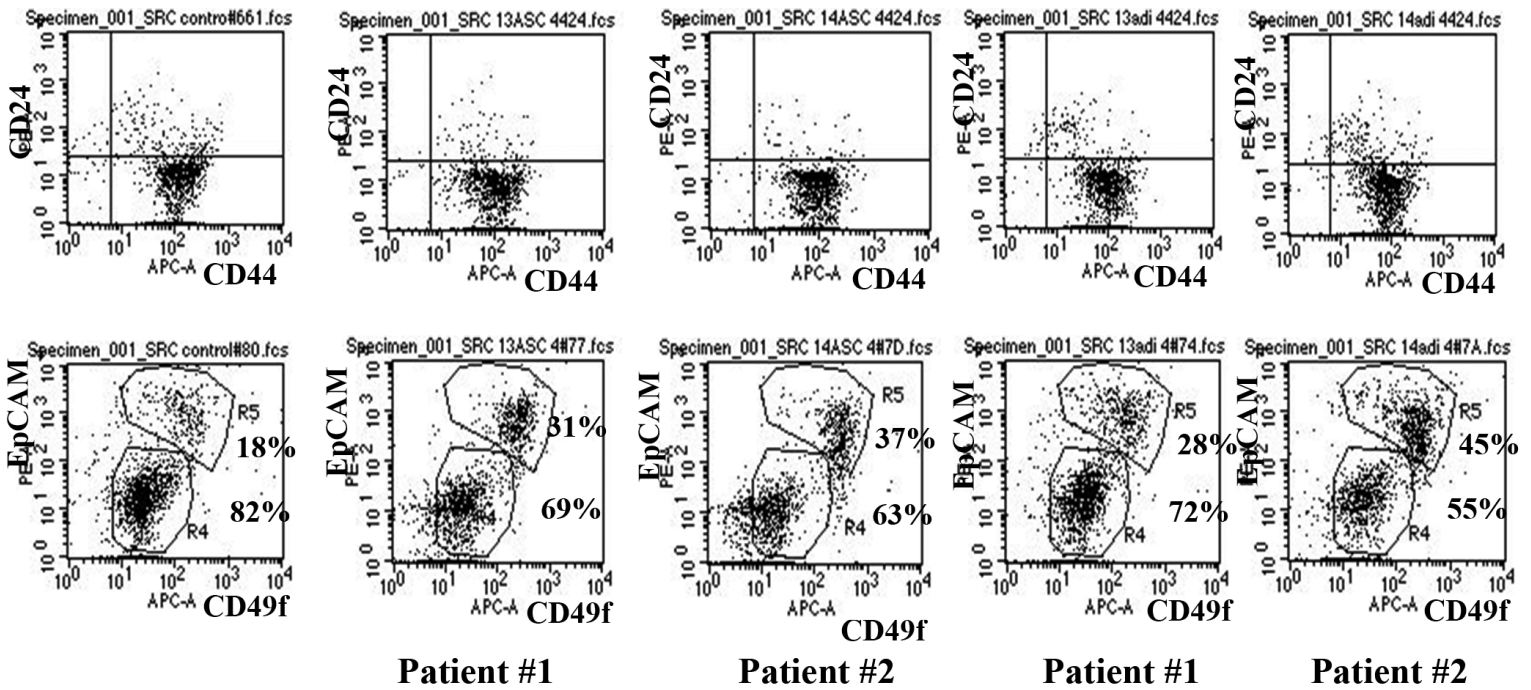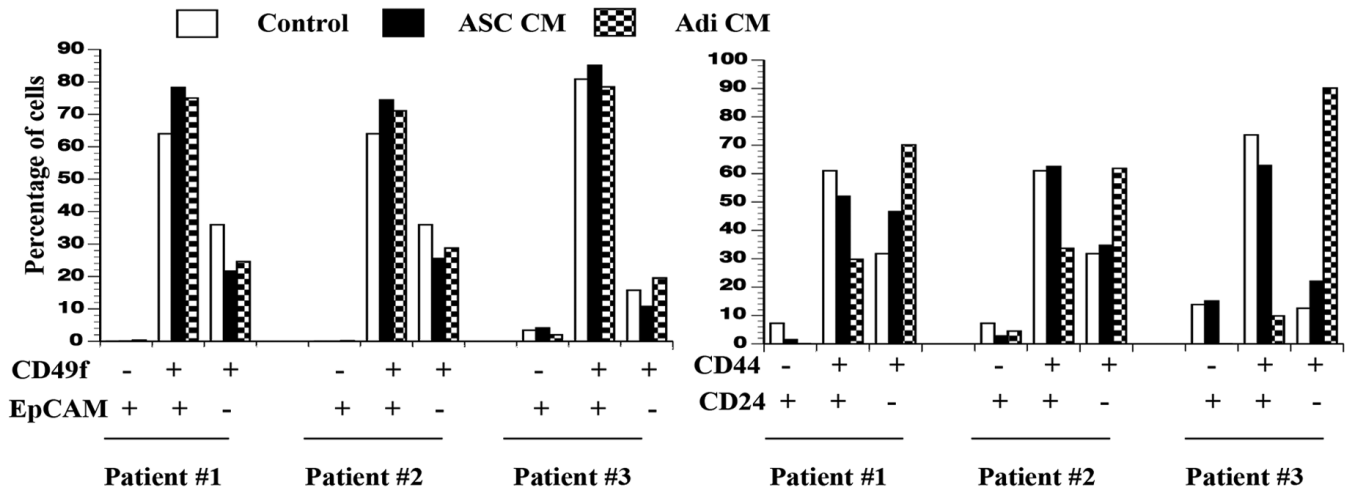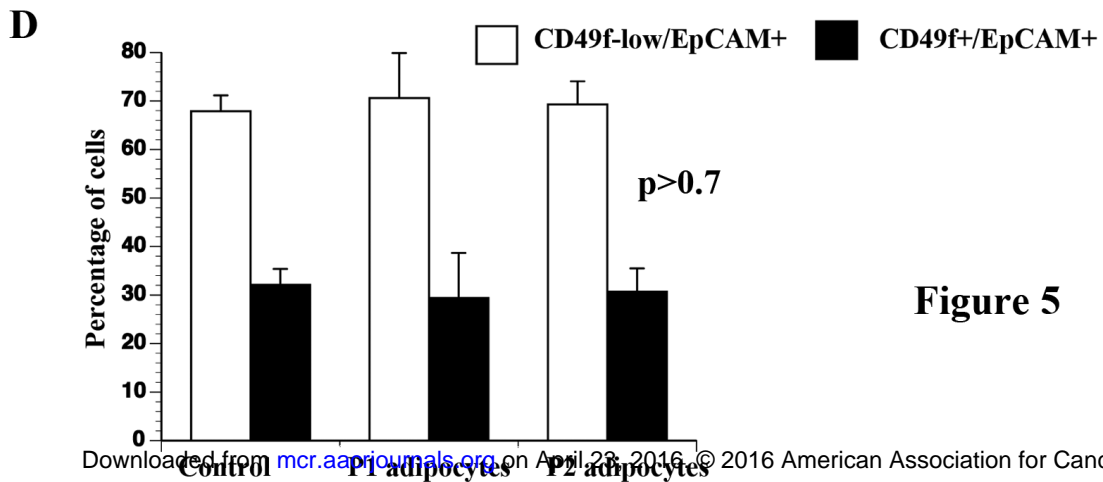