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PRINCIPAL INVESTIGATOR: Andrew J. Armstrong, M.D.

CONTRACTING ORGANIZATION: Duke University
Durham, NC 27705

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Andrew J. Armstrong, M.D.

E-Mail: andrew.armstrong@duke.edu

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14. ABSTRACT

The purpose of this DOD PRTA is to investigate the role of epithelial plasticity in the promotion of metastasis in advanced prostate cancer (PC) through the interrogation of biomarkers in primary PC, metastases, and circulating tumor cells (CTCs). Epithelial plasticity biomarkers inclusive of epithelial-mesenchymal transition (EMT) and stemness phenotypes were evaluated in a prospective correlative study of men with metastatic castration-resistant PC in which CTCs were collected and analyzed. These results unequivocally demonstrated that the preponderance (>80%) of CTCs from these men co-expressed both epithelial (cytokeratin, EpCAM, E-cadherin) and mesenchymal (N- and O-cadherin, vimentin) and stemness (CD133) proteins. These results are published in Molecular Cancer Research and provide the strongest direct evidence to date for the existence of EMT in human cancer. In year 1, we have also begun to analyze EMT marker expression in a tissue microarray of radical prostatectomy specimens linked to long-term follow up. Initial results suggest feasibility/validity, and we are in the process of antibody optimization for additional studies of EMT/stemness as it relates to prostate cancer recurrence. We have also begun a collaborative research process with Veridex to develop a novel CTC capture ferrofluid to identify non-epithelial CTCs from the blood of men with CRPC.

15. SUBJECT TERMS
Circulating tumor cells, prostate cancer, epithelial plasticity, epithelial mesenchymal transition, metastasis, lethal phenotype, biomarkers, training award

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INTRODUCTION

In 2011, over 75 U.S. men will die every day from prostate cancer (PC)(1). Many deaths could potentially be prevented through identification and treatment directed at high risk disease. Currently, clinical/pathologic measures (i.e. PSA, stage, grade) provide no biologic insights into the process by which PC cells metastasize and become lethal. The measurement of circulating tumor cells (CTCs) in men with PC represents one biomarker with prognostic and predictive implications(2). Many patients with metastatic PC, however, have undetectable CTCs, limiting clinical utility. We have identified epithelial-mesenchymal transitions (EMT) in experimental models of PC in which the cellular phenotype undergoes reversible (plastic) changes from an epithelial to a mesenchymal nature facilitating metastatic spread, followed by epithelial reversion in the target metastatic organ(3). While in the active process of metastasis, CTCs may possess a mesenchymal/plastic phenotype, and thus may not be captured by existing epithelial-based CTC technologies. In this PRTA annual report, we provide evidence for the common co-expression of epithelial and mesenchymal/EMT biomarkers in CTCs from patients with metastatic castration-resistant prostate cancer (CRPC) as well as the common expression of stem cell biomarkers in these CTCs(4). This data provides strong evidence for the importance of EMT to prostate cancer metastasis in humans. We are also in the process of investigating these biomarkers in localized prostate cancers prior to metastasis in order to correlate their expression with long term recurrence and metastasis risk. Based on these results, we have developed a novel CTC capture method based on this EMT biology to identify non-epithelial CTCs which are previously underdetected using conventional CTC assays; this method will be further investigated in years 2-5. Finally, in this PRTA, we will investigate broad RNA expression profiles and DNA copy number changes in CTCs vs. paired metastases from men with CRPC in order to identify targets for therapeutic intervention, better understand the process of metastatic dissemination, and the phenotypic plasticity and monoclonality of prostate cancer metastases. Together these aims may provide insight into metastasis biology in PC and lead to the identification of relevant targets for therapies directed against this lethal metastatic process.

BODY

Task 1: To link evidence of epithelial plasticity with adverse clinical features and PSA-based outcomes using a Durham VA Hospital prostate cancer tissue microarray and the SEARCH database (Months 1-36)

Key Research Accomplishments: During year 1, the primary aim was to obtain IRB approval at the Durham VA Medical Center and to contruct and validate the tissue microarray of radical prostatectomy (RP) specimens. In this TMA, 207 men contributed RP tissues with linkage to clinical and pathologic data as well as outcomes data over a 4-5 year follow-up period. We obtained approval for this work and completed the construction of this TMA and linked clinical data through the SEARCH database as described in specific aim 1 of this PRTA. In order to assess EMT biomarkers in prostate tissue, we began by testing a positive control biomarker, Ki-67, which has been shown in multiple studies to correlate with adverse pathologic features and PSA recurrence after surgery. In evaluating this biomarker, the TMAs were stained using a standard Ki-67 antibody (Dako IgG1 clone MIB-1, 1:50 dilution) and evaluated fashion using a 0-100% scoring system based on expression frequency. Scoring was performed by a trained GU pathologist who was blinded to outcome, who provided a scoring sheet that was then linked to the TMA key and outcomes data. These studies demonstrated that very few men had a high

Ki-67 index (11/207 had Ki-67>1%). We did find a nonsignificant relationship between Gleason sum (≤6, 7, 8-10) and maximum mean Ki-67 (0.52, 0.8 7, 0.92 per cent, ANOVA p=0.23) indicating some modest validity to this biomarker in our TMA. In addition, when men were sorted by NCCN/D'Amico risk categories based on composite PSA/stage/Gleason profiles, we found a non-significant relationship between risk score (low, intermediate, high) and maximum mean Ki-67 score (0.67, 0.82, 1.13 percent, ANOVA p=0.13). A weak association between Ki67 category (zero (reference), low (0.5%), intermediate (1%), and high (5%)) and PSA recurrence was noted (HR vs reference of 0.9 9, 1.1, 2.18, p=0.08, figure 1). We are in the process of rescoring the Ki-67 based on a 0-100% scale using the epithelial component only; scoring the stromal component may have falsely lowered these scores compared to historic data using this biomarker. This may provide a greater differential scoring between samples and a greater accuracy of Ki-67 expression in carcinoma cells. Given the need for careful optimization of this TMA and positive control antigens prior to the application to EMT antigens, year 1 has focused on this process intensely.

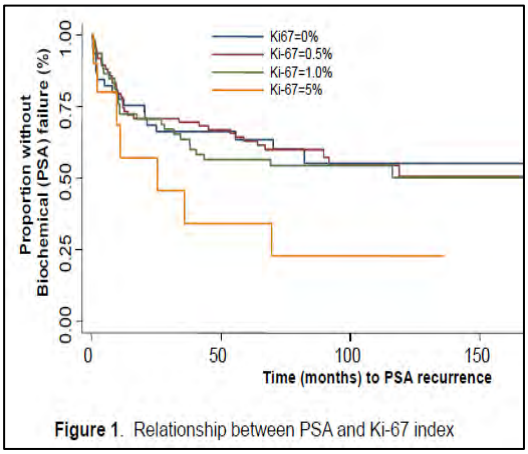

Figure 1. Relationship between PSA and Ki-67 index

In year 2, following final optimization of this TMA, we will update SEARCH outcomes to include a greater number of metastatic and recurrence events. We have already developed and validated several EMT markers that will then be applied in the following order to the TMA in order to study the association of marker expression with PSA recurrence and other clinical/pathologic variables: 1) vimentin (epithelial component only and total), 2) N-cadherin, 3) E-cadherin, 4) SNAIL (SNAI-1), 5) ZEB-1, 6) O-Cadherin, 7) CD133, 8) TWIST. Additional antibodies will be ascertained as part of this analysis over time will include novel FGFR2 isotype specific antibodies that are able to recognize the extracellular domain of the IIIc and IIIb variants of this receptor, respectively. Given the association of epithelial states with FGFR2 vIIIb expression and mesenchymal states with FGFR2 vIIIc expression, this is of great interest. This antibody has been developed through a expression vector followed by column purification and antibody generation (polyclonal) in rabbits over the past year. In year 2, this antibody will be evaluated in both positive and negative control cells prior to its application to the TMA project.

Task 2: To identify the presence of mesenchymal and stem cell markers on circulating prostate cancer cells (CTCs) derived from men with castration-resistant prostate cancer and associated with adverse clinical outcomes (Months 1-18).

Key Research Accomplishments: We have successfully conducted this task, leading to the attached published manuscript (**Armstrong et al, Mol Cancer Res 2011(4)**) describing in detail our findings of EMT and stem cell markers in the majority of CTCs from men with metastatic castration-resistant prostate cancer and women with metastatic breast cancer. This data provides among the strongest evidence to date for the existence of EMT (epithelial-mesenchymal transition) in human cancer. Given the importance of EMT to cancer metastasis and the lethal phenotype, as well as to the generation of stemness and chemoresistance, we believe these findings have profound implications for cancer biology. They suggest that EMT plays a strong role in prostate cancer metastasis during castration-resistant progression. The findings also suggest that our existing CTC assays may underdetect circulating tumor cells with more mesenchymal phenotypes, given that they are likely to have reduced or absent EpCAM expression. Please see manuscript for a detailed discussion of these implications and results. We continue this task into year 2 as we examine additional biomarkers of EMT and stemness in CTCs beyond those initially examined, including aldehyde dehydrogenase, beta-catenin, FGFR2 isotypes, SNAIL, ZEB-1, and TWIST. Please see the attached manuscript which provides details as to the methods used and results obtained.

Task 3: To refine the circulating tumor cell detection technology to capture circulating prostate tumor cells based on a mesenchymal surface marker rather than an epithelial marker (Months 37-60). This corresponds to pilot study 2 of SA2.

Key Research Accomplishments: We have begun the process of developing a collaborative research relationship with Veridex/Johnson and Johnson to develop a second generation CTC detection/capture assay based on task 2 above and the attached manuscript. These efforts are supported by the DOD PRTA (salary support). We have acquired a novel CTC detection instrument with a novel ferrofluid targeted at N-cadherin rather than EpCAM. Over the next several years, we will begin and conduct steps to optimize and validate this novel CTC assay using healthy volunteers (negative controls), spiked tumor samples (positive controls), and patients with advanced prostate cancer. This work has led to the development of a **patent** focused on the novel detection of circulating tumor cells using an EMT antigen-based ferromagnetic capture method (submitted 9/24/10, international application number PCT/US10/50223). In addition, this work has led to the development of a research collaboration agreement with Veridex/Johnson and Johnson towards the development of a novel CTC capture method based on these findings using N-cadherin or O-cadherin based ferrofluids to capture CTCs from the blood. This work is ongoing.

Task 4: To detect similarities in RNA expression profile and patterns of oncogenic expression in circulating tumor cells and matched metastatic prostate cancer tumor tissue (Months 1-54).

The major obstacle to performing RNA expression analysis from CTCs taken from patients with CRPC has been the isolation of a pure population of cells from whole blood that is devoid of leukocytes. Through a prolonged trial and error process intended to optimize this methodology, we have begun to have success in isolating these pure cellular populations. Our current operating procedures include drawing blood into EDTA vacutainers, followed by an initial red cell lysis followed by CD45 magnetic bead negative exclusion of contaminating leukocytes . This enriched CTC population is then further enriched and purified through FACS, gating against a tumor antigen (ie EpCAM) and negative excluding CD45 a second time. There is a high degree of CTC loss in this process; however, we are currently able to isolate a pure population of about 3-10% of the original spiked CTC population in simulated runs, which produces sufficient numbers of cells for RNA Sequencing. A schema of this plan is shown in **figure 2.**

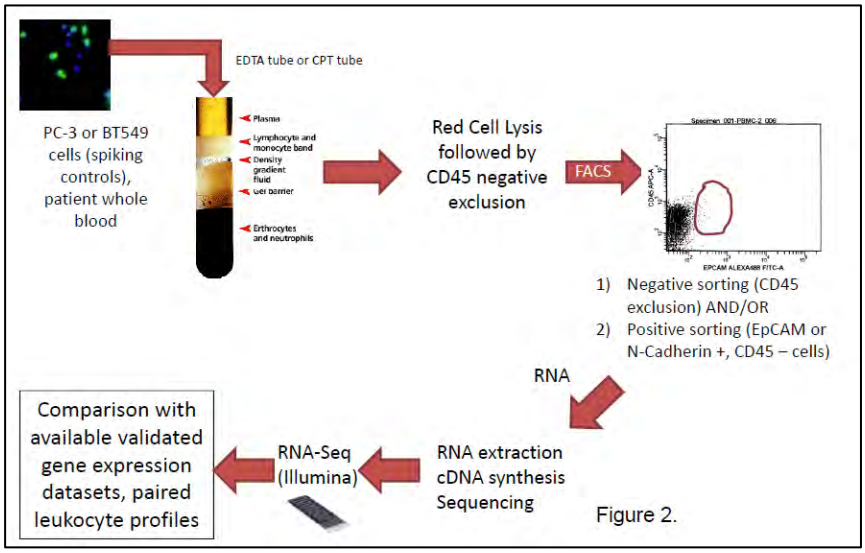

Figure 2.

Over the next 2-4 years, we will develop a partnership with Illumina to be able to conduct CTC RNA Sequencing (single cell) using several different methods including the above method as well as other methods to isolate pure CTC populations, and compare RNA expression profiles of CTCs to that of the patient's own control leukocyte RNA expression. This will be further explored in future grants as well given the need for additional funding (Prostate SPORE, R01, DOD/PCF), and Dr. Armstrong will be applying in the fall of 2011 for independent peer-reviewed funding to support the supplies and analysis needed for these studies. Currently, the preliminary data for these projects and

the initial optimization steps are being supported by Dr. Armstrong and the PRTA in terms of effort and technician support. No supplies/equipment for this task is provided by the PRTA.

Task 5: To estimate the clonality and heterogeneity of circulating tumor cells as compared with metastatic sites by DNA array-based comparative genomic hybridization (Months 1-54).

Key Research Accomplishments: In order to ascertain copy number changes at the DNA level in individual and rare cells, a number of optimization steps are needed as identified in the original SOW. These include the development of array-based comparative genomic hybridization (CGH) assays that are able to accurately and reproducibly ascertain copy number changes in small number of cancer cells. In order to accomplish this, we initially began work with a single cell oligonucleotide CGH (SCOMP) approach using BAC-arrays (bacterial artificial chromosomes) in collaboration with the Duke Center for Human Genetics (DCHG) and Simon Gregory, PhD, a renowned genetic researcher. We applied SCOMP initially to control DNA (male Y-chromosomes) with amplification and compared with non-amplified control DNA, in order to ascertain the potential biases in copy number changes that accompany DNA amplification through PCR. By identifying chromosomal regions that were subject to amplification bias, we were then able to co-hybridize tumor cell-derived DNA with control DNA to control for this bias. This was successful performed using whole cell lines (T47D breast cancer cell lines) that had well-characterized copy number alterations and a reproducible and accurate copy number profile was obtained. We then performed serial dilutions on the T47 cell lines from 1000 cells down to 5 cells in order to ascertain if this CGH profile was stably ascertained with the small numbers of cells that are typically acquired in the circulation of patients with metastatic cancer (ie CTCs). We were able to demonstrate that the arrayCGH profile of 5 T47D cells matched that of 1000 cells, even for smaller regions of chromosomal breakpoints. However, given that this procedure was technically difficult to reproduce and maintain over time, we explored a second procedure (whole genome amplification kit, WGA, Sigma-Aldrich), which uses fragmented genomic DNA and PCR-based amplification to develop an arrayCGH library that has been well validated now at the single cell level (reference: N Navin et al, Nature 2011).

Figure 3. Whole genome amplification of CTCs (WGA)

We have now successfully applied this WGA method to control DNA and T47-based DNA to obtain arrayCGH profiles from a small number of cells (20-200 cells) with reliable profiles. Due to some increased noise-to-signal generated by this process, we are in the process of developing methods to reduce aberrant DNA caused by the WGA method through

sonication and retesting. These optimization steps are critical for the next 1-2 years as we begin to apply these methods to patient samples. We have successfully applied these methods to spiked samples of T47D cells into whole blood with serial dilutions and FACS-based separation methods using anti-EpCAM antibodies and CD45 leukocyte depletion. Results are shown in **figure 3** demonstrating a consistent arrayCGH signal down to 20 cells spiked into whole blood.

The next steps will involve repeating these spiking experiments using PC3 or other prostate cancer cells lines with known CGH profiles, optimizing the WGA protocol with sonication of DNA to remove unwanted DNA fragments that contribute to the increased noisiness in the assay, and then application of these techniques to patient samples based on our IRB-approved clinical protocol. DOD PRTA funds support the effort of Dr. Armstrong and one technician for this work but do not support the actual supplies.

Task 6: To develop skills necessary to succeed as a leader in clinical and translational research and obtain independent peer-reviewed research funding to support a long term career in research (Months 1-60)

During year 1, Dr. Armstrong applied for the Duke Clinical Research Training Program in order to develop the additional skills needed as a clinical-translational researcher in oncology. Given that he has already received a master's degree in clinical investigation in addition to his oncology fellowship training, this coursework will consist of specialized classes in genomic medicine, proteomics, pharmacology, longitudinal data analysis, and other courses to further enhance this training as it directly relates to the projects described in this PRTA. Courses will start in the fall of 2011 and consist of 2 credits per semester.

Dr Armstrong is a full Duke IRB board member and a member of the Duke Cancer Institute's Cancer Protocol Committee, which provides scientific review pre-IRB review for cancer protocols. This practice-based training in clinical research is ongoing and provides much needed experiential education in the conduct of clinical trials and research design and safety oversight.

Dr. Armstrong participates regularly in a number of educational and research based meetings at Duke, including the prostate cancer journal club (monthly), clinical research staff meetings (oversight of trial conduct, data and safety monitoring, weekly), the Garcia-Blanco laboratory meetings (weekly to monthly), the prostate cancer SPORE meetings (monthly), new protocol meetings (to review internal and external proposals, monthly), Translational Science meetings through the Department of Pharmacology and Cell Biology (monthly), Oncology Grand Rounds (weekly), urology tumor board (monthly). Dr. Armstrong participated in 2010 in a Duke Clinical Research Leadership Retreat (3 days), sponsored by the Dean's Office, which provided valuable feedback and education in a range of leadership topics from conflict resolution to giving feedback to leading a diverse team of staff and peers. Dr. Armstrong serves on the oncology fellowship committee, and is now a mentor to a medical oncology fellow (Rhonda Bitting), who is taking on a new project in circulating tumor cell biology linked to the projects described in this PRTA. He also serves as a mentor to a Duke undergraduate (Avinav Ettyreddy) in the Garcia-Blanco lab who is working on a project related to the development of antibodies against FGFR2 splice variants that will allow for the detection of these splice variants in CTCs and human cancer samples.

In addition, Dr Armstrong participates in the following national-level programs that provide training in leadership and clinical practice: ASCO (Chair of a Clinical Science Symposium in 2010), NCCN (Prostate Cancer guidelines member), GU Symposium (ASCO-ASTRO-SUO), and the Prostate Cancer Foundation annual scientific retreat. He attended the DoD IMPACT meeting in 2011 as well and presented an educational talk on "Prostate Cancer 101" in addition to presenting several posters and leading a walking tour of the posters. In year 2, Dr.

Armstrong will continue with these educational and scientific endeavors as part of his clinical research training and academic mission.