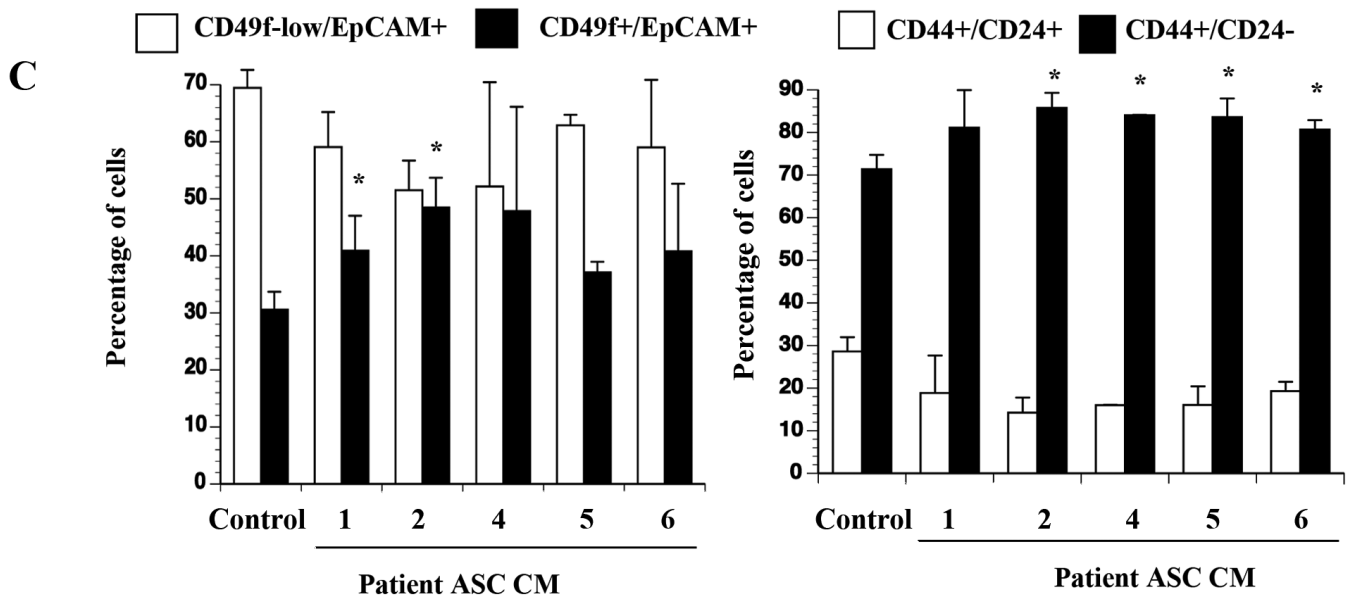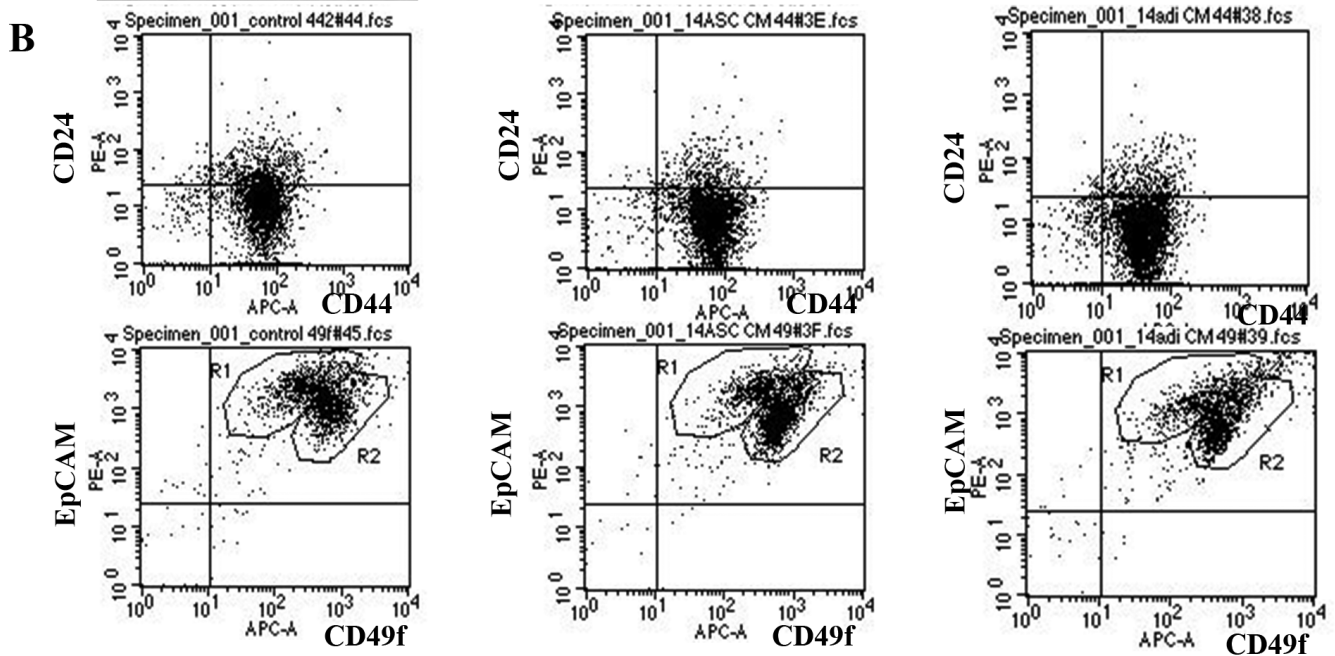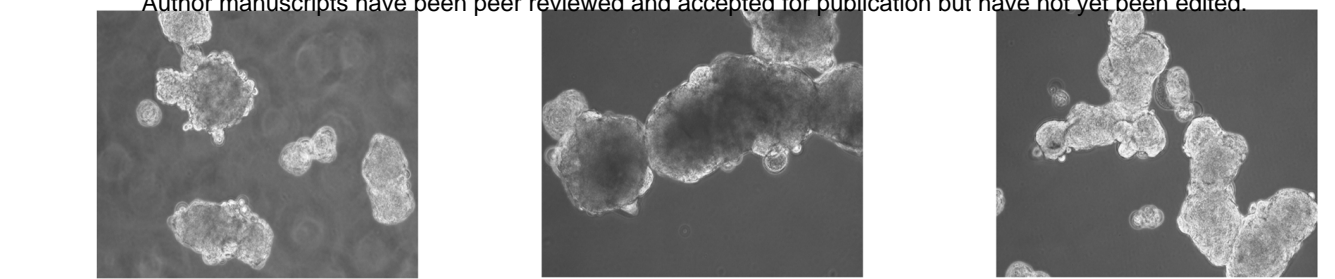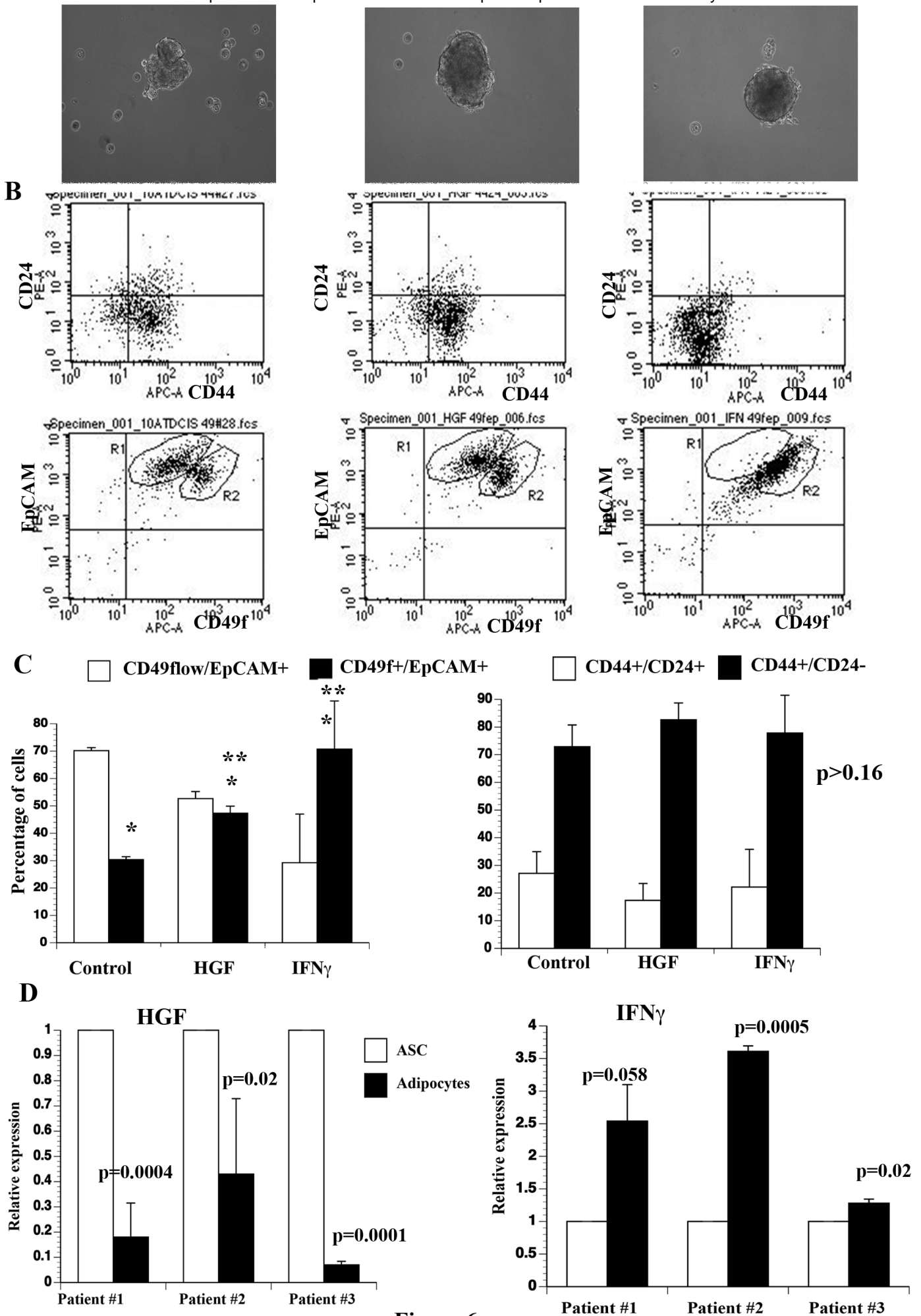
Molecular Cancer Research

Distinct Effects of Adipose-derived Stem Cells and Adipocytes on Normal and Cancer Cell Hierarchy

Manjushree Anjanappa, Riesa Burnett, Michael A Zieger, et al.

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