KEY RESEARCH ACCOMPLISHMENTS

- **Accepted manuscript** in Molecular Cancer Research (Armstrong et al, attached, published online first June 10, 2011) related to task 2. This data presented the strongest evidence to date for the existence of EMT in human cancer, including men with metastatic prostate cancer and women with metastatic breast cancer. This paper was highlighted in a number of press releases and journals.
- Development of **methods** to isolate individual circulating tumor cells for genetic and genomic analyses (tasks 4-5) and to optimize single cell arrayCGH profiling of rare cells
- Attainment of **IRB approval** at the Durham VAMC (task 1) and the Duke University IRB (tasks 2-3) along with continuing reviews
- **Validation** of tissue microarray linked to SEARCH database using a control biomarker (Ki-67) that will allow for interpretation of the relationship between EMT biomarkers in surgical prostatectomy specimens to be ascertained and correlated with clinical outcome
- Inclusion as a major project (one of four) in the Duke Prostate Cancer SPORE team, with grant submission deadline in September 2011, based on the work accomplished as part of this DOD PRTA.
- Ongoing training in clinical research and clinical oncology as stated above in task 6, with successful application to the Duke Clinical Research Training Program for ongoing clinical research training.
- **Accepted manuscript** highlighting and reviewing the importance of EMT in prostate cancer (Armstrong et al, Asian Journal of Andrology, 2010)(5)
- Development of a **patent** focused on the novel detection of circulating tumor cells using an EMT antigen-based ferromagnetic capture method (submitted 9/24/10, international application number PCT/US10/50223).
- Development of a **research collaboration agreement** with Veridex/Johnson and Johnson towards the development of a novel CTC capture method based on these findings using N-cadherin or O-cadherin based ferrofluids to capture CTCs from the blood. This work is ongoing.
- Presentation of data at national meeting: AACR meeting on EMT (Washington, DC February 2010 invited speaker and poster presenter) entitled “Circulating Tumor Cells from Patients with Metastatic Breast and Prostate Cancer Express Vimentin and N-Cadherin”.
- Presentation of data at national meeting: ASCO 2010 GU Symposium poster abstract 172 entitled “Plasticity, stemness, and aggressive behavior in preclinical models and circulating prostate cancer cells: importance of the transitional phenotypic state to lethal cancer biology”. Attached as an appendix(6).

REPORTABLE OUTCOMES

1) **Manuscript: Armstrong** et al, Molecular Cancer Research 2011 (online 6/10/11)
2) **US Patent:** submitted 9/24/10 for the detection of circulating tumor cells based on EMT antigen-based capture
3) **Research Collaboration Agreement:** with Veridex/J&J to develop a next-generation CTC detection assay based on our preliminary data and published data from this PRTA. This has included the provision and development of a research Cellsearch® machine and Celltracks® Analyzer and two novel ferrofluids for clinical testing.

4) **ASCO GU Symposium poster/abstract 2010 (abstract 172)**
5) **Oral podium presentation, AACR Conference on EMT 2010 (Washington, DC)**
6) **Manuscript (review): Armstrong AJ and Garcia-Blanco M, "**Epithelial-mesenchymal transition in prostate cancer: providing new targets for therapy". Asian J Androl 2010;12.
7) **Development of Tissue Microarray and validation with SEARCH Database (Durham VA Medical Center)**
8) **Development of methods for single cell isolation and arrayCGH DNA profiling from whole blood**
9) **Grant proposals that arose from this award:** Sidney Kimmel Scholar Award (not funded), Prostate Cancer SPORE project (pending September 2011), U01 (PI: Dewhirst) award for Tumor Microenvironment research (pending)
10) **Application to Duke CRTP program, Summer 2011**

CONCLUSIONS

In this annual report for the DOD PRTA, we have provided strong evidence for the existence of epithelial-mesenchymal transition in human cancer, particularly prostate cancer. The findings of EMT and stemness markers in the majority of circulating tumor cells from men with metastatic prostate cancer and women with metastatic breast cancer has several important implications: 1) EMT and stemness pathways likely play an important role in cancer progression, metastasis, and lethality and suggests routes for new therapeutic approaches; and 2) current methods to detect CTCs that rely solely on epithelial characteristics (ie EpCAM) may miss a large number of non-epithelial tumor cells that have undergone EMT during circulatory spread and transit. We intend to apply our findings directly toward therapeutic target identification and technology development. We will do this by developing and optimizing methods for single cell (CTC) capture and isolation from whole blood for DNA and RNA profiling, by developing and optimizing methods to capture CTCs using a novel EMT antigen-based ferrofluid to identify novel cell phenotypes that would otherwise be missed by current methods (ie N-cadherin or O-cadherin based ferrofluids), and by investigating the relevance of these EMT and stemness markers in primary and metastatic samples to patient outcomes and clinical factors. By identifying novel cell phenotypes and pathways that contribute to lethal cancer progression, the hope is to use these biomarkers early to detect and thus intervene with novel EMT-based therapeutic approaches to prevent or significantly delay metastasis.

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(4) Armstrong AJ, Marengo MS, Oltean S et al. Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers. Mol Cancer Res 2011.

(5) Armstrong AJ, Freedland SJ, Garcia-Blanco M. Epithelial-mesenchymal transition in prostate cancer: providing new targets for therapy. Asian J Androl 2011; 13(2):179-180.

(6) Armstrong AJ, Oltean S, Marengo M et al. Plasticity, stemness, and aggressive behavior in preclinical models and circulating prostate cancer cells: importance of the transitional phenotypic state to lethal cancer biology. Genitourinary Cancers Symposium . 2-20-0010.
Ref Type: Abstract

Appendices

A. ASCO GU Symposium Abstract

Plasticity, stemness, and aggressive behavior in preclinical models and circulating prostate cancer cells: Importance of the transitional phenotypic state to lethal cancer biology.

Sub-category:
Prostate Cancer: Early/Localized disease, Locally Advanced/Recurrent/Advanced disease, and Biology
Category:
Genitourinary Cancer
Meeting:
2010 Genitourinary Cancers Symposium
Session Type and Session Title:
General Poster Session C: Prostate Cancer
Abstract No:
172
Author(s):
A. J. Armstrong, S. Oltean, M. Marengo, L. Chen, G. Kemeny, C. I. Herold, P. K. Marcom, D. J. George, M. Garcia-Blanco; Duke University Medical Center, Durham, NC; University of Bristol, Bristol, United Kingdom; Duke University, Durham, NC; Beth Israel Deaconess Medical Center, Boston, MA

Abstract:

Background: Epithelial plasticity is defined as the ability of cells to interconvert between phenotypic states. We sought to characterize plasticity in the Dunning rat tumor model and circulating tumor cells (CTCs) from men with castration-resistant metastatic prostate cancer (CRPC). Our previous work revealed epithelial plasticity in this system and a propensity for these cells to undergo epithelial-mesenchymal (EMT) or MET transitions, suggesting a relationship to metastatic potential. **Methods:** We characterized tumorigenic and metastatic behavior of Dunning clonal sublines which differ in epithelial phenotype and FGFR2 splice variant expression. We evaluated differences in gene expression and evaluated the ability of soluble factors to mediate phenotypic transitions. We then evaluated CTCs in patients with metastatic CRPC or breast cancer for co-expression of cytokeratin with a measure of plasticity, vimentin, using the Veridex CellSearch collection methodology based on EpCAM ferromagnetic capture. **Results:** We found that mesenchymal-like AT3 cells could shift into a transitional state, characterized by co-expression of epithelial and mesenchymal FGFR2 isoforms. Transitional AT3 cells were highly malignant, forming tumors and producing frequent metastases. Moreover, this epithelial phenotype was inducible by soluble factors and expressed high levels of CD44H and CD133, cell surface markers associated with a stem-like cell phenotype, with significant up-regulation of stem cell pathways compared to the mesenchymal-like AT3 cells. Finally we found CTCs from patients with CRPC and hormone-refractory breast cancer co-expressed cytokeratin and vimentin in approximately 75% of CTCs from 20 consecutive patients. **Conclusions:** We have identified reversible transitional phenotypes in preclinical models of PC with state-specific expression of stemness genes and proteins that may contribute to metastasis and aggressive behavior, and have identified markers of plasticity in CTCs from patients with two types of epithelial cancers, suggesting that these transitional cells are important for metastasis.

B. Manuscript (Armstrong et al, Molecular Cancer Research 2011)

Molecular Cancer Research

Angiogenesis, Metastasis, and the Cellular Microenvironment

Circulating Tumor Cells from Patients with Advanced Prostate and Breast Cancer Display Both Epithelial and Mesenchymal Markers

Andrew J. Armstrong[1,2,3,4,6], Matthew S. Marengo[5,7], Sebastian Oltean[5,7], Gabor Kemeny[5,7], Rhonda L. Bitting[1,2,3], James Turnbull[6], Christina I. Herold[1], Paul K. Marcom[1,6], Daniel George[1,2,3,4,6], and Mariano A. Garcia-Blanco[1,3,5,7]

Abstract

During cancer progression, malignant cells undergo epithelial-mesenchymal transitions (EMT) and mesenchymal-epithelial transitions (MET) as part of a broad invasion and metastasis program. We previously observed MET events among lung metastases in a preclinical model of prostate adenocarcinoma that suggested a relationship between epithelial plasticity and metastatic spread. We thus sought to translate these findings into clinical evidence by examining the existence of EMT in circulating tumor cells (CTC) from patients with progressive metastatic solid tumors, with a focus on men with castration-resistant prostate cancer (CRPC) and women with metastatic breast cancer (BC). We showed that the majority (>80%) of these CTCs in patients with metastatic CRPC coexpress epithelial proteins such as epithelial cell adhesion molecule, cytokeratins (CK), and E-cadherin, mesenchymal proteins, including vimentin, N-cadherin and O-cadherin, and the stem cell marker CD133. Equally, we find that more than 75% of CTCs from women with metastatic BC coexpress CK, vimentin, and N-cadherin. The existence and high frequency of these CTCs coexpressing epithelial, mesenchymal, and stem cell markers in patients with progressive metastases has important implications for the application and interpretation of approved methods to detect CTCs. *Mol Cancer Res; 9(8); 1–11. ©2011 AACR.*

Introduction

Most metazoan cells can be classified as either epithelial or mesenchymal based on morphology, behavior, and molecular signatures. In adult animals, epithelial and mesenchymal cells usually remain in 1 phenotypic state; that is, epithelial cells do not change their properties and become mesenchymal. During development, however, epithelial cells of the early embryo give rise to all 3 embryonal layers (endoderm, mesoderm, and ectoderm), which include mesenchymal cells (1). Therefore, these early embryonal cells have the ability to transition between epithelial and mesenchymal states, a property we define as epithelial plasticity (for a slightly different definition see ref. 2). Indeed, observations in embryos showed epithelial-mesenchymal transitions (EMT) and mesenchymal-epithelial transitions (MET; ref. 3), which may be viewed, perhaps naively, as forward and reverse directions of the same reaction mechanism (for review, see refs. 2, 4).

Although it is convenient to present EMT/MET as a reversible reaction between binary states, there are suggestions that intermediate states exist and that these may play important roles. The importance of EMT and MET in cancer progression is now widely, albeit not unanimously, accepted (see recent reviews refs. 4–8). EMT is observed in human cancer cells *in vitro* and in xenografts, and in the leading edge of invasive carcinomas *in vivo* (5, 6). In human prostate carcinoma, loss of E-cadherin expression and overexpression of N-cadherin, which indicates the presence of an EMT, independently correlates with high Gleason score and systemic and metastatic recurrence after surgery, linking EMT to more aggressive clinical behavior (9–12). In addition, recent studies have shown the importance of

Authors' Affiliations: [1]Department of Medicine; [2]The Duke Prostate Center and [3]Duke Cancer Institute; [4]Department of Surgery; [5]Center for RNA Biology and [6]Oncology Clinical Trials Shared Resource, Duke Cancer Institute; and [7]Department of Molecular Genetics and Microbiology, Duke University Medical Center, North Carolina

Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

M.S. Marengo, S. Oltean, and G. Kemeny contributed equally to this work.

Current address for S. Oltean: Microvascular Research Laboratories, Department of Physiology and Pharmacology, University of Bristol, Southwell Street, BS2 8EJ Bristol, United Kingdom.

Current address for C.I. Herold: Division of Medical Oncology, Department of Medicine, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, Boston 02215.

Corresponding Author: Andrew J. Armstrong, DUMC Box 102002, Durham, NC 27710. Phone: 919-668-8797; Fax: 919-668-7117; E-mail: andrew.armstrong@duke.edu or Mariano A. Garcia-Blanco, DUMC Box 3053, Research Drive, Durham, NC 27710. Phone: 919-613-8632; Fax: 919-613-8646; E-mail: garci001@mc.duke.edu

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N-cadherin expression during castration-resistant metastatic progression in preclinical models of prostate cancer and in human metastases. These translational studies have suggested a link between loss of epithelial markers, gain of mesenchymal markers, and the induction of signaling pathways that promote survival and androgen-receptor independent growth (13). In breast cancer, a similar link has been established between EMT markers in primary and disseminated bone marrow tumor cells and aggressive clinical behavior (14–18). Likewise, evidence for MET was obtained from microscopic analysis of colorectal carcinoma metastases, which adopted epithelial characteristics of the noninvasive regions of the primary tumor (19). In prostate cancer, attachment of metastatic cells to bone cells correlates with expression of E-cadherin (20). These and many other studies describe the existence of these transitions during carcinogenesis and raise questions about their functional importance. There is strong evidence that EMT is important for metastatic behavior and chemoresistance (18, 21); however, the importance of MET has been more difficult to ascertain. Previously, we found that the preponderance of MET events among lung metastases in rats bearing AT3 rat prostate adenocarcinoma tumors suggested an important functional relationship between the capacity to revert to a more epithelial state and metastatic growth in the lung parenchyma (22, 23).

A strict view of epithelial plasticity in cancer posits that a mesenchymal-like state reached post-EMT is the driver of malignant fitness. Indeed, there is strong evidence that the mesenchymal properties of invasiveness and motility are required for metastases (see above) and that EMT leads to expression of cancer stem cell markers, including CD44 (24). Nonetheless, observations above suggest that mesenchymal properties per se are not sufficient for optimal malignant behavior (19, 22, 23, 25). A broader interpretation suggests that the ability to easily transition between epithelial-like and mesenchymal-like states, which we define as phenotypic plasticity, may be linked to stem cell–like properties and is a more important determinant of aggressive metastatic behavior than the properties of the end states. In a preclinical model examining the importance of stem cells and cancer growth, subcutaneous injection of CD133-positive cells, which are predicted to be stem cell like, into immunodeficient mice causes tumor growth, whereas injection of CD133-negative cells do not (26). A recent clinical study that measured mRNAs coding for stem-like markers in the bloodstream of patients with resected colorectal cancer found that the expression of CD133, CEA, and cytokeratin (CK) RNAs was associated with recurrent disease and an overall poor prognosis (27).

Therefore, we posit that the most plastic cells will be those that inhabit transitional or intermediate states with properties of both epithelium and mesenchyme, and that these transitional cells will be particularly malignant and stem like. To test this proposal in human disease, we sought evidence for markers of both mesenchymal and stemness phenotypes in circulating tumor cells (CTC) from patients with metastatic castration-resistant prostate cancer (CRPC) and metastatic breast cancer (BC).

Materials and Methods

Analysis of human circulating tumor cells

Patients eligible for the CTC biomarker protocols included (i) men with mCRPC, with metastatic progression by prostate-specific antigen (PSA; 2 consecutive rises over nadir separated by more than 1 week) or radiologic criteria (Response Evaluation Criteria in Solid Tumors or new bone scan lesions), a PSA 5 or more, age 18 years or older; or (ii) women with mBC with disease progression or with initiation of a new systemic therapy, who were older than 18 years of age, and who were at least 7 days from treatment with an anthracycline-containing regimen. All subjects provided informed consent as part of an Institutional Review Board-approved prospective clinical protocol. Blood (15 mL) was collected from patients and processed within 48 hours at the Duke University, Department of Molecular Pathology and Clinical Pathology Laboratory using the Cell Search System (Veridex, Raritan). Veridex profile kits, which isolate epithelial cell adhesion molecule (EpCAM)-positive cells by using a ferromagnetic immunoabsorbtion assay without additional staining, were used to collect CTCs. An additional tube was collected and processed in parallel for CTC enumeration by using the Veridex CellSearch method using the standard test kit. Following profile kit processing, the isolated cells were either processed immediately or stored overnight in 4% paraformaldehyde (PFA) and processed the next day. An initial wash using a bench top magnet to enrich the EpCAM-bound cells was conducted to further isolate CTCs, with resuspension of the cell pellet after magnet release into 100 μL PBS. Immunostaining was done on teflon-coated slides. Briefly, cells were pipetted into the wells of the slides and left to settle for ~30 minutes followed by standard immunostaining procedures with careful aspiration to minimize cell loss at room temperature. Following 4% PFA fixation, permeabilization with PBT (PBS with 0.2% v/v Triton), and blocking with 10% goat serum for 30 minutes, triple immunostaining was done using CD45 antibody labeled with Alexa 647, CK labeled with Alexa 555 and either Vimentin (BD Biosciences), N-cadherin (BD Biosciences), O-cadherin (Invitrogen), or CD133 (Novus Biologics) labeled with Alexa 488. Supplementary Table S1 provides specifics on the control cells, dilutions, and products used. Nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI) was then done. A CTC was defined as an intact cell, containing a nucleus and expressing CK but lacking CD45 expression. Control cells were evaluated in parallel with each patient sample for immunofluorescent staining intensity and scoring (see Supplementary Table S1 for controls used and Supplementary Fig. S1 for images of these real-time controls). Human peripheral blood mononuclear cells (PBMC), obtained by Ficoll purification of buffy coats from normal American

Red Cross donors, were kindly provided by Micah Luftig (Duke) and used as positive control cells for CD45 expression and negative controls for CK and cadherin proteins. In addition, the coexpression of E-cadherin (BD Biosciences) with N-cadherin on EpCAM-captured, CD45-negative DAPI-positive cells, irrespective of their CK expression, was examined. In this situation, all CD45-negative cells were manually enumerated and the proportion of CD45-negative nucleated cells that expressed E- or N-cadherin were scored. To ensure that the antibodies against E- and N-cadherin (Supplementary Table S1) did not cross-react with their respective antigens, a series of control cells with known E- and N-cadherin expression levels were examined by Western blot analysis against E- and N-cadherin and actin as a loading control.

The slides were mounted with gel/mount media (Biomeda). The slides were analyzed with an Olympus IX 71 epifluorescence microscope, and images were acquired using an Olympus DP70 digital camera. Image processing was done with DP Controller software (Olympus). All fields on each slide were analyzed sequentially, with each CK-positive–nucleated cell that was CD45 negative being counted as a CTC. Standardized exposure times optimized for each antibody were used consistently throughout the analysis for case and control cells.

Immunohistochemical analysis of metastases

Under the same informed consent protocol, men undergoing CTC collection additionally consented to have a radiologic-guided metastatic biopsy for analysis of biomarker expression by immunohistochemistry (IHC). Samples were obtained through core needle biopsies during light sedation, and immediately formalin fixed and paraffin embedded. For analysis, slides were deparaffinized, rehydrated, and endogenous peroxidase was inactivated for 30 minutes in 0.3% H_2O_2 (hydrogen peroxide) in methanol. Specific antigen retrieval steps were done for individual antigens. Three markers were evaluated by IHC: vimentin (M7020; Dako, 1:150; antigen retrieval with pepsin treatment at 37°C for 15 minutes), CK cocktail (18-0132; Invitrogen, 1:50 and 349205; BD Biosciences 1:50, antigen retrieval with pepsin treatment at 37°C for 15 minutes) and CD45 (M0701; Dako, 1:200; antigen retrieval with sodium citrate 10 mmol/L, pH 6.0 at 100°C for 30 minutes). Primary antibody was incubated for 60 minutes at room temperature. Dako Envision horseradish peroxidase secondary antibody was used for 30 minutes at room temperature and the signal was detected with 3,3′-diaminobenzidine reagent (Vector kit SK 4100). Slides were counter stained with hematoxylin and eosin and assessed by a trained pathologist for expression using appropriate positive (localized prostate tissue microarray sections) and negative controls (mock antibody) for each marker.

Statistical analyses

We used simple descriptive statistics to estimate the prevalence of mesenchymal and CD133 antigen coexpression on EpCAM-captured CTCs, summarizing these findings on both an intraindividual level and within group (CRPC and BC) level. To compare CTC count (standard CellSearch method) against the proportion of CTCs that coexpress vimentin, N- and O-cadherin, or CD133, linear regression analysis was done. Goodness of fit was tested by ANOVA.

Results

To examine the coexpression of mesenchymal and/or stemness antigens on CTCs from patients with metastatic disease, we took advantage of the existing Food and Drug Administration (FDA)-approved capture method to initially capture and isolate cells from whole blood. CTCs have both independent prognostic and predictive significance in multiple epithelial malignancies, including mCRPC and mBC (28, 29), and can be collected, isolated, and analyzed for a variety of biomarkers relevant to cancer biology (30–32). The approved technology for CTC capture relies on the expression of EpCAM on the surface of epithelial cells, and thus currently measured CTCs must be epithelial like. Yet, these cells have escaped from the primary tumor, possibly as a result of an EMT/invasiveness program, and may have mesenchymal properties.

To test for the existence of mesenchymal-like CTCs, blood was collected from 41 men with mCRPC and 16 women with mBC (see baseline characteristics for the patients in Table 1 and Supplementary Table S2) and CTCs were processed by using the CellSearch EpCAM-based immunocapture method and profiled for expression of CD45 (PTPRC) (a leukocyte marker), CK, and E-cadherin (CDH1; epithelial markers), vimentin (VIM), N-cadherin (CDH2), and O-cadherin (CDH11; mesenchymal markers), and CD133 (a stem cell marker) by immunofluorescence (IF; refs. 10, 33; Supplementary Table S1). Leukocytes were defined as nucleated (DAPI positive), CD45-positive and CK-negative cells (Fig. 1A), whereas CTCs were defined as nucleated (DAPI positive), CD45-negative and CK-positive cells (Fig. 1B–G). Among CTCs, we identified subsets of cells that additionally expressed vimentin (Fig. 1C) or N-cadherin (Fig. 1D and E) or O-cadherin (Fig. 1F and G). Real-time positive and negative control cells were used at the time of individual patient CTC analysis to determine positive or negative IF expression using identical exposure, antibody concentration, and camera settings without alteration. Real-time control cell images for each corresponding antigen and subject depicted in Figures 1, 3, and 4 are provided in Supplementary Figure S1.

Among men with mCRPC, we found that CTCs coexpressed vimentin and CK in 10/10 (100%) patients, and by this criterion 108/126 (86%) of enumerated CTCs ~~were transitional~~ (Fig. 1B–C, Table 2 and Supplementary Table S3, and also see Supplementary Fig. S1 for control cells and Supplementary Fig. S2 for additional examples). Biopsies of bony metastases done within 1 week of initial CTC collection in 2 of these patients (patients 6 and 7 in Table 1) revealed no vimentin expression in the CK-positive

Table 1. Baseline demographic and clinical characteristics of the men with metastatic CPRC in this study ($n = 41$)

Demographics	$n = 41$
Median age, years (range)	71 (50–89)
Race, ethnicity	
White, non-Hispanic	73%
Black, non-Hispanic	27%
Baseline disease history	
Median Gleason score (range)	8 (5–10)
Median baseline PSA (ng/dL, range)	248.9 (14.0–13,419.5)
Median baseline pain (range)[a]	0 (0–7)
Median Karnofsky performance status (range)	90 (60–100)
Median number of prior hormonal therapies (range)	2.5 (0–5)
Prior chemotherapy	68%
Prior bisphosphonates	73%
Sites of metastatic disease	
Visceral (lung + liver)	54%
Lymph node only	0%
Metastatic to bone:	
Metastatic to bone with lymph nodes (no visceral metastases)	24%
Metastatic to bone without lymph nodes (no visceral metastases)	22%

[a]Pain is scored as a linear analog scale (0–10 range).

tumor foci, but strong vimentin expression in the surrounding bone stroma, which lacks CK expression (Fig. 2). These same patients had CTCs taken at the same time as the CT-guided tumor biopsy that commonly expressed coexpressed CK and vimentin. These findings are consistent with invasion and metastasis by CTCs that subsequently ~~are~~ undergo EMT/MET or exist in a transitional state; an alternative explanation may be that vimentin coexpression may be heterogeneous in metastases, similar to CTC expression.

Among the next cohort of 11 men with mCRPC, we found CTCs coexpressing N-cadherin and CK in 11/11 (100%) patients, and by this criterion 205/244 (84%) of CTCs were identified as coexpressing these markers (Fig. 1D and E, Table 2 and also see Supplementary Table S3, Supplementary Fig. S1 for real-time control cells and Supplementary Fig. S3 for additional examples). The expression of N-cadherin among CTCs varied from undetectable, determined by the real-time negative controls described in Supplementary Table 1, to very strong (Fig. 1D). This variation was observed among CTCs from individual patients as can be seen by examination of 3 CTCs from patient 11 (Fig. 1E). Although we noted heterogenous vimentin expression among patient and control leukocytes, we did not observe N-cadherin expression among patient or control leukocytes.

Among 10 women with mBC, 9 had detectable CTCs and of these, we found evidence of vimentin coexpression in 7 (78%) patients, and 55/88 CTCs overall (63%) coexpressed vimentin (Supplementary Fig. S2, Table 2 and Supplementary Table 4, and controls in Supplementary Fig. S1). Among another 6 women with detectable CTCs and mBC, 4 had evidence of CK and N-cadherin coexpression, and overall 78/95 CTCs (82%) had N-cadherin expression, with significant heterogeneity in expression in a given individual (Table 2, Supplementary Fig. S3 and controls in Supplementary Fig. S1). These data indicate that the majority of CTCs in patients with mBC and mCRPC coexpress epithelial (EpCAM and CK) and mesenchymal (vimentin, N-cadherin) markers, and thus exist in a transitional phenotypic state, similar to that observed in our preclinical models.

Given these findings, we next sought to better characterize the dual expression of the epithelial E-cadherin with the more mesenchymal N-cadherin on circulating CD45-negative–nucleated cells. Western blot analysis of control cells (T47D, BT549, PC-3, and PBMC cells) indicated a lack of cross-reactivity of our E-, N-, or O-cadherin antibodies against their respective antigens (data not shown), providing assurance that these antibodies are able to measure the intended target antigen. In an additional cohort of 6 men with progressive metastatic CRPC, we characterized all CD45-negative cells that were initially captured by using the standard CellSearch method. Given the limited number of proteins that may be evaluated using IF in an individual cell, we examined the presence of both E- and N-cadherin expression on these CD45-negative cells but ~~are~~ not able to additionally measure CK expression and therefore cannot define a CTC as described above. Interestingly, as shown in Table 3, Figure 3, and Supplementary Figure 1, we found clear evidence for 4 distinct subtypes of CD45-negative cells, including those cells that coexpressed both E- and N-cadherin (20%–71% of cells examined in a given subject), cells that expressed E- but not N-cadherin (2%–22% of cells), cells that expressed N- but not E-cadherin (0%–45% of cells), and cells that lacked both cadherin family members (25%–48% of cells). The proportion of CD45-negative cells that expressed N-cadherin was similar to the proportion that expressed E-cadherin (61% vs. 53%), although this varied greatly in individual men (48%–72% vs. 23%–75%, respectively). Finally, we noted that the subtype of E-cadherin–negative/N-cadherin–positive cells was relatively rare among these EpCAM-captured cells except in 2 men (subjects 39 and 40) who had progressive disease on their most recent treatment with the mTOR inhibitor temsirolimus as part of an experimental protocol. Although these analyses are limited to those cells captured based on EpCAM expression, the results indicate that CTCs coexpress epithelial and mesenchymal proteins and suggest the existence of currently undetected CTCs with

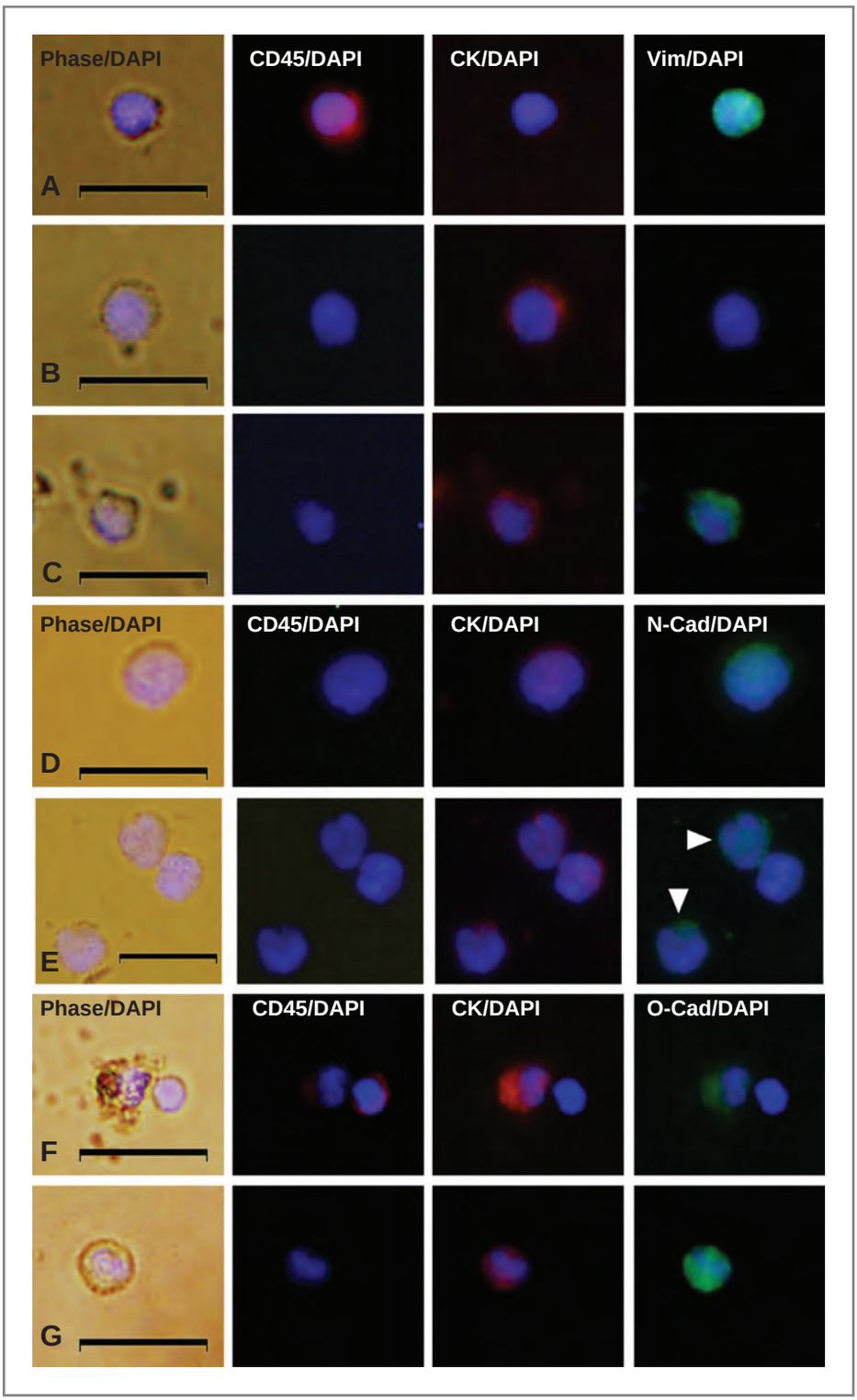

Figure 1. Coexpression of epithelial and mesenchymal proteins in CTCs from men with metastatic castration-resistant prostate cancer (mCRPC). All panels represent merged images derived from phase/DAPI, CD45/DAPI, CK/DAPI, and either vimentin (Vim)/DAPI, N-cadherin (N-cad)/DAPI expression, or O-cadherin (O-cad)/DAPI as indicated. Shown are examples of (A) a leukocyte with CD45 expression, (B) a CTC with no vimentin expression, (C) a CTC with vimentin expression, (D) a CTC with N-cadherin expression, (E) 3 CTCs, 2 with N-cadherin expression (arrowheads), (F) a CTC with O-cadherin expression and a nearby leukocyte, and (G) an additional CTC with O-cadherin expression. Scale bars represent 20 μm and were added from an image taken at identical magnification and resolution. Control cells were assayed in parallel at the same time of CTC collection and analysis with each set of patient samples and are shown in Supplementary Figure S1.

reduced or absent epithelial markers. Methods that evaluate whole blood for nonleukocyte-nucleated cellular populations without initial EpCAM-based capture may thus be able to identify these additional cells.

Given that prostate cancer has a tendency to metastasize to bone, we next hypothesized that adhesion molecules that favor an osteoblastic tumor microenvironment may be visualized on CTCs from men with CRPC. O-cadherin

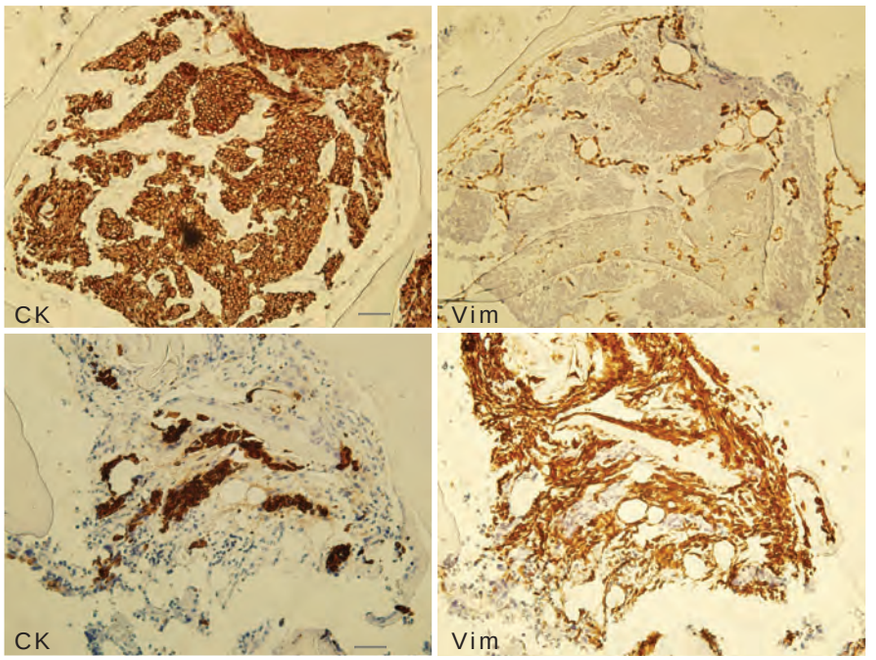

Figure 2. Expression of vimentin and CK in prostate cancer metastases. Images are taken from a CT-guided targeted bone metastasis biopsy at the same time as circulating tumor cells were collected and evaluated for vimentin coexpression by immunofluorescence as described. Images are from patient 6 (top) and patient 7 (bottom), with CK (left), and vimentin (right) expression assayed by IHC (20× magnification). Scale bars in the CK panels represent 50 μm and were added from an image taken at identical magnification and resolution.

has been recently linked to metastasis to bone in preclinical models of prostate cancer (34, 35), and we thus ~~wanted~~ to examine its coexpression with CK in CTCs. Indeed, we found coexpression in 6 of 6 men detectable CTCs by EpCAM-based ferromagnetic capture, and O-cadherin was expressed in 64%–96% of CTCs~~, and any O-cadherin expression in 100% of all men with CRPC~~ (e.g., shown in Fig. 1F and G, summarized in Table 2, and Supplementary Table S3 with controls in Supplementary Fig. S1). These findings suggest that prostate cancer CTCs coexpress adhesion molecules that may promote homing to bone and homotypic binding to osteoblastic cells.

Given the expression of the stem cell–associated antigen CD133 in putative prostate cancer stem cells and other cancer stem cell populations (27, 33, 36), we investigated CD133 expression in CTCs from men with mCRPC. We found CD133 to be expressed in 11/11 (100%) men with CTCs, and in 127/153 (83%) of CTCs from these men (Fig. 4, Table 2, Supplementary Fig. S4 and controls in Supplementary Fig. 1). These data suggest that CTCs from patients with common epithelial malignancies coexpress both epithelial and mesenchymal markers, suggesting that EMT/MET transitions may be contributing to metastatic progression. In addition, in men with metastatic CRPC, the coexpression of the stemness antigen CD133 in the majority of CTCs suggests that these cells may have acquired properties of stemness during their migration into the bloodstream (21, 24).

Discussion

In these studies, we have identified biomarkers suggestive of epithelial plasticity and stemness in CTCs from patients with common metastatic epithelial malignancies, including breast and prostate cancer. The identification of both epithelial and mesenchymal phenotypes among CTCs in a significant subset of patient samples offers several important clinical opportunities. These data suggest that CTCs may undergo phenotypic changes from epithelial to more mesenchymal transitional states during metastatic transit, whereas metastases themselves may be more epithelial in phenotype and marker expression. Our findings also suggest that in addition to cells expressing both epithelial and mesenchymal markers, there may be an unknown number of CTCs that are more mesenchymal-like and thus are EpCAM negative. These cells will be missed by the FDA-approved CellSearch method, the Adna Test (AdnaGen AG) system, and current microfluidic technologies, which enrich for CTCs by immunoabsorbtion of cells expressing MUC1 or EpCAM (37). Indeed, recent studies in breast cancer have suggested that "normal" type breast cancer cell lines that overexpress EMT and stem cell antigens ($CD44^+$, $CD24^-$) may lack EpCAM and are thus not detectable by currently approved CTC detection systems (38). Therefore, it is possible that the number of CTCs in patients with metastatic cancer is much higher than currently appreciated.

It is well appreciated that cells induced to undergo EMT activate stem cell pathways (24). Indeed, a recent study

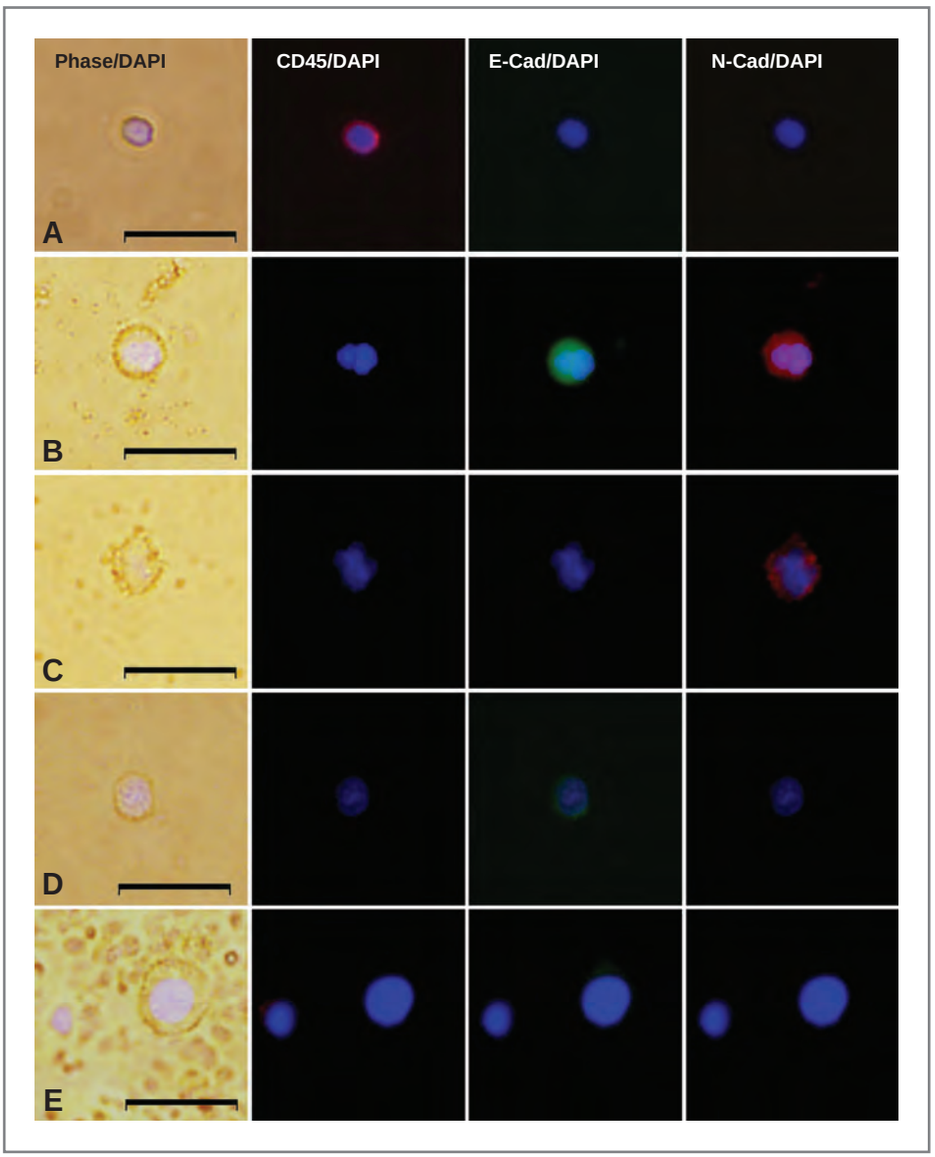

Figure 3. E-cadherin and N-cadherin coexpression among CD45-negative–nucleated cells from men with metastatic CRPC. All panels represent merged images derived from phase/DAPI, CD45/DAPI, E-cadherin (E-cad)/DAPI, and N-cadherin (N-cad)/DAPI expression as indicated. A, a leukocyte with CD45 expression, (B) a CD45-negative–nucleated cell, with E-cadherin and N-cadherin coexpression, (C) a CD45-negative–nucleated cell with N-cadherin expression and no E-cadherin expression, (D) a CD45-negative–nucleated cell with E-cadherin expression and no N-cadherin expression, and (E) a nucleated cell lacking expression of CD45, E-cadherin, and N-cadherin. Scale bars represent 20 μm and were added from an image taken at identical magnification and resolution. Control cells were assayed in parallel at the same time of CTC collection and analysis with each set of patient samples and are shown in Supplementary Figure S1.

showed a striking relationship between prostate cancer–associated fibroblast chemokine expression of interleukin-6 or TGF-β and the acquisition of tumor invasiveness, metastatic propensity, EMT antigen expression, and stemness characteristics (39). Our findings suggest that CTCs captured by using an epithelial-based capture assay, the current FDA standard method, express the stemness marker CD133. These findings are consistent with the results of Mani and colleagues (24) showing a relationship between EMT and stemness states in breast cancer models. It is tempting to speculate that these CTCs may represent transitional cells with both epithelial and mesenchymal phenotypes, and this heterogeneity or plasticity may also extend to stem cell markers and to treatment-induced effects. For example, we observed an increased predominance of N-cadherin expression in CTCs from 2 men who were progressing on their most recent treatment with an mTOR inhibitor, suggesting that certain systemic agents may alter CTC phenotype. An alternative explanation to this transitional state theory is that tumor heterogeneity for these markers exist, without the need to transition phenotypically. Further preclinical and clinical studies of metastatic tumors in which the EMT/MET process is experimentally disrupted or induced may shed further light on these observations.

Our working model of plasticity also predicts that cells with maximal stem cell character, which by definition will be highly malignant, should display both epithelial and mesenchymal traits, because they inhabit intermediate states in the epithelial-mesenchymal axis. Here, we show that patients with metastatic breast and prostate carcinomas have CTCs that exist in phenotypic states that may be inter-

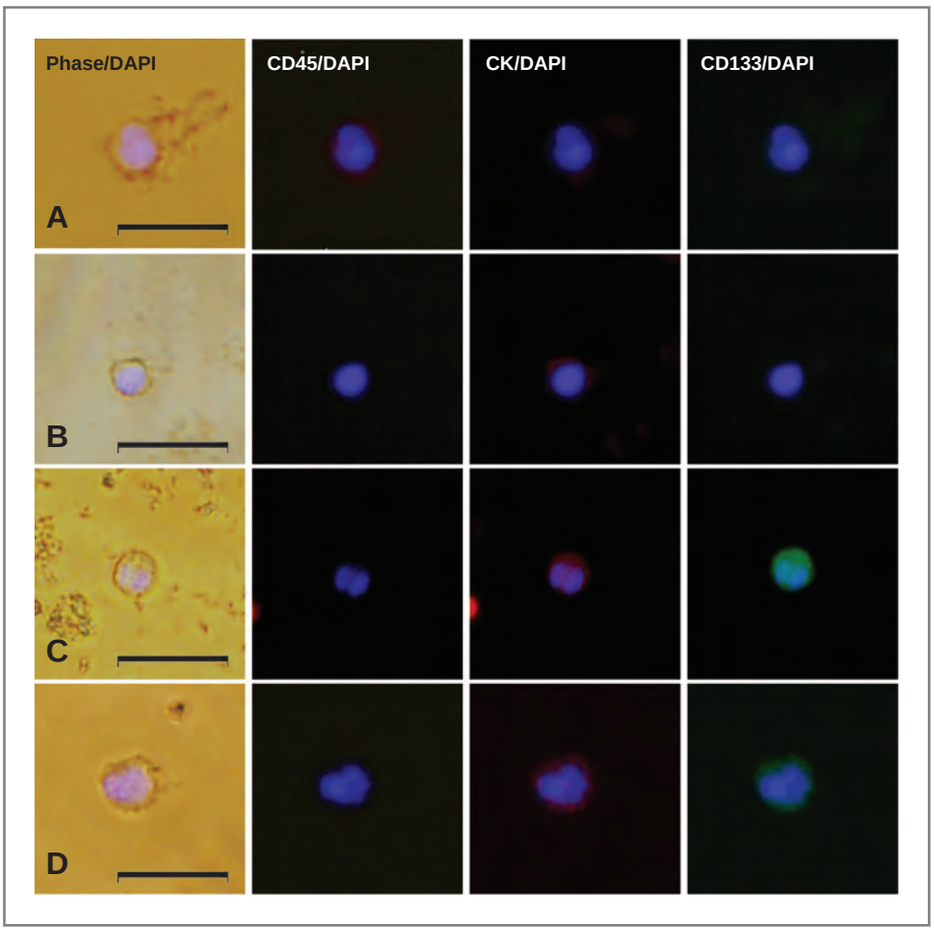

Figure 4. Expression of a stem-like cell marker CD133 in CTCs from men with mCRPC. All panels represent merged images derived from phase/DAPI, CD45/DAPI, CK/DAPI, and CD133/DAPI expression as indicated. Shown are examples of (A) a leukocyte with CD45 expression, (B) a CD133-negative CTC, (C) a CD133-positive CTC, and (D) an additional example of a CD133-positive CTC. Scale bars represent 20 μm and were added from an image taken at identical magnification and resolution. Control cells were assayed in parallel at the same time of CTC collection and analysis with each set of patient samples and are shown in Supplementary Figure S1.

mediate to epithelial and mesenchymal states. Although the enumeration of EpCAM-captured CTCs or CD133-positive CTCs correlates with disease progression, there is great heterogeneity to the number of CTCs isolated from individual patients with metastatic carcinomas, and thus a further refinement in the methods of CTC detection by using EMT antigen–based capture methods may result in improved prognostication CTC identification (27–29). Aktas and colleagues recently showed that a population of cells enriched in CTCs expressed RNAs encoding mesenchymal markers;

Table 2. Prevalence of EMT and stemness antigen marker expression by immunofluorescence in circulating tumor cells (intact, nucleated CK^+, $CD45^-$, DAPI + cells) from the circulation of men with metastatic CRPC and women with metastatic BC

Antigen	*n*	Marker-positive CTCs (%)	Patients with marker-positive CTCs (%)
Vimentin (CRPC)	10	108/126 (86%)	10/10 (100%)
Vimentin (BC)	10	65/97 (65%)	7/10 (70%)
N-Cadherin (CRPC)	11	205/244 (84%)	11/11 (100%)
N-Cadherin (BC)	6	78/95 (82%)	4/6 (67%)
O-Cadherin (CRPC)	6	107/120 (89%)	6/6 (100%)
CD133 (CRPC)	11	127/153 (83%)	9/11 (82%)

NOTE: Columns on the right indicate the number of manually scored CTCs scoring positive for each marker and the number of patients in each group who have at least 1 CTC that stains positive for a given marker.

Table 3. Prevalence of E-cadherin and N-cadherin expression on CD45-negative–nucleated cells isolated from the circulation using an EpCAM-based ferrofluid (CellSearch method) among men with progressive metastatic CRPC

Subject number	Clinical CTC	CD45-negative cells	E + N + cells (%)	E − N − cells (%)	E + N − cells (%)	E − N + cells (%)
35	7	24	17 (71%)	6 (25%)	1 (4%)	0 (0%)
36	55	23	11 (48%)	8 (35%)	4 (17%)	0 (0%)
37	112	122	82 (67%)	30 (25%)	4 (3%)	6 (5%)
38	1000	65	27 (42%)	22 (34%)	14 (22%)	2 (3%)
39[a]	45	44	9 (30%)	21 (48%)	1 (2%)	13 (30%)
40[a]	16	83	18 (22%)	26 (31%)	2 (22%)	37 (45%)
Summary	1235	361	164 (45%)	113 (31%)	26 (7%)	58 (16%)

NOTE: Clinical CTC = enumeration by using the FDA-approved method. Each column represents the number and percent of CD45-nucleated cells that were identified based on dual marker expression.
[a]Indicates 2 patients with metastatic CRPC who were progressing on therapy with the mTOR inhibitor temsirolimus.

however, this study could not prove coexpression of epithelial and mesenchymal markers in the same cell (40). On the basis of the results presented here, further studies to explore methods to capture cells based on these markers in addition to EpCAM are warranted to investigate their relevance to metastatic progression and chemoresistance.

There are several inherent limitations to this work. First, we have not shown true stemness among CTCs, which would require further experimental evidence of serial clonal passage and transplantation, or prolonged culture of CTCs from patients. This is not technically feasible with current methods. Our markers of stemness are correlative in nature only, and may be associated with properties other than stemness. We also acknowledge that coexpression of epithelial and mesenchymal markers does not, in itself, represent plasticity, as we are unable to observe this dynamic *in vivo* in patients. Our clinical observations suggest plasticity based on the coexpression in real time on CTCs during the process of metastasis, and the lack of expression of vimentin in paired metastases from the same patients. The importance of this plasticity to highly aggressive metastatic behavior can only be tested through experimental manipulation of preclinical systems in which either EMT/MET is prevented; future experiments will need to address this issue. Finally, these studies have not correlated coexpression of EMT factors on CTCs with clinical outcomes; these prognostic studies require large appropriately powered studies and patients with long-term follow-up, such as has been recently reported with CD133-positive colorectal CTCs and postoperative outcomes (27). Our findings, however, suggest that the measurement of CTCs collected through both EpCAM-enriched and EMT antigen–enriched methods may complement each other in providing prognostic or predictive information during systemic therapy that should be prospectively evaluated.

Finally, CTCs expressing mesenchymal or stem like markers expression, which comprise the majority of cells isolated in this study, and additional cells that may go undetected due to EpCAM loss, represent a therapeutic problem. It has been well documented that EMT alters drug sensitivity (21, 41, 42) and it has been challenging to direct therapy to cancer cells with stem cell–like properties, perhaps because of their recalcitrance to undergo apoptosis (43). Although recent studies suggest both a screening method and actual compounds (e.g., salinomycin) that can selectively target cancer stem cells (21), these aggressive cells still represent a formidable challenge. Our findings suggest that these cell types may be highly prevalent among patients with metastatic epithelial tumors, and suggest methods for the improved detection of these cells *in vivo* to assist in developing novel therapeutic strategies.

Disclosure of Potential Conflicts of Interest

A.J. Armstrong, S. Oltean, D. George, and M.A. Garcia-Blanco are listed as inventors in a related patent application (application number PCT/US10/50233) filed on September 24, 2010.

Authors' Contributions

A.J. Armstrong, S. Oltean, D. George, and M.A. Garcia-Blanco conceived of the original study. A.J. Armstrong and M.A. Garcia-Blanco directed the research. G. Kemeny, S. Oltean, and M.S. Marengo carried out laboratory experiments. A.J. Armstrong, J. Turnbull, C.I. Herold, P.K. Marcom, and D. George carried out clinical procedures and recruitment. While all coauthors contributed to the writing of the manuscript, A.J. Armstrong, M.S. Marengo, and M.A. Garcia-Blanco did the majority of writing and editing.

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C. Asian Journal of Andrology Manuscript (2010)

Asian Journal of Andrology (2010), 1–2

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RESEARCH HIGHLIGHT

Epithelial–mesenchymal transition in prostate cancer: providing new targets for therapy

Andrew J Armstrong, Stephen J Freedland and Mariano Garcia-Blanco

Asian Journal of Andrology advance online publication, ** ** 2010; doi:10.1038/aja.2010.169

The ability of epithelial cells to undergo phenotypic transitions during embryogenesis, wound healing and malignant progression is now widely accepted as a core biological process termed epithelial–mesenchymal transition (EMT).[1] During cancer progression, the process of EMT has been associated with the acquisition of stemness properties, treatment resistance and metastatic progression, hallmarks of malignancy.[2,3] Indeed, induction of EMT in breast cancer model systems generates properties of self-renewal, metastasis and chemotherapy resistance, demonstrating causality.[2,3] The hijacking of this core biological process during malignant progression is likely related to key initiating events, including genetic mutations and activation of oncogenic pathways that in turn regulate cell proliferation, migration, epithelial–mesenchymal signaling and cross-talk, and angiogenesis.[4] In prostate cancer, this link between EMT and progression has been circumstantial, but definitive evidence is emerging.[5,6] For instance, new data indicate that expression of the fusion protein TMPRSS2-ERG, which is created through interstitial deletion of chromosome 22 in over half of all prostate cancers,[7] can lead to activation of the beta-catenin pathway and EMT.[8] Additionally, overexpression of the polycomb-repressive complex protein EZH2 has been linked to EMT, metastasis and castration resistance.[9] In prostate cancer model systems, activated stromal signaling from the tumor microenvironment may induce EMT and stemness properties in prostate cancer, contributing to castration resistance and metastatic progression through this molecular cross-talk, often mediated by cytokines and other paracrine factors.[10] We have recently shown that several EMT factors, such as vimentin and N-cadherin, are commonly expressed in circulating tumor cells from men with castration-resistant metastatic prostate cancer.[11] This E-cadherin to N-cadherin switch has been associated with EMT in many tumor types, and in prostate cancer has previously been associated with relapse after surgery and development of metastatic disease.[5]

In a very exciting manuscript in 7 November 2001 advance online publication of *Nature Medicine*, Tanaka *et al.* found that N-cadherin may be more than just a marker of EMT; it may be a useful therapeutic target.[12] In a series of elegant preclinical and clinical correlative studies, they found that N-cadherin was upregulated in castration-resistant prostate cancer cell lines, and increased in expression during serial passage of prostate cancer cell lines in the setting of castration. They next found that N-cadherin expression was increased in clinical metastases from men with castration-resistant disease compared with benign prostate conditions or localized or hormone-sensitive disease, although nearly one-third of men with hormone-naive disease expressed N-cadherin, implying likely some degree of pre-existing expression in tumors rather than complete induction during castration-resistant progression. More importantly, in a series of experiments in which N-cadherin was ectopically expressed and in which N-cadherin was silenced, Tanaka *et al.* demonstrated that N-cadherin induced prostate cancer cells toward castration-resistant growth, promoted muscle invasion, and led to loss of E-cadherin and increased expression of vimentin, and a more mesenchymal phenotype. RNAi-mediated knockdown of N-cadherin reduced invasion and castration-resistant tumor growth, implying that N-cadherin is both necessary and sufficient for malignant growth in these model systems. All of these features point toward a causal role of N-cadherin. Interestingly, N-cadherin expression led to loss of androgen receptor expression, suggesting an inverse role between EMT and androgenic signaling in prostate cancer. Finally, Tanaka *et al.* demonstrated the possibility of targeting N-cadherin through the generation of two monoclonal antibodies against extracellular N-cadherin domains. Anti-N-cadherin antibody administration reduced expression of other EMT markers, tumor growth and invasion, and delayed castration resistance, lymph node metastases and angiogenesis. These changes were accompanied by increased E-cadherin expression. Forced N-cadherin over expression was associated with BCL-2 overexpression, transforming growth factor-beta 1 signaling, and IL-8 and IL-6 expression, all factors implicated in castration-resistant prostate cancer and tumor metastasis.[10] Moreover, targeting antibodies against N-cadherin reduced Akt activation and IL-8 and IL-6 expression during tumor regression, suggesting at least one oncogenic mechanism behind the downstream effects of N-cadherin expression and the mediation of treatment resistance.

Collectively, this work suggests that N-cadherin, an EMT factor, is important in castration-resistant progression in prostate cancer, as well as in contributing to metastasis and the lethal phenotype. It is interesting to speculate that the switch to N-cadherin expression in prostate cancer may induce homotypic adhesion to neural structures, which are known to express N-cadherin, as well as to activated prostate stroma, which may also undergo N-cadherin induction.[13,14] This cross-talk and change in adhesion

Department of Surgery, Durham VA Medical Center, Duke Prostate Center, Division of Urologic Surgery, and Departments of Surgery and Pathology, Duke University Medical Center, Durham, NC 27710, USA
Correspondence: Dr A J Armstrong (andrew.armstrong@duke.edu)

preference may lead to perineural invasion, nodal and disseminated hematogenous metastases. Combined with the existing data linking EMT to stemness, chemoresistance and metastasis in multiple epithelial tumors, the new data by Tanaka *et al.* suggest a new approach targeting EMT directly using N-cadherin monoclonal antibodies or potentially small molecule inhibitors of EMT drivers as a novel approach to prostate cancer therapy and for other solid tumors. Further proof of this will require testing of N-cadherin antibodies in additional preclinical models of prostate cancer that more closely resemble human disease as well as in orthotopic models of prostate cancer that undergo hematogenous rather than lymph node spread. It would be of great interest if N-cadherin antibodies were able to reduce the circulating tumor cell count and development of new metastatic foci in these settings, given the detection of this antigen on circulating tumor cells.[11] Finally, further testing of the safety of N-cadherin targeting will be needed, given its normal role in development, adhesion and mesenchymal cell function.

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PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference *(if desired) (12 characters maximum)* 028193-9082 WO00

Box No. I TITLE OF INVENTION

BIOMARKERS FOR CIRCULATING TUMOR CELLS

Box No. II APPLICANT ☐ This person is also inventor

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

DUKE UNIVERSITY
2812 Erwin Road, Suite 306
Durham, North Carolina 27705
United States of America

Telephone No.

Facsimile No.

Applicant's registration No. with the Office

E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send, notifications issued in respect of this international application to that e-mail address if those offices are willing to do so.

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This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

SINGER, Christopher P.
Michael Best & Friedrich LLP
100 East Wisconsin Avenue, Suite 3300
Milwaukee, WI 53202-4108
United States of America

Telephone No. (312) 222-0800

Facsimile No. (414) 277-0656

Agent's registration No. with the Office 48,701

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E-mail address: mkeipdocket@michaelbest.com

☐ **Address for correspondence:** Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GARCIA-BLANCO, Mariano A.
Duke University
2812 Erwin Road, Suite 306
Durham, North Carolina 27705
United States of America

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
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- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

ARMSTRONG, Andrew J.
Duke University
2812 Erwin Road, Suite 306
Durham, North Carolina 27705
United States of America

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
- ☒ the United States of America only
- ☐ the States indicated in the Supplemental Box

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

GEORGE, Daniel J.
Duke University
2812 Erwin Road, Suite 306
Durham, North Carolina 27705
United States of America

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: US

State *(that is, country)* of residence: US

This person is applicant for the purposes of:
- ☐ all designated States
- ☐ all designated States except the United States of America
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OLTEAN, Sebastian
Duke University
2812 Erwin Road, Suite 306
Durham, North Carolina 27705
United States of America

This person is:
- ☐ applicant only
- ☒ applicant and inventor
- ☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality: US

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This person is applicant for the purposes of:
- ☐ all designated States
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☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No. V DESIGNATIONS

The filing of this request **constitutes under Rule 4.9(a) the designation** of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.

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(The check-boxes above may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.)

Box No. VI PRIORITY CLAIM

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application *(day/month/year)*	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application: regional Office	international application: receiving Office
item (1) 27 January 2010 (27/01/2010)	61/298,845	US		
item (2) 26 February 2010 (26/02/2010)	61/308,780	US		
item (3) 01 March 2010 (01/03/2010)	61/309,131	US		

☐ Further priority claims are indicated in the Supplemental Box.

☐ The **International Bureau** is requested to obtain from a digital library, a certified copy of the earlier application(s) *(if the earlier application(s) is available to it from a digital library)* identified above as:*

☐ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

☒ The **receiving Office** is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) *(if the earlier application(s) was filed with the Office which for the purposes of this international application is the receiving Office)* or to obtain a certified copy of the earlier application(s) from a digital library and transmit a copy of it to the International Bureau *(if the earlier application(s) is available to the receiving Office from a digital library)*, identified above as:*

☐ all items ☐ item (1) ☐ item (2) ☒ item (3) ☐ other, see Supplemental Box

* Where the certified copy of the earlier application(s) is not stored in a digital library under the number of the earlier application indicated above but under the application number of another application which also claims priority from it, indicate that number in the supplemental sheet (item 4).

Restore the right of priority: the receiving Office is requested to restore the right of priority for the earlier application(s) identified above or in the Supplemental Box as item(s) (_____). (*See also the Notes to Box No. VI; further information **must** be provided to support a request to restore the right of priority.)*

Incorporation by reference: where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(if more than one International Searching Authority is competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used)*:

ISA/ US

BIOMARKERS FOR CIRCULATING TUMOR CELLS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to United States Provisional Patent Application No. 61/298,845 filed January 27, 2010; United States Provisional Patent Application No. 61/308,780 filed February 26, 2010; and United States Provisional Patent Application No. 61/309,131 filed March 1, 2010, which are all incorporated herein by reference in their entireties.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with government support under federal grant number 5R33CA097502 from the NIH (NCI), and federal grant number 5Kl2CAlO063904 from the NIH (NCI). The U.S. Government has certain rights to this invention.

SEQUENCE LISTING

[0003] The sequence listing is filed with the application in electronic format only and is incorporated by reference herein. The sequence listing text file "B2442027.txt" was created on September 24, 2010 and is 131,287 bytes in size.

FIELD

[0004] The disclosure relates to methods for the detection and prognosis of cancer. Moreover, the disclosure provides methods for detecting circulating tumor cells (CTCs) that include the identification, detection, and optional enumeration of one or more biomarkers associated with CTCs that can be used in methods relating to a prognosis, diagnosis, or the treatment of cancer in a subject.

BACKGROUND

[0005] Most metazoan cells can be classified as either epithelial or mesenchymal based on morphology, behavior and molecular signatures. Epithelial cells are generally polar in the apico-basal direction, adherent to adjacent cells in the plane perpendicular to the polarity, and non-motile in the polar direction. Mesenchymal cells, in contrast, lack polarity, do not form tight interactions with neighboring cells, and are motile. In adult animals epithelial and mesenchymal cells remain stably in one state or the other; that is, an epithelial cell does not

change its properties and become mesenchymal. During development, however, epithelial cells of the early embryo give rise to all three embryonal layers (endoderm, mesoderm and ectoderm), which include mesenchymal cells (Hay, E.D., et al. *Am. J. Kidney Dis.* **1995**, *26*, 678-690). Therefore, these early embryonal cells have the ability to transition between epithelial and mesenchymal states, a property sometimes referred to as epithelial plasticity. Embryos have been shown to undergo epithelial-mesenchymal transitions (EMTs) as well as mesenchymal-epithelial transitions (METs) (Acloque, H., et al. *J. Clin. Invest.* **2009**, *119*, 1438-1449).

[0006] Circulating tumor cells (CTCs) are cells that have detached from a primary tumor and circulate in the bloodstream. CTCs may constitute seeds for subsequent growth of additional tumors (metastasis) in different tissues. Thus, detection of CTCs can provide for diagnosis and/or prognosis for overall survival and therapeutic implications in subjects with cancers such as metastatic prostate and breast cancer. The number of CTCs in any patient sample (e.g., a blood sample) can be very small, which can make detection difficult. Current methods for detecting CTCs are based on the detection of epithelial cell adhesion molecule (EpCAM) expression, which is a biomarker associated with epithelial cells. Such methods can under-detect CTCs under circumstances where cells undergo a decrease or loss of EpCAM expression, such as biologic processes including EMT. Because of the important role CTCs can play in the diagnosis, monitoring, and prognosis of disease in patients having cancer, any shortcoming in the detection technology needs to be addressed by the art.

[0007] Accordingly, there is a need for methods and systems for detecting CTCs that do not rely on existing capture technologies, and methods for correlating CTC detection to diagnosis, monitoring, and prognosis of disease in cancer patients.

SUMMARY

[0008] In an aspect, the disclosure provides a method for detecting a circulating tumor cell (CTC) in a biological sample, the method comprising detecting at least one epithelial mesenchymal transition (EMT) biomarker in the biological sample.

[0009] In an aspect, the disclosure provides a kit for detecting a circulating tumor cell (CTC) in a biological sample, the kit comprising an antibody to at least one EMT biomarker and instructions for use.

[0010] In an aspect, the disclosure provides a method of predicting responsiveness of a subject having cancer to a course of cancer treatment, the method comprising: determining the

level or presence of expression of at least one EMT biomarker to obtain an EMT biomarker profile and/or optionally a gene expression pattern for a CTC; and predicting the responsiveness of the subject to the cancer drug based on the EMT biomarker profile and/or optional gene expression pattern. In some embodiments the method includes: determining the level or presence of expression of at least one EMT biomarker in a sample from the subject to obtain a biomarker profile and optionally a gene expression pattern in a CTC for the subject; identifying the type of cancer from the biomarker profile and/or optional gene expression pattern, and optionally characterizing the stage of the cancer; and predicting responsiveness of the subject to the cancer drug based on any one of the biomarker pattern, the optional gene expression pattern, the type of cancer, or the stage of the cancer. Embodiments of this aspect can include detecting a number of cells captured and enumerated from a blood sample using at least one EMT biomarker applied to a sample from the subject. These cells that express the EMT biomarker are thereby captured using the EMT biomarker and could then be used to obtain a gene expression pattern in CTCs for the subject; to predict responsiveness of the subject to the cancer drug based on the obtained gene expression pattern, and for the detection of other biomarkers in these CTCs to assist in guiding therapy of that subject. These cells could also be used to measure the level of the specified EMT biomarker or other EMT biomarkers.

[0011] In an aspect, the disclosure provides a method of assessing the number of CTCs using both the traditional EpCAM based capture methodology and an EMT-marker based capture methodology. This EMT-based capture may replace or complement existing CTC capture technologies. The further capture, enumeration, and characterization of these CTCs using EMT antigen capture may further targeting delivery of a cancer drug in a subject having cancer comprising administering to the subject a cancer drug linked to an antibody specific for at least one EMT biomarker or specific drugs based on a gene expression profile or presence of this EMT biomarker.

[0012] In an aspect, the disclosure provides a method of estimating the prognosis of a subject with cancer as well as permitting a further characterization of CTCs that may predict for therapeutic responsiveness, the method comprising: determining the level of or presence of expression of at least one EMT biomarker in a sample from the subject to determine the number of CTCs in the subject and to obtain a gene expression pattern for the subject; and providing a prognosis to the subject based on the gene expression or biomarker profile pattern obtained.

[0013] In an aspect, the disclosure provides a method for monitoring progression of cancer in a subject undergoing therapeutic treatment, the method comprising detecting the level of expression or presence of expression of at least one EMT biomarker and the quantification of CTCs captured using this method in blood samples taken from the subject at a first and a second time; and comparing the first and second levels of expression; wherein a detected difference in the level of expression of the at least one EMT biomarker in the first and second samples over time indicates a change in the progression status of the cancer.

[0014] In an aspect, the disclosure provides a method for detecting cancer in a subject, the method comprising determining the presence of CTCs that express at least one EMT biomarker in a sample from the subject as compared to a normal or control sample, wherein an increased level of at least one EMT biomarker indicates presence of cancer progression or metastatic spread in the subject.

[0015] In an aspect, the disclosure provides a method of treating cancer in a subject comprising administering to the subject a cancer drug linked to an antibody that specifically binds at least one EMT biomarker.

[0016] Other aspects and embodiments of the disclosure will become apparent by consideration of the detailed description and accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] **Figure 1**. **(A)** depicts a schematic representation of the IIIb and IIIc alternatively spliced isoforms of FGFR2. **(B)** is a schematic of the pRIIIcl^2 minigene and the fluorescence read-out. **(C)** is an RT-PCR analysis of the reporter (upper panel) and endogenous FGFR2 (lower panel). **(D)** are epifluorescence and phase-contrast pictures of clones AT3-M and AT3-T.

[0018] **Figure 2. (A)** depicts examples of clusters of DsRED positive cells formed by AT3-M cells upon treatment with conditioned media from clone AT3-T. **(B)** depicts flow cytometry analysis of the same experiment.

[0019] **Figure 3. (A)** depicts growth curves for clones AT3-T and AT3-M. **(B)** is graph of growth of AT3-M, AT3-T, and DT cells in soft agar. **(C)** depicts a sacrifice curve for rats injected with AT3-M or AT3-T cells. **(D)** depicts a comparison of tumor volumes resulting from AT3-T and AT3-M injection.

[0020] **Figure 4. (A)** a representative example of cells that express both RFP and GFP at the periphery of an AT3-M tumor stably transfected with Gint and pRIIIcl^2 reporters. **(B)** a

representative example of a section from an AT3-T tumor stably transfected with GFP and pRIIIcl^2 reporters.

[0021] **Figure 5** a representative example of cells that express both RFP and GFP at the periphery of an AT3-M tumor stably transfected with Gint and pRIIIcl^2 reporters.

[0022] **Figure 6. (A)** representative pictures of cells for the scratch-wound assay. **(B)** a quantification of migration. **(C)** an invasion assay using Matrigel coated membranes. **(D)** a quantification of invasion assay results.

[0023] **Figure 7** are metastatic foci in lungs from animals with tumors from either AT3-T or AT3-M clones (stably transfected with GFP and pRIIIcI^2 reporters). **(A)** (upper panel) is an example of a section exhibiting the pattern for clone AT3-T (i.e. GFP+, DsRED+) in a metastatic focus and (lower panel) an example of a section exhibiting a plastic pattern for clone AT3-T (i.e. GFP+, DsRED-) in a metastatic focus. **(B)** (upper panel) is an example of a section exhibiting the pattern for clone AT3-M (i.e. GFP+, DsRED-) in a metastatic focus and (lower panel) an example of a section exhibiting a plastic pattern for clone AT3-M (i.e. GFP+, DsRED+) in a metastatic focus.

[0024] **Figure 8A** a membrane with serial two-fold dilutions of whole cell Iysates cut in half and immunoblotted for CD133 (upper panel) or β-actin (lower panel). **(B)** a membrane with serial twofold dilutions of whole cell lysates cut in half and immunoblotted for CD44 (upper panel) or β-actin (lower panel).

[0025] **Figure 9** depicts a model comparing stem cell-like character and epithelial mesenchymal phenotype.

[0026] **Figure 10** depicts CTCs from patients with prostate adenocarcinoma. **(A)** illustrates an example of a leukocyte from a human peripheral blood mononuclear cell (PMBC) sample: CD45 (+), CK (-), and vimentin (+). **(B)** illustrates an example of a CD45 (-), CK (+), and vimentin (-) cell from a patient with metastatic breast cancer. **(C)** illustrates an example of a CD45 (-), CK (+), vimentin (+) from a patient with metastatic breast cancer (mBC). **(D)** illustrates an example of a CD45 (-), CK (+), vimentin (+) from a patient with metastatic progressive castrate-resistant prostate cancer (mCRPC).

[0027] **Figure 11** depicts immunofluorescent images of CTCs from patients with mCRPC and mBC.

[0028] **Figure 12** depicts immunofluorescent images of CTCs from patients with mCRPC and mBC.

[0029] **Figure 13** depicts immunofluorescent images of CTCs from patients with mCRPC and mBC.

[0030] **Figure 14** depicts immunofluorescent images of CTCs from patients with mCRPC and mBC.

[0031] **Figure 15** depicts immunofluorescent images of CTCs from patients with mCRPC and mBC.

[0032] **Figure 16** depicts immunofluorescent images of CTCs from patients with mCRPC and mBC.

DETAILED DESCRIPTION

[0033] Before any embodiments are described in detail, it is to be understood that the claims are not limited to the details of construction and the arrangement of components set forth in the following description or illustrated in the included drawings.

[0034] In a general sense, the disclosure provides biomarkers that have been identified to be associated with circulating tumor cells (CTCs). As described herein, one or more biomarkers of epithelial mesenchymal transition (EMT) are detectable on CTCs of patients afflicted with common epithelial malignancies. These transitional cells often display stem cell-like characteristics (stemness) and/or plasticity. Further, the disclosure provides description that metastatic propensity and epithelial phenotypic changes correlate with alternative splicing of the FGFR2 gene. The disclosure also provides that, as illustrated in the non-limiting Examples, transitional cells are found in cancer patients where many CTCs co-expressed biomarkers associated with epithelial and mesenchymal cells.

[0035] Thus, as described below EMT biomarker expression can be used to detect and quantify CTCs in a biological sample. Accordingly, methods comprising detection of EMT biomarker expression, or detection of CTCs, or a combination thereof, can be used to assess cancer prognosis, tumor invasiveness, risk of metastasis, or to stage tumors. As one of skill in the art will appreciate, any suitable method for evaluating EMT biomarker expression can be used to evaluate EMT biomarker expression according to the methods described herein including, but not limited to, detection with antibodies, real time RT-PCR, Northern analysis, Western analysis, and flow cytometry.

[0036] As described herein the ability for a cell to transition easily between epithelial-like and mesenchymal-like states (phenotypic plasticity) is a relevant determinant of malignant fitness more so than the properties of the end states. While these epithelial transitions are

phenotypic, the propensity to transition (plasticity) among carcinoma cells may be determined by genotype. The majority of plastic cells may inhabit transitional intermediate states with properties of both epithelium and mesenchyme, and that these transitional cells may be particularly malignant. Such cells may be detected in: (1) tumors where the cancer cells have mixed histology, which indeed have been observed and have been classified as highly aggressive (e.g., clonal sarcomatous carcinomas of epithelial origin, which exhibit an extremely aggressive behavior, such as sarcomatoid renal cell carcinoma and carcinosarcoma of the prostate); and (2) cancer cells co-expressing epithelial and mesenchymal markers, as described herein.

[0037] The disclosure, as illustrated by the non-limiting embodiments in the Examples, provides for identification of cells that possess an intermediate phenotype – expressing epithelial and mesenchymal isoforms of FGFR2, having epithelial-like morphology and gene expression patterns, while also displaying mesenchymal cell-like migration, tumor formation, and metastases. In embodiments, these cells are identified in patients with advanced cancer, metastatic adenocarcinoma, and metastatic breast and prostate carcinomas. In some embodiments, the cells comprise CTCs. In some embodiments the CTCs co-expresses biomarkers including, for example, EpCAM, cytokeratin, and vimentin, which identify cells as both epithelial- and mesenchymal-like. In some embodiments, these CTCs in intermediate phenotypic states are identified by detecting EMT biomarkers and provide a diagnosis and/or prognosis of the state and/or degree of malignancy of a cancer.

[0038] In an aspect the disclosure provides a method for detecting CTCs in a biological sample, the method comprising detecting at least one epithelial mesenchymal transition (EMT) biomarker in the biological sample. In some embodiments such as illustrated in the Examples, biomarkers of EMT are present on the CTCs of patients with common epithelial malignancies. In some embodiments methods that include detection and identification of alternative splice variants of the FGFR2 gene are used to correlate to metastatic propensity and epithelial phenotypic in a CTC.

[0039] Thus, EMT biomarker expression may be used to detect CTCs. EMT biomarker expression, or detection of CTCs, or a combination thereof, may be used to assess cancer prognosis, tumor invasiveness, risk of metastasis, or to stage tumors. As mentioned above, the methods described herein can include any suitable method for evaluating EMT biomarker expression including, but not limited to, detection with antibodies, real time RT-PCR, Northern analysis, magnetic particles (e.g., microparticles or nanoparticles), Western analysis, and any

method or system involving flow cytometry. In some embodiments, the methods and EMT biomarkers can be used in a commercially available system such as a system that has been approved by a regulatory agency (e.g., FDA) including, for example, CellSearch® technology (Veridex LLC). Thus, the methods can incorporate standard protocols that are known in the art. For example, embodiments comprising CellSearch® technology can include detecting the presence of an EMT biomarker, and correlated to quantifying the number of circulating tumor cells (CTCs) a biological sample, (e.g., blood collected from women in need of a new treatment regimen for metastatic breast cancer, or men in need of treatment for mCRPC). Typical protocols can include drawing blood sample sizes of about 15 mL that can be collected at any particular time (suitably when the patient starts the new therapy, and then again at three to four week intervals). The number of CTCs can be correlated with disease response or progression as determined by standard radiology studies (e.g., CT scans) performed every nine to 12 weeks.

[0040] In an aspect, the disclosure relates to a method for detecting a circulating tumor cell (CTC) in a biological sample, wherein the method comprises detecting at least one epithelial mesenchymal transition (EMT) biomarker in the biological sample. As noted above, a biological sample can be from any tissue or fluid from an organism. In some embodiments the biological sample is from a bodily fluid or tissue that is part of, or associated with, the lymphatic system or the circulatory system of the organism. In some embodiments the biological sample is a blood sample.

[0041] The epithelial mesenchymal transition (EMT) and cellular plasticity biomarkers used in the methods described herein are associated with circulating tumor cells (CTCs). Accordingly, in various embodiments the methods include detecting the presence of one or more EMT biomarker and correlating that detection with the presence of a CTC, optionally quantifying the number of CTCs in the sample. As discussed herein, EMT biomarkers can include any detectable biomolecule that is associated with a transitional cell that exhibits characteristics (e.g., phenotype, or surface antigen or gene expression profiles, etc.) of plasticity, stem-like properties, invasiveness, and/or chemo-resistance of a cell. In some non-limiting embodiments, the EMT biomarker includes any of vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms (such as, for example FGFR2 that includes or excludes either exon IIIc or exon IIIb), or CD133, or any combination of two or more thereof. In some embodiments, the EMT biomarker can include one or more of vimentin (polypeptide SEQ ID NO: 14 encoded by polynucleotide SEQ ID NO: 13), N-cadherin (polypeptide SEQ ID

NO: 2 encoded by polynucleotide SEQ ID NO: 1; polypeptide SEQ ID NO: 16 encoded by polynucleotide SEQ ID NO: 15), O-cadherin (polypeptide SEQ ID NO: 4 encoded by polynucleotide SEQ ID NO: 3; polypeptide SEQ ID NO: 18 encoded by polynucleotide SEQ ID NO: 17), E-cadherin (polypeptide SEQ ID NO: 12 encoded by polynucleotide SEQ ID NO: 11; polypeptide SEQ ID NO: 24 encoded by polynucleotide SEQ ID NO: 23), FGFR2 (polypeptide SEQ ID NO: 8 encoded by polynucleotide SEQ ID NO: 7; polypeptide SEQ ID NO: 10 encoded by polynucleotide SEQ ID NO: 9; polypeptide SEQ ID NO: 22 encoded by polynucleotide SEQ ID NO: 21), and CD133 (polypeptide SEQ ID NO: 6 encoded by polynucleotide SEQ ID NO: 5; polypeptide SEQ ID NO: 20 encoded by polynucleotide SEQ ID NO: 19). In some embodiments, the EMT biomarker can include one or more of N-cadherin, for example human N-cadherin (for example SEQ ID NO: 16, CCDS ID No: CCDS11891.1); O-cadherin, for example human O-cadherin (for example SEQ ID NO: 18, CCDS ID No: CCDS10803.0); E-cadherin, for example human E-cadherin (for example SEQ ID NO: 24, CCDS ID No: CCDS10869.1); CD133, for example human CD133 (for example SEQ ID NO: 20, CCDS ID No: CCDS47029.1); FGFR2, for example human FGFR2 (for example SEQ ID NO: 22, CCDS ID No: CCDS31298.1); and vimentin, for example human vimentin (for example SEQ ID NO: 14, Accession No. BC000163). It will be understood by one of skill in the art that when reference is made to polynucleotides that encode polypeptides in the above embodiments as well as embodiments throughtout, the polynucleotide can be disclosed as either an RNA (e.g., mRNA) or a DNA (e.g., cDNA).

[0042] The EMT biomarkers can be associated with any organism (ortholog) and in certain embodiments are EMT biomarkers associated with a human. Any portion or the entirety of an EMT biomarker can be used for detecting in the methods described herein such as, for example, an epitope of an EMT biomarker protein that binds to an antibody, or a nucleic acid sequence of an EMT biomarker an expressed or transcribed mRNA molecule that is complementary to a reporter nucleic acid probe or primer. In some embodiments, the methods provide for detecting expression of at least two EMT biomarkers. In certain embodiments, expression of vimentin and E-cadherin are detected. In certain embodiments, expression of N-cadherin and O-cadherin are detected. This measure may be used alone or in combination with another method to detect CTCs. In certain embodiments, the methods described herein may be used as a supplemental method in conjunction with CellSearch® Circulating Tumor Cell Test (noted above). Thus, embodiments provide for a method as part of a dual or complementary detection system that can be used to detect and optionally quantify CTCs in a sample (e.g.,

comprising the detection of EpCAM and at least one EMT biomarker). The expression of at least one EMT biomarker may be used to isolate CTCs. The expression of at least one EMT biomarker may be used to count or provide a relative number or amount of CTCs, using any known method for correlating detection of a biomarker to a cell, such as a CTC. CTCs may be detected at the time of, prior to, or after metastasis.

[0043] Cancers may include, but are not limited to, breast cancer, colon cancer, lung cancer, prostate cancer, testicular cancer, brain cancer, skin cancer, rectal cancer, gastric cancer, esophageal cancer, sarcomas, tracheal cancer, head and neck cancer, pancreatic cancer, liver cancer, ovarian cancer, lymphoid cancer, cervical cancer, vulvar cancer, melanoma, mesothelioma, renal cancer, bladder cancer, thyroid cancer, bone cancers, carcinomas, sarcomas, and soft tissue cancers. Thus, the disclosure is generally applicable to any type of cancer in which expression of an EMT biomarker occurs. In certain embodiments, the cancer is a solid tumor malignancy. In certain embodiments, the cancer is breast, colon, or prostate cancer.

[0044] Expression of at least one EMT biomarker may be detected using any suitable method known in the art, including but not limited to, binding with antibodies or fragment thereof, antibodies tethered to or associated with an imaging agent, expression reporter plasmids, flow cytometry, and any suitable array scanner technology. The antibody or fragment thereof may suitably recognize a particular intracellular protein, protein isoform, or protein configuration.

[0045] As used herein, an “imaging agent” or “reporter molecule” is any entity which enhances visualization or detection of the cell to which it is delivered. Any type of detectable reporter molecule/imaging agent can be used in the methods disclosed herein for the detection of one or more EMT biomarker. Such detectable molecules are known in the art and include, for example, magnetic beads, fluorophores, radionuclides, nuclear stains (e.g., DAPI). For example, an imaging agent can include a compound that comprises an unstable isotope (i.e., a radionuclide) or a fluorescent moiety, such as Cy-5, Alexa 647, Alexa 555, Alexa 488, fluorescein, rhodamine, and the like. Suitable radionuclides include both alpha- and beta-emitters. In some embodiments, the targeting vehicle is labeled. In other embodiments, suitable radioactive moieties include labeled polynucleotides and polypeptides which can be coupled to the targeting vehicle. In some embodiments, the imaging agent comprises a radionuclide such as, for example, a radionuclide that emits low-energy electrons (e.g., those that emit photons with energies as low as 20 keV). Such nuclides can irradiate the cell to which they are delivered without irradiating surrounding cells or tissues. Non-limiting

examples of radionuclides that are can be delivered to cells include ^{137}Cs, ^{103}Pd, ^{111}In, ^{125}I, ^{211}At, ^{212}Bi and ^{213}Bi, among others known in the art. Further imaging agents suitable for delivery to a cell in accordance with some embodiments include paramagnetic species for use in MRI imaging, echogenic entities for use in ultrasound imaging, fluorescent entities for use in fluorescence imaging (including quantum dots), and light-active entities for use in optical imaging. A suitable species for MRI imaging is a gadolinium complex of diethylenetriamine pentacetic acid (DTPA). For positron emission tomography (PET), ^{18}F or ^{11}C may be delivered. Other non-limiting examples of reporter molecules are discussed throughout the disclosure.

[0046] In an aspect, the disclosure provides a kit for detecting CTCs in a sample. In embodiments, the kit comprises an antibody to at least one EMT biomarker. The antibody in the kit can be connected to or associated with an imaging agent. In embodiments, the kit can comprise an antibody to at least one EMT biomarker, wherein the antibody is associated a magnetic bead. The magnetic bead may be used for ferromagnetic separation and enrichment of CTCs.

[0047] Aspects also relate to methods of predicting responsiveness of a subject to a cancer drug. The methods may comprise determining the level of expression of at least one EMT biomarker in a sample from the subject. The level of expression of at least one EMT biomarker may be used to obtain a gene expression pattern in CTCs for the subject. The methods may further comprise predicting responsiveness of the subject to the cancer drug based on the gene expression pattern obtained. Genome variation in CTCs from the subject may also be determined.

[0048] Also provided are methods of providing a cancer prognosis to a subject. The methods may comprise determining the level of expression of at least one EMT biomarker in a sample from the subject. The level of expression of at least one EMT biomarker may be used to determine the number of CTCs in the sample. The CTCs may be captured using at least one EMT biomarker. The level of expression of at least one EMT biomarker may be used to determine a gene expression pattern in the CTCs for the subject. A prognosis may be provided to the subject based on the gene expression pattern obtained.

[0049] Also provided are methods for following the progress of cancer in a subject. The methods may comprise determining the level of expression of at least one EMT biomarker in samples from the subject at a first and a second time, and comparing the first and second levels of expression. The level of expression of at least one EMT biomarker in the sample may be

determined over time, such as following initiation of a new cancer therapy. The level of expression of at least one EMT biomarker in the sample may be used to determine the number or amount of CTCs. An increase between the first and second levels may indicate progression of the cancer. A decrease between the first and second levels may indicate remission or response of the cancer to the therapy. No difference between the first and second levels may indicate arrest or stability in the progression of the cancer.

[0050] Also provided are methods of screening for cancer in a subject. The methods may comprise determining the level of expression of at least one EMT biomarker in a sample from the subject. The level of expression of at least one EMT biomarker may be used to determine the amount or number of CTCs in the subject. The level of expression of at least one EMT biomarker may be compared to a normal or control sample. An increased level of at least one EMT biomarker may indicate presence of cancer in the subject.

[0051] Also provided are methods of arresting cell growth or inducing cell death of a cancer cell expressing an EMT biomarker. The methods include contacting the cancer cell with a conjugate capable of mediating intracellular delivery of an agent, such as the antibodies to EMT markers described herein. The agent is capable of arresting or attenuating the growth of the cell or inducing cell death through any mechanism after agent internalization. The cancer cell may be contacted with the conjugate *in vitro*, *in vivo*, or *ex vivo*. These methods may be useful in treating cancer by directly targeting cancer cells expressing an EMT biomarker for delivery of agents capable of decreasing or arresting cell growth or inducing cell death.

[0052] The disclosure also provides for targeted therapeutic methods and molecules that comprise an anti-cancer agent linked to a binding agent that targets at least one EMT as described herein. In some embodiments the link between the anti-cancer agent and the binding agent is a covalent bond. In some embodiments the link is formed by strong electrostatic interactions (hydrogen bonds, hydrophilic/hydrophobic interaction, or oppositely charged moieties, and the like). Any anti-cancer agent can be used in such molecules and therapeutic methods, and can be selected by one of skill in the art based on the type of cancer to be treated, the progress/stage of the cancer, potential adverse drug interactions, dosage requirements, administration schedule, and the like.

EXAMPLES

Example 1. Materials and Methods

[0053] *Plasmids and cell culture.* The minigene used (pRIIIcl^2) was previously described (S. Oltean et al., *Proc Natl Acad Sci USA* 2006, 103, 14116, incorporated herein by reference in its entirety). All cell lines were cultured in low glucose DMEM (Invitrogen) with 10% FBS and 15 IJg/mL blasticidin. Single cell progenies were isolated from a population of AT3 cells stably transfected with pRIIIcl^2 minigene by limiting dilution to produce a concentration of 1 cell/10 wells and plated on 96-well plates. Cells were counted using a hemocytometer to obtain an initial concentration of 1 x 10^5 cells/mL. Through a series of progressive dilutions a final concentration of 1 cell/mL was obtained and 100 IJI were pipetted in each well of three 96-well plates. All wells were monitored through bright field microscopy, those appearing to contain more than one cell were excluded, and those containing single cells were further cultured into 25 mL flasks. 16 of an expected 27 clones were obtained using this procedure in a first round.

[0054] To measure cell population growth rate *in vitro*, cells were plated at 50,000/well in 6-well dishes. Viable cells were counted using Trypan Blue staining at 24, 48, 72, and 96h.

[0055] *Animals and tumor cell implantation.* Cells were trypsinized, washed, and resuspended in PBS at a final concentration of 3 x 10^5 cells/mL, and kept on ice for less than 30 minutes before implantation. Cells (3 x 10^5) were injected subcutis in both flanks of Copenhagen 2331 rats (Harlan Labs, Indianapolis, IN; 75-90 g, 2 months of age). Animals were continuously monitored for tumor growth. All animal procedures were approved by the Duke University Institutional and Animal Care and Use committee and followed NIH guidelines. Sacrifice curves were compared using a Mantel-Haenszel logrank test. Tumor volume was compared using an unpaired t test. Prism 4.0c for the Macintosh (Graphpad, La Jolla, CA) was used for statistical analyses.

[0056] *Histological sections and analysis.* Excised tumors and lungs were washed in PBS at room temperature. Depending on the size of the lungs, they were frozen either together or separately. The tumor sections and the lungs were placed in cryomolds, embedded in optimal-cutting-temperature tissue sectioning medium (Sakura Finetek, Torrance, CA), snap-frozen in liquid nitrogen, and stored at 80°C. Slides for fluorescence imaging were prepared as follows: the tissue was incubated for 2-3 h at -20°C to equilibrate the temperature and then sectioned with a microtome. The sections (15 µm) were placed on glass slides, fixed in 4% (wt/vol) paraformaldehyde for 30 min at room temperature, and rinsed in PBS at room temperature. The

slides were mounted with gel/mount media (Biomeda, Foster City, CA). The sections were analyzed by using an Olympus (Melville, NY) IX 71 epifluorescence microscope, and images were acquired by using an Olympus DP70 digital camera. Image processing was done with DP Controller software (Olympus). For hematoxylin-eosin staining after fluorescence imaging, the slides were incubated in warm water for 15-20 minutes for the cover slip to come off, slides were dried, and staining was performed according to standard procedure.

[0057] *RNA extraction from tumor sections.* Sections were fixed in 4% (wt/vol) paraformaldehyde for 5 minutes, rinsed in PBS, and imaged. DsRED+ and DsRED- regions of the sections were marked on the slide. The slide was immersed in warm water for 5 minutes to remove the coverslip and the DsRED+ and DsRED- regions scraped off. RNA isolation was further performed as described before (N. Masuda, T. Ohnishi, S. Kawamoto, M. Monden, K. Okubo, Nucleic Acids Res 1999, 27, 4436, incorporated herein by reference in its entirety). Briefly, samples were treated with proteinase K in digestion buffer containing SDS, and further isolation of RNA was performed using the RNeasy kit (QIAGEN, Valencia, CA).

[0058] *Immunoblots.* Cells were collected from confluent 25 cm^2 tissues flasks by scraping, washed in PBS, and lysed in sample buffer. Whole cell lysates were serially diluted in sample buffer, fractionated via 7.5% SDS-PAGE, and transferred to PVDF. Membranes were cut in half. The bottom half was probed with anti-β-actin at 1:1000 or 1:5000 (Santa Cruz Biotechnology, CA, 47778) as an internal loading control, while the top half was probed with anti-CD 133 (Santa Cruz Biotechnology, CA, 30219) at 1:200 or anti-CD44 (Santa Cruz Biotechnology, CA, 7946) at 1:200.

[0059] *Gene expression analysis.* Triplicate cultures of AT3-M and AT3-T cells were grown to -60% confluency. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA), and triplicate samples were submitted to the Duke Microarray Facility. Gene expression analysis was performed using the R027K rat spotted arrays 3.0 (Operon, Huntsville, AL). Bioinformatical analysis of expression differences between AT3-M and AT3-T cells was done using the GeneSpring GX software version 7.3.1 (Agilent Technologies, Durham, NC). The data files (representing signals for 26,986 gene probes in all six data points, three for AT3-M and three for AT3-T) were normalized using the feature: per Spot and per Chip - intensity dependent (Iowess) normalization. The resulting gene list was used to determine the significantly differentially expressed genes between AT3-M and AT3-T using the "Filtering on Volcano plot" feature with the following characteristics: (1) Test type: Parametric test, don't

assume variances equal; (2) Multiple testing correction: None; (3) Fold Difference: Twofold or greater and a P-value cutoff of 0.05.

[0060] *Analysis of human circulating tumor cells.* Patients eligible for the CTC biomarker protocols included (1) men with progressive CRPC, with metastatic progression by PSA (two consecutive rises over nadir separated by >1 week) or radiologic criteria (RECIST or new bone scan lesions), a PSA ≥5, age ≥18 years; or (2) women with mBC with disease progression or with initiation of a new systemic therapy, who were >18 years of age, and who were at least 7 days from treatment with an anthracycline-containing regimen. Blood (15 mL) was collected from patients and processed within 48 hours at the Duke University CTC lab using the Cell Search System (Veridex, Raritan, NJ). Veridex profile kits were used, which isolate EpCAM positive cells without additional staining. The isolated cells were either processed immediately or stored overnight in 4% paraformaldehyde and processed the next day. Immunostaining was done on teflon coated slides. Briefly, cells were pipetted into the wells of the slides and left to settle for ~30 minutes followed by standard immunostaining procedures with careful aspiration to minimize cell loss. An initial ferromagnetic wash using a benchtop magnet was performed to further isolate CTCs, with resuspension of the cell pellet after magnet release 100 uL PBS. Following 4% PFA fixation and permeabilization with PBT (PBS with 2% Triton) and blocking with 10% goat serum for 30 minutes, triple immunostaining was performed using CD45 antibody (AbCam #33533-50) labeled with Alexa 647, cytokeratin (AbD Serotec #MCA 1907HT) labeled with Alexa 555, and Vimentin (BD Biosciences, San Jose, CA #550513) labeled with Alexa 488. Nuclear staining with 4',6-diamidino-2phenylindole (DAPI) was then performed. A CTC was defined as an intact cell by microscopic examination, containing an intact nucleus and expressing cytokeratin but lacking CD45 staining, using appropriate controls (see Table 1 for antibodies and controls). Human peripheral blood mononuclear cells (PBMCs), obtained by Ficoll purification of buffy coats from normal donors, were kindly provided by Micah Luftig (Duke University, Durham NC) and used as control cells for CD45 expression. Linear regression analysis was performed to compare CTC count (standard Cellsearch method) against the proportion of CTCs that co-express vimentin. Goodness of fit was tested by analysis of variance.

Table 1. EMT/Stemness Antigens to be assessed in CTCs.

Antigen	Product	Positive Control	Negative Control	Leukocyte Expression	Dilution
Vimentin	BD Biosciences, mouse monoclonal IgG1	PBMCs, PC-3, DUl45	T47D, LnCAP	Yes	2:225
N-cadherin	DAKO, mouse monoclonal IgG1, 6G11	Sarcoma, rat brain, PC-3	DU145, T47D, mock	No	4:225
Cytokeratin (pan)	AbD Serotec, mouse monoclonal IgG1, MCAI907HT, clone AEI/AE3	T47D, DUl45	PC-3, PBMCs	No	2:45
CD45	Invitrogen, mouse IgG1, HI30, MHCD4500	PBMC	PC-3, DUl45	Yes	1:45
CD133	Santa Cruz mouse monoclonal IgG, sc-130127	CaCo-2 colon cancer cells	Mock	Variable	4:225

[0061] The slides were mounted with gel/mount media (Biomeda, Foster City, CA). The slides were analyzed with an Olympus (Melville, NY) IX 71 epifluorescence microscope, and images were acquired using an Olympus DP70 digital camera. Image processing was done with DP controller software (Olympus). All fields were analysed, with each cytokeratin positive nucleated cell that was CD45 negative being counted as a CTC. Positive control cells for each antibody included PC-3 cells for vimentin, peripheral blood mononuclear cells (PBMCs) for CD45, and T47D breast cancer cell lines for cytokeratin. A similar volume of reaction mix without antibody was used for negative controls.

[0062] Media exchange experiments. The cells of AT3-T or AT3-M clones were plated at a concentration of 150,000 cells/2 mL of media in 6-well plates and allowed to incubate for 24 h. The conditioned media was then filtered using a 0.22 μm filter, and then immediately allowed to incubate with cells of the other clone, which was plated at the same concentration and had its media aspirated and cells washed with 2 mL of PBS. All cells with media replaced were incubated for 72 h, and phase and epifluorescent microscopy was used to monitor cell phenotypes 24, 48, and 72 h after treatment. Control plates, in which media was conditioned, cells washed with PBS and media added back to the same cells, were also used.

[0063] *Scratch-wound assay.* Cells were plated and left to grow to nearly 100% confluency in 6-well dishes. A wound was simulated by scratching the cells with a sterile 200 IJI pipette tip. The wells were washed twice with PBS and fresh media added. Pictures were taken in the same marked spot at 0, 24, and 48 h. Percent migration was calculated as (width at 0 h - width at 24 or 48 h) 1 width at 0 h x 100. Relative migration was compared using two-way analysis of variance via Prism 4.0c for the Macintosh (Graphpad, La Jolla, CA).

[0064] *Matrigel assay.* Matrigel assay was performed per manufacturer's indications (BO Biosciences). Briefly, after rehydration, 2 x 10^5 cells were plated either in the control or in the matrigel-coated inserts and incubated for 22 h. Following incubation, the non-invading cells from the upper-part of the inserts were removed using cotton-tipped swabs. The cells from the lower part of the membrane were stained with hematoxylin-eosin, membranes were removed, placed on a slide and observed under the microscope.

[0065] *Immunohistochemical (IHC) analysis of metastases.* Under the same informed consent protocol as the analysis of human circulating tumor cells described above, men undergoing CTC collection additionally consented to have a radiologic-guided metastatic biopsy for analysis of biomarker expression by IHC. Samples were obtained through core needle biopsies during light sedation, and immediately formalin-fixed and paraffin embedded. For analysis, slides were deparaffinized, rehydrated, and endogenous peroxidase was inactivated for 30 min. in 0.3% H_2O_2 (hydrogen-peroxide) in methanol. Specific antigen retrieval steps were performed for individual antigens. Three markers were evaluated by IHC: vimentin (M7020, Dako, 1:150; antigen retrieval with pepsin treatment at 37ºC for 15 minutes), cytokeratin cocktail (18-0132, Invitrogen, 1:50 and 349205, BD Biosciences 1:50, antigen retrieval with pepsin treatment at 37ºC for 15 minutes), and CD45 (M0701, Dako, 1:200; antigen retrieval with sodium citrate 10 mM, pH 6.0 at 100ºC for 30 minutes). Primary antibody was incubated for 60 minutes at room temperature. Dako Envision horseradish peroxidase secondary antibody was used for 30 minutes at room temperature and the signal was detected with DAB reagent (Vector kit SK 4100). Slides were counter stained with hematoxylin and eosin and assessed by a trained pathologist for expression using appropriate positive (localized prostate tissue microarray sections) and negative controls (mock antibody) for each marker.

[0066] *Statistical analyses.* To determine the significantly differentially expressed genes between AT3-M and AT3-T the GeneSpring GX “Filtering on Volcano plot” feature was used with the following characteristics: (1) Test type: Parametric test, don’t assume variances equal;

(2) Multiple testing correction: None; (3) Fold Difference: Twofold or greater and a P-value cutoff of 0.05. To compare CTC count (standard Cellsearch® method) against the proportion of CTCs that co-express vimentin, N-cadherin, or CD133, linear regression analysis was performed. Goodness of fit was tested by analysis of variance.

Example 2. Isolation of individual AT3 clones that inhabit an intermediate phenotypic state

[0067] The alternative splicing of FGFR2 transcripts, which produces either FGFR2-IIIb or -IIIc variants in epithelial and mesenchymal cells respectively, is exquisitely regulated (Figure 1A). In Figure 1A is a schematic representation of the IIIb and IIIc alternatively spliced isoforms of FGFR2. FGFR2 contains an extracellular domain (with three IgG-like domains), a transmembrane domain (TM), and two intracellular tyrosine kinase domains. The IIIb isoform is found in epithelial cells while the IIIc isoform in mesenchymal cells. Exons IIIb and IIIc are regulated coordinately to provide mutually exclusive expression of the two isoforms and transcripts including both exons are destabilized by nonsense-mediated decay. We have previously used FGFR2 alternative splicing reporters, in particular constructs that measure the epithelial-specific silencing of exon IIIc (e.g., pRIIIcl^2 in Figure 1B), to report on the phenotypic state of cells *in vitro* and *in vivo*. In Figure 1B is a schematic of the pRIIIcl^2 minigene and the fluorescence read-out. The minigene contains the DsRED open reading frame interrupted by exon IIIc and flanking introns of the FGFR2 gene. In epithelial cells exon IIIc is skipped, DsRED open reading frame is formed and results in fluorescence signal. In mesenchymal cells, exon IIIc is included and the DsRED open reading frame is disrupted, resulting in low or close-to-background fluorescence signal. The pRIIIcl^2 splicing reporter, which produces a variant red fluorescence protein (DsRED) when exon IIIc is silenced, revealed MET in primary tumors derived from AT3 cells implanted in the flanks of Copenhagen white rats. While most tumors contained MET foci, each tumor had very few foci and these were not randomly distributed but rather were associated with collagenous stroma. In contrast to the low frequency of MET in primary tumors, a high incidence of MET among lung metastases in these animals was observed, suggesting an unexpected association between the more epithelial phenotype and aggressive behavior. These studies could not ascertain whether the epithelial-like AT3 cells found in the lungs had undergone MET in the primary tumors or during the process of metastasis.

[0068] In an attempt to find post-MET cells *in vitro*, limiting dilution was used to obtain clones from AT3 cells stably transfected with the pRIIIcl^2 reporter. A total of 16 clones of a

maximum calculated recovery of 27 were obtained, which is ~ 60% cloning efficiency. Eleven of these sixteen clones expressed RIIIcl^2 transcripts (italicized in Table 2), and of these, eight expressed DsRED (Table 2). Some of the clones had an epithelial-like morphology (cells with cobblestone appearance and adherent to each other), while others had a mesenchymal-like morphology (spindle-shaped), as well as clones that displayed a mixed phenotype. It is important to note that given the high cloning efficiency and the high frequency of DsRED+ clones, it is highly unlikely for these epithelial-like clones to come from a very small population within the parental AT3 cells. Rather, the process of subcloning induced a phenotypic transition in a significant number of the AT3 cells.

Table 2. Properties of AT3 clones.

AT3 Clones	**Cellular morphology[3]**	**DsRED expression[2]**	**Detection of exon lllc skipping among Rlllcl3 transcripts[1]**	**FGFR2 transcipts detected[3]**
1	*Epithelial*	*High*	+	*IIIc*
2	*Epithelial*	*High*	+	*IIIc > IIIb*
3	Epithelial	Low	ND	IIIc > IIIb
4	Epithelial	Low	ND	IIIc
5	*Epithelial*	*High*	+	*IIIc > IIIb*
6	Mesenchymal	Low	ND	IIIc
7	Mixed	Low	ND	IIIc
8	*Mixed*	*High*	+	*IIIc*
9	Mixed	Low	ND	IIIc
10	*Mixed*	*High*	+	*IIIc*
11	*Mesenchymal*	*Low*	-	*IIIc*
12	*Mesenchymal*	*Low*	-	*IIIc*
13	*Epithelial*	*High*	*- 1*	*IIIc > IIIb*
14	*Epithelial*	*Low*	-	*IIIc*
15	*Epithelial*	*High*	+	*IIIc*
16	*Mixed*	*High*	*- 2*	*IIIc*

[1]See Figure 1C. A "+" indicates detection of RIIIcl^2 transcripts missing exon IIIc, a "-" all RIIIcl^2 transcripts include exon IIIc, ND means that no RIIIcl^2 transcripts were detected.
[2]Determined by epifluorescence microscopy (high is defined as fluorescence above background of naive AT3 cells and low undistinguishable from the same cells).
[3]Discussed further herein and illustrated in Figure 1C.

[0069] All of the clones obtained by limiting dilution were analyzed to determine the splicing status of RIIIcl^2 and endogenous FGFR2 transcripts. We could not detect exon IIIc skipping among pRIIIcl^2 transcripts or any evidence of exon IIIb inclusion among endogenous FGFR2 transcripts in clones with a mesenchymal-like morphology (Figure 1C and Table 2). Figure 1C shows RT-PCR analysis of the reporter (upper panel) and endogenous FGFR2

(lower panel). Primers used for the reporter are designed in the DsRED regions flanking exon IIIc. RT-PCR shows a higher percentage of the skipped product in clone AT3-T compared to clone AT3M. Reactions that did not include RT (-RT) reveal a contaminating product that is out-competed by the presence of a bona fide cDNA template (AT3-M lanes). Since exons IIIb and IIIc differ in size by only 3 nucleotides, analysis of the presence of IIIb or IIIc exons in FGFR2 gene was done by using primers in the flanking exons and specific restriction digestion of the resulting RT-PCR products. Exon IIIb is digested by Aval (A) and IIIc by HincII (H). There is a higher percentage of exon IIIb in clone AT3-T. The RT-PCR are replicates from three different cultures of the two clones. These clones did not express detectable levels of DsRED (Figure 1D and Table 2). Figure 1D shows epifluorescence and phase-contrast pictures of clones AT3-M and AT3-T shows the difference in fluorescence intensity and morphology between the two clones. Epifluorescence pictures were taken at the same exposure. All pictures were acquired at 200X magnification. While the skipping of exon IIIc among pRIIIcl^2 transcripts from epithelial-like clones could be expected, the observation that all of these clones both skipped and included exon IIIc was unexpected (Figure 1C, Table 2 and data not shown). Analysis of endogenous FGFR2 transcripts revealed that four of the clones with epithelial morphology and DsRED expression had clear evidence of coexpression of both IIIb and IIIc isoforms (Table 2, and Figures 1C and 1D). As shown in Figure 1, AT3-T cells expressed epithelial and mesenchymal isoforms of FGFR2. The expression of DsRED in all the cells suggested that each cell in the culture was expressing both isoforms (Figure 1C).

[0070] We followed two clones with epithelial morphology, high DsRED levels and co-expression of FGFR2-IIIb and -IIIc transcripts (clone 2 and clone 5 (clone 5 herein AT3-T)) and noted that the phenotypic characteristics described above were stable for over six months. Equally, we followed clone 11 (clone 11 herein AT3-M) and clone 12 for six months, and noted that the mesenchymal morphology, undetectable DsRED expression and exclusive production of FGFR2-IIIc were also stable. We concluded from these observations that AT3 cells were plastic and were coaxed by sub-cloning to populate intermediate phenotypic states, with properties of epithelial and mesenchymal cells.

[0071] A media exchange experiment was used to investigate whether or not the splicing of RIIIcI^2 transcripts in the DsRED expressing clones was regulated by soluble factors.. Media conditioned by DsRED expressing clones (clone 5 in Table 2) was filtered and added to DsRED negative clones (clone 11 in Table 2). DsRED+ cells were observed among DsRED- cells incubated with DsRED+ conditioned media (Figure 2). Figure 2A shows examples of

clusters of DsRED positive cells formed by AT3-M cells upon treatment with conditioned media from clone AT3-T. Media was conditioned for 24 h, filtered and added on AT3-M cells. Pictures (acquired at 200X) are taken 48 h following media exchange. Figure 2B shows results from flow cytometry analysis of the same experiment. Left upper panel represents clone AT3-M conditioned with media from the same clone, as a negative control. Right upper panel represents clone AT3-T, which is DsRED positive. The lower panel represents clone AT3-M 48 h after conditioned media from clone AT3-T was added. Different lots of fetal bovine serum caused variation in this effect. This effect was quantified by flow cytometry and these data suggested that about half of the DsRED- cells were induced to express DsRED at levels equivalent to those seen in DsRED+ cells (Figure 2). The changes observed were not due to prolonged culture of the cells in the same wells because conditioned media from a separate DsRED- culture did not induce DsRED expression. As shown in Figure 2, AT3-T conditioned media induced AT3-M cells to express DsRED. These observations suggest that soluble factors secreted by the DsRED+ clones or dilution of factors extant in the DsRED- conditioned media may contribute to plasticity.

Example 3. AT3-M and AT3-T cells are tumorogenic

[0072] The initial characterization of the AT3-T revealed that these transitional cells grew slower and reached a lower confluent density than the AT3-M (Figure 3A). Figure 3A shows growth curves for clones AT3-T and AT3-M. Cells were plated at 0 h time-point, trypsinized, and counted at the indicated times. Data are the mean ± S.D. (n = 3). To investigate their growth *in vivo* AT3-M and AT3-T cells were co-transfected with pGint a plasmid that expresses EGFP (herein GFP) in both mesenchymal and epithelial cells, and sorted stable populations of each cell line using flow cytometry for uniform GFP intensity. The GFP expressing cells maintained the morphological characteristics, the differential DsRED expression, and the differences in the splicing of pRIIIcl[2] and FGFR2 transcripts first observed after sub-cloning.

[0073] We injected 3 x 10^5 GFP-expressing AT3-T or AT3-M cells subcutis in both flanks of Copenhagen white 2331 male rats. All of the animals developed bilateral tumors, indicating that both AT3-M and AT3-T cells were highly tumorogenic in these syngeneic rats. As a humane endpoint, rats were sacrificed when tumor length estimated by palpation reached 1 cm. The *in vivo* growth curves for the AT3-M and AT3-T tumors were significantly different, as determined by a logrank test ($p = 0.0020$; Figure 3B). Figure 3B is a sacrifice curve for rats

injected with AT3-M or AT3-T cells. Figure 3C shows comparison of tumor volumes resulting from AT3-T and AT3-M injection. The Y-axis represents tumor volumes at the time of sacrifice of the animals and the X-axis days from the time of implantation to the time of sacrifice. Average tumor volumes and average days until sacrifice are represented with S.D. bars. Some points represent more than one tumor with the same volume on the same day. Tumor volume was measured (Figure 3C) and although most AT3-T animals were sacrificed later, there was no significant difference in tumor size ($p = 0.76$). As shown in Figure 3, AT3-T cells grew more slowly than the mesenchymal-like AT3-M cells *in vitro* and *in vivo*, but both were equally tumorogenic. We concluded that whereas AT3-T cells grew more slowly *in vitro* and *in vivo* relative to their more mesenchymal siblings, these transitional cells were capable of forming tumors.

Example 4. Both AT3-M and AT3-T are plastic

[0074] Since the implanted AT3-M and AT3-T cells could be tracked by GFP expression, and epithelial character could be interrogated by DsRED expression, the plasticity of the tumors were able to investigated. The overwhelming majority of cells in AT3-M tumors expressed GFP but not DsRED (Figure 4A). As shown in Figure 4, tumors from both AT3-T and AT3-M clones have evidence of plasticity. Figure 4A shows representative example of cells that express both RFP and GFP at the periphery of an AT3-M tumor stably transfected with Gint and pRIIIcl^2 reporters. Pictures were taken at 200X magnification. To compensate for a low RFP signal, the color curve of the entire picture was adjusted. Nonetheless, groups of cells were observed expressing both GFP and DsRED in many AT3-M tumor sections, especially near the tumor capsule, (Figure 4A; see also Figure 5). Figure 5 shows a representative example of cells that express both RFP and GFP at the periphery of an AT3-M tumor stably transfected with Gint and pRIIIcI^2 reporters. Pictures were taken at 200X magnification. In this version, overall RFP signal was not adjusted via color curve after the image was captured. RFP positive cells were clearly above background level.

[0075] Many sections from AT3-T tumors co-expressed GFP and DsRED; however, large areas were observed that expressed GFP but not DsRED in all 64 sections surveyed (Figure 4B). Figure 4B shows representative example of a section from an AT3-T tumor stably transfected with GFP and pRIIIcl^2 reporters. Pictures were taken at 200X magnification. RNA extracted from these regions of AT3-T tumors confirmed the presence of the pRIIIcl^2

transcripts. Both AT3-T and AT3-M cells were plastic and produced tumors with cells that displayed a range of epithelial-mesenchymal properties.

***Example 5. AT3-T cells are motile* in vitro *and metastatic* in vivo**

[0076] Comparison of AT3-T and AT3-M mobility and invasive potential was performed in culture. Motility was measured in culture by a "wound closure" assay, and no significant motility difference ($p = 0.59$) was found between cell lines 24 and 48 hours after a scratch-wound had been made in the cultures (Figure 6). Figure 6A shows representative pictures for the scratch-wound assay (experiment done in triplicate for each clone). Pictures were taken at 40X magnification. Figure 6B shows quantification of migration as explained in Methods. Mean and SO values were derived from triplicate experiments. Figure 6C shows invasion assay using Matrigel coated membranes. Representative pictures of each clone and for both control membranes and Matrigel-coated membranes ($n = 5$). Cells were stained with hematoxylin-eosin. Pictures were taken at 40X magnification. Figure 6D shows quantification of invasion assay results. Mean and SD values were derived from five individual experiments. To gauge invasive properties of the cells we measured the number of cells traversing through Matrigel membranes in a 22-hour period. The same number of AT3-T and AT3M cells was observed on the Matrigel membranes suggesting that the two cell lines were equally capable of invading this membrane (Figure 6). While a higher number of cells from clone AT3-T were observed on the control membrane compared to clone AT3-M, these studies nevertheless indicated that the more epithelial AT3-T cells had similar motility and invasive potential as the AT3-M cells. As shown in Figure 6, AT3-M and AT3-T cells exhibited similar migration *in vitro*.

[0077] In order to assess invasiveness *in vivo* lungs from the twenty animals harboring AT3-M and AT3-T tumors were examined for presence of metastatic foci. No macroscopic metastatic nodules were observed in any of the lungs, which was likely due to the sacrificing protocol used on the animals when the tumors reached a specified size instead of using survival as the end-point. The GFP expression from the Gint reporter was examined to evaluate the presence of micrometastases by epifluorescence microscopy. To assure a comprehensive evaluation, 7-8 equally spaced sections from each lung were surveyed (total of 150 sections for each clone). The presence of metastatic foci was determined by GFP fluorescence, followed by counter-staining of the sections with hematoxylineosin (Figure 7). Figure 7A shows (upper panel) an example of a section exhibiting the expected pattern for clone AT3-T (i.e. GFP+,

DsRED+) in a metastatic focus, and (lower panel) an example of a section exhibiting a plastic pattern for clone AT3-T (i.e. GFP+, DsRED-) in a metastatic focus. Figure 7B shows (upper panel) an example of a section exhibiting the expected pattern for clone AT3-M (i.e. GFP+, DsRED-) in a metastatic focus, and (lower panel) an example of a section exhibiting a plastic pattern for clone AT3-M (i.e. GFP+, DsRED+) in a metastatic focus. As shown in Figure 7, metastatic foci in lungs from animals with tumors from either AT3Tor AT3-M clones (stably transfected with GFP and pRIIIcI^2 reporters) had evidence of plasticity. Metastatic foci were found in 7 out of 10 lungs for clone AT3-M and 6 out of 10 lungs for clone AT3-T.

[0078] Evaluation of the plasticity of the metastatic foci using the combined output of the GFP and DsRED reporters revealed plastic foci (DsRED+ for AT3-M and DsRED- for AT3-T) in the case of both clones: 3 out of 12 for clone AT3-T and 13 out 16 for clone AT3-M (Figure 7). These studies indicated phenotypic plasticity for the AT3-M cells and suggested it for the AT3-T cells. Importantly, both cell lines were metastatic despite differences in the original epithelial vs. mesenchymal phenotype.

[0079] *Plasticity and metastatic behavior of cancer cells*. Both the mesenchymal AT3-M and the more epithelial AT3-T cells metastasized efficiently. The drivers of metastasis, however, may be different in these two cells. The gene expression comparison between the AT3-M and AT3-T clones revealed at least one intriguing possibility: microarray analysis showed a 12-fold increase in the expression of junctional adhesion molecule C (JAM-C) in AT3-T compared to AT3-M, and this was confirmed by RT-PCR and immunoblot analysis. JAMs were present in leukocytes and at the tight junctions of epithelial and endothelial cells and have been shown to be involved in transendothelial migration of monocytes. JAM-C is expressed in several cell lines with high metastatic potential and knock-down of this molecule in the HT1080 human fibrosarcoma line significantly decreases its metastatic properties *in vivo*. Moreover, JAM-C is also present in the gene sets associated with stemness that had significant overlaps with genes that define clone AT3-T. Therefore clone AT3-T, by over-expression of different adhesion molecules may acquire metastatic capabilities. In addition, the overexpression of the downstream Hedgehog pathway effector GLI3 may be significantly upregulated in the more epithelial and stem cell-like AT3-T cells as compared to the more mesenchymal AT3-M cells. Hedgehog signaling has been linked to EMT, stemness, and metastasis/aggressiveness in several tumor types, and thus differential expression or regulation of developmental programs may underly these phenotypical differences across these cell lines. Increased expression of Patched, a Hedghog pathway component, has been linked to prostate tumors during

progression to androgen independence and in circulating tumor cells of men with metastatic castration-resistant prostate cancer.

Example 6. AT3-T cells display a stem cell-like gene expression signature

[0080] AT3-T cells sometimes formed tight clusters resembling protospheres. While sphere formation is not an exclusive property of stem cells, it has been associated with stemness in many different systems. Given these observations and the high tumorogenicity of AT3-T and AT3-M cells, they were tested for the expression of markers associated with cancer stem-like cells. Also included were the parental AT3 cells and another Dunning tumor cell line, DT cells, which display epithelial markers and are only weakly tumorogenic in Copenhagen white rats. The DT cells expressed very low levels of CD44 and CD133, which are associated with highly malignant cancer stem-like cells (Figure 8). CD133 was detectable in DT lysates only when four fold more lysate was loaded. The mesenchymal-like AT3 cells expressed much higher levels of both CD44 and CD133 than the DT cells (note that the lanes for the DT samples are overloaded in Figure 8A), which is consistent with recent reports that EMT induces stemness in mammary epithelial carcinoma cells. Figure 8A shows a membrane with serial twofold dilutions of whole cell lysates was cut in half and immunoblotted for CD133 (upper panel) or β-actin (lower panel). Size markers are in kDa. A faster migrating CD133 band repeatably detected only in DT lysates is marked (*), suggesting possible post-translational regulation. Figure 8B shows a membrane with serial twofold dilutions of whole cell lysates was cut in half and immunoblotted for CD44 (upper panel) or β-actin (lower panel). Representative blots from two independent sets of lysates are shown. AT3-T expressed CD44 and CD133. Interestingly, the AT3-T cells expressed overall higher levels of CD44 and CD133 than the more mesenchymal AT3-M. Moreover, AT3-T cells expressed a higher ratio of CD44H to CD44E when compared to AT3-M cells. The CD44H isoform has been associated with malignancy while CD44E is not. This suggests a more complex relationship between epithelial transitions and acquisition of stem cell-like properties. Consistent with expression of stem-like markers, both AT3-M and AT3-T cells formed colonies in soft agar and tumors when injected into Copenhagen white rats, and these tumors led to extensive metastases similar to parental AT3 cells (Figure 3B).

[0081] To further explore these connections between transitions and stemness, global gene expression in AT3-M and AT3-T cells was compared. This analysis showed that 422 genes were differentially expressed (≥ 2-fold; p-value < 0.05) in these two cells (Table 3). Many of

the genes that were upregulated in AT3-T relative to AT3-M were preferentially expressed in epithelial cells and vice versa for those preferentially expressed in mesenchymal cells (Table 4). There were exceptions to this, however. Expression of the gene disintegrin-like and metalloprotease was consistent with a mesenchymal phenotype, but this mRNA level was 4-fold higher in AT3-T compared to AT3-M. Integrin β-4, normally associated with epithelial-like cells, was expressed 3-fold lower in AT3-T compared to AT3-M. These observations were consistent with the characterization of AT3-T cells as displaying more epithelial features than AT3-M cells and as populating an intermediate phenotypic state.

Table 3.

x Fold change (AT3-T/AT3-M)	Gene Symbol (Human)	Gene Symbol (Rat)
0.00771	P2RX5	P2rx5
0.011	CCNB1lP1	#N/A
0.0296	STRA6	Stra6
0.0327	G0S2	G0s2
0.0835	SERPINF1	Serpinf1
0.101	GSTA1	#N/A
0.107	RSNL2	Clip4
0.115	ADAMTS7	#N/A
0.134	GZMB	#N/A
0.137	SPON2	#N/A
0.156	MMP3	#N/A
0.191	ATP8A1	#N/A
0.197	EVPL	Evpl
0.21	LGALS3BP	Lgals3bp
0.216	SERPINB2	Serpinb2
0.219	NETO2	Neto2
0.223	PTX3	#N/A
0.23	SERPINB7	Serpinb7
0.233	RASIP1	#N/A
0.235	OMD	#N/A
0.239	HLA-G	#N/A
0.239	HLA-A	#N/A
0.247	CD97	Cd97
0.251	GJA4	Gja4
0.254	DSU	#N/A
0.257	MGLL	Mgll
0.261	SPHK1	#N/A
0.268	HRBL	Zcwpw1
0.268	ZCWPW1	Zcwpw1
0.27	ENPP3	Enpp3
0.275	PTGS1	Ptgs1
0.278	RAMP1	Ramp1
0.281	DHRS3	Dhrs3
0.282	FAM117A	Fam117a

x Fold change (AT3-T/AT3-M)	Gene Symbol (Human)	Gene Symbol (Rat)
0.284	TUBB2A///TUBB2B	Tubb2b
0.284	TUBB2B	Tubb2b
0.285	C10orf10	LOC500300
0.289	SYTL2	#N/A
0.291	SLC39A4	Slc39a4
0.292	CHRD	Chrd
0.292	GIP	Gip
0.293	CKLF	Cklf
0.294	PLAU	Plau
0.295	GUF1	#N/A
0.307	CGI-38	Tppp3
0.311	LECT2	Lect2
0.318	NQO2	#N/A
0.32	C11orf75	RGD1309410
0.324	DOCK2	#N/A
0.325	LGALS2	#N/A
0.326	CASP4	Casp1
0.326	LTBP4	Ltbp4
0.334	HSPB1	Hspb1
0.335	ITGB4	Itgb4
0.34	BPHL	Bphl
0.341	FOXF2	#N/A
0.345	MYH1	#N/A
0.345	SMAD6	Smad6
0.348	TGFB1	Tgfb1
0.351	MMP10	#N/A
0.363	MMP9	Mmp9
0.363	COL18A1	Col18a1
0.366	HES1	#N/A
0.369	SLC35D2	#N/A
0.377	ADORA2B	Adora2b
0.377	COL3A1	Col3a1
0.379	DPEP2	Dpep2
0.382	GPR153	Gpr153_predicted
0.383	LOC55908	#N/A
0.389	SELPLG	#N/A
0.394	P2RX1	Atp2a3
0.394	ATP2A3	Atp2a3
0.394	ADD3	Add3
0.395	TSPAN9	Tspan9
0.399	LOC54103	#N/A
0.4	BFSP2	#N/A
0.4	FLJ14213	RGD1309969
0.4	PGGT1B	Pggt1b
0.401	HCN2	Hcn2
0.403	C2orf33	RGD1310230
0.404	TMEPAI	#N/A

x Fold change (AT3-T/AT3-M)	Gene Symbol (Human)	Gene Symbol (Rat)
0.405	INHA	Inha
0.406	HPSE	#N/A
0.409	CRY1	Cry1
0.413	IL3RA	ll3ra
0.413	CDC42EP1	#N/A
0.416	ARG1	Arg1
0.417	MAPK14	Mapk14
0.419	FLJ22028	#N/A
0.421	GALR2	Galr2
0.422	TSPAN8	Tspan8
0.422	FAM77C	RGD1561205
0.422	USP2	Usp2
0.422	LAMA3	#N/A
0.424	CCNE1	Ccne1
0.424	NSF	Nsf
0.428	ST3GAL5	St3gal5
0.429	SYNJ2	Synj2
0.43	ADA	Ada
0.43	PCBP3	Pcbp3
0.433	ZNF43	#N/A
0.433	C14orf130	Ubr7
0.436	SOS2	#N/A
0.436	RASSF3	#N/A
0.436	GLMN	Glmn
0.438	OSR2	Osr2
0.44	AGTPBP1	Agtpbp1
0.444	DBNDD2	RGD1311642
0.445	SGCB	#N/A
0.446	HBLD2	Isca1
0.448	SCARB1	Scarb1
0.448	EVI2A	Evi2a
0.448	AP4M1	#N/A
0.451	IGF2BP3	#N/A
0.452	FLJ10404	Ddx41
0.454	TGFB2	Tgfb2
0.459	PASK	Pask
0.461	C19orf37	Zfp428
0.462	BMP1	Bmp1
0.464	PTPN13	Ptpn13
0.47	PTPRG	#N/A
0.47	EFNB1	Efnb1
0.472	PER2	Per2
0.472	IRS3L /// LOC442715	Irs3
0.472	HRBL	Irs3
0.472	MAP3K3	Kcnh6
0.472	WDR68	Kcnh6
0.472	KCNH6	Kcnh6

x Fold change (AT3-T/AT3-M)	Gene Symbol (Human)	Gene Symbol (Rat)
0.472	CCDC44	Kcnh6
0.473	CIB2	Cib2
0.475	MPZL1	Mpzl1
0.475	FADS2	#N/A
0.48	ZNF185	#N/A
0.482	SLC29A1	Slc29a1
0.487	RUNX3	Runx3
0.488	NINJ1	Ninj1
0.489	RASL11B	Rasl11b
0.49	ECE2	Ece2
0.49	TNNC2	Tnnc2
0.491	WASPIP	Wipf1
0.492	FN1	Fn1
0.494	NDE1	Nde1
0.494	CAMK2G	Camk2g
0.495	CUTL1	Cux1
0.495	ABHD6	Abhd6
0.495	PTPN14	Ptpn14
0.497	FLJ13946	#N/A
0.498	BAIAP2	Baiap2
0.499	MSL3L1	Msl3l1
0.499	DYNLT1	Dynlt1
0.499	GSTM3	Gstm5
2	CHES1	Foxn3
2.004	AQR	Agr /// Znf770
2.006	EPN1	Epn1
2.011	PPBP	Ppbp
2.019	SLC35D1	#N/A
2.022	PTPRC	#N/A
2.031	USP47	Usp47
2.041	DHX29	#N/A
2.047	HMOX1	#N/A
2.05	CAV1	Cav1
2.053	BUB1B	Bub1b
2.069	KCNIP4	#N/A
2.072	--	#N/A
2.072	ADAM10	#N/A
2.073	KIAA1155	#N/A
2.074	PSTPIP2	#N/A
2.083	MAML1	#N/A
2.084	RAB32	#N/A
2.089	FAM111A	#N/A
2.095	ATRNL1	#N/A
2.101	PPIC	Ppic
2.101	CHD4	Chd4
2.109	IDE	Ide

x Fold change (AT3-T/AT3-M)	Gene Symbol (Human)	Gene Symbol (Rat)
2.117	PITPNM3	#N/A
2.121	NFE2L1	Nfe2l1
2.121	MFSD1	#N/A
2.133	KITLG	Kitlg
2.161	ING3	Ing3
2.167	CD24	#N/A
2.169	IDS	#N/A
2.177	MGC3196	LOC686289 /// LOC690285
2.185	FBXL11	Fbxl11
2.185	--	Fbxl11
2.191	ZC3H12A	#N/A
2.195	RKHD2	#N/A
2.201	LAMC2	Lamc2
2.217	KIF11	Kif11
2.242	SNAPC5	Snapc5
2.252	THRAP3	#N/A
2.261	HS6ST1	#N/A
2.264	OXCT1	#N/A
2.266	TEK	#N/A
2.268	HIST2H4///H4/o/// LOC648164	#N/A
2.271	TMF1	Tmf1
2.273	ZBTB7B	Zbtb7b
2.274	CAMSAP1L1	RGD1310950
2.279	CYP3A5	Cyp3ai
2.279	CYP3A7	Cyp3a9
2.279	CYP3A4	Cyp3a9
2.282	PENK	Penk1
2.283	KIAA2010	Smek1
2.284	CHRNA1	#N/A
2.299	BAT3	Bat3
2.302	ROM1	Rom1
2.306	HOXB8	#N/A
2.309	KLK14	#N/A
2.31	SUV39H1	#N/A
2.315	LOC440354///BOLA2/// LOC595101	RGD1564579
2.315	UBN1	Ubn1
2.323	C1orf103	#N/A
2.333	EYA2	Eya2
2.347	MT2A	#N/A
2.353	KIAA1815	Ermp1
2.355	SETD1B	#N/A
2.369	MPHOSPH1	Kif20b
2.38	EFNA1	Efna1
2.392	ABCF2	Abcf2

x Fold change (AT3-T/AT3-M)	Gene Symbol (Human)	Gene Symbol (Rat)
2.397	LIMA1	Lima1
2.418	EXTL3	Extl3
2.418	ARL6IP2	Arl6ip2
2.442	GRAMD3	Gramd3
2.456	JARID1A	Jarid1a
2.476	ARHGEF9	Arhgef9
2.485	CAD	Cad
2.493	RAI17	#N/A
2.526	KIAA0284	#N/A
2.529	SGPP1	Sgpp1
2.531	ABCB1	#N/A
2.531	ABCB1///ABCB4	#N/A
2.542	KIF1C	#N/A
2.553	KIAA0020	LOC499339
2.563	ADAM15	Adam15
2.577	UBE1	Uba1
2.577	INE1	Uba1
2.58	GRIP2	Grip2
2.59	PPEF1	#N/A
2.619	SC65	Sc65
2.62	FER1L3	#N/A
2.62	NOC3L	#N/A
2.62	RBP4	#N/A
2.645	SPINK4	Spink4
2.653	ATXN2L	#N/A
2.711	AHCYL1	Ahcyl1
2.723	TUBB3	Tubb3
2.723	MC1R	Tubb3
2.729	AGPAT7	Lpcat4
2.749	HOXC11	#N/A
2.766	APH1A	Aph1a
2.785	CNOT1	RGD1308009
2.785	CSNK2A2	RGD1308009
2.794	STAC	#N/A
2.904	STAG1	#N/A
2.942	MBNL1	#N/A
2.982	MNT	Mnt
3.007	RANBP5	Ipo5
3.014	HERC1	Herc1
3.065	ALDOC	Aldoc
3.122	KIAA0460	--
3.174	FLT3	#N/A
3.278	CXCL6	Cxcl6
3.366	GLI3	#N/A
3.489	SSR3	#N/A
3.585	BCAN	Bcan
3.824	FKBP10	Fkbp10

x Fold change (AT3-T/AT3-M)	Gene Symbol (Human)	Gene Symbol (Rat)
3.903	GSTK1	Gstk1
3.931	PSCDBP	#N/A
3.974	ALCAM	Alcam
4.056	ADAMTS13	
4.203	SPRR2B	#N/A
4.276	GPR126	#N/A
5.169	SULF1	Sulf1
5.529	TFF1	Tff1
6.52	PTN	Ptn
8.591	MLF1	Mlf1
9.012	THBS2	Thbs2
10.79	HEPH	Heph
12.53	JAM3	Jam3

Table 4. Examples of epithelial or mesenchymal genes in the expression data analysis of clones AT3-T and AT3-M.

Gene name	x Fold change in AT3-T vs. AT3-M
Junctional adhesion molecule C	12.53
Disintegrin-like and metalloprotease	4.05
Activated leukocyte cell adhesion molecule	3.97
Tubulin	2.73
Epithelial protein lost in neoplasm	2.39
Laminin	2.20
TGFβ2	0.45
MMP9	0.36
Collagen, type XVIII	0.36
MMP10	0.35
Integrin β4	0.33

Gene name	x Fold change in AT3-T vs. AT3-M
TGFβ1	0.31
Urokinase plasminogen activator	0.29
MMP3	0.15

[0082] Two gene sets were assembled: one composed of gene products upregulated in AT3-T (relative to AT3-M) and the second of those downregulated in AT3-T (relative to AT3-M). The two gene sets were compared for overlap with 5,452 gene sets from the Molecular Signature Database collections (Gene Set Enrichment Analysis (GSEA) http://www.broad.mit.edu/gsea/). Analysis of genes over-expressed in AT3-T relative to AT3-M for overlap with 5,452 gene sets from the Molecular Signature Database collections via Gene Set Enrichment Analysis (GSEA) did not show any significant enrichment of sets associated with EMT or MET. In this regard, both AT3-M and AT3-T resembled the mesenchymal-like, parental AT3 line. Among the 15 most significant overlaps for the genes overexpressed in AT3-T there were three sets of genes activated in hematopoetic stem cells ($p = 3.24 \times 10^{-8}$), neural stem cells ($p = 3.07 \times 10^{-7}$) and embryonal murine stem cells ($p = 5.14 \times 10^{-6}$), (Table 5) while among the 20 most significant overlaps for the genes that are relatively downregulated in AT3-T cells were two gene sets associated with development of mature cell types. Expression of the downstream hedgehog pathway effector GLl3 was found to be 3.4-fold overexpressed in AT3-T cells compared to AT3-M cells, indicating that regulation of this developmental/stemness pathway in prostate cancer may be tied to the underlying phenotypic state during EMT/MET, similar to what has been reported in other tumors. These data indicated that AT3-T cells have gene expression profiles similar to stem cells, and, in concordance with the analysis of CD44 and CD133 protein expression, suggested that AT3-T cells exist in a more stem cell-like state than the more mesenchymal AT3-M cells.

Table 5.

GSEA Collections:	C1, C3, C2, C5, C4				
# overlaps shown:	20				
# gene sets in collections:	5452				
# genes in comparison	127				

(N)					
# genes in collections (N)	39655				
gene set name	# genes in gene set (k)	Description	# genes in overlap (k)	k/K	p value
TATAAA_V$TATA_O1	1333	Genes with promoter regions [- 2kb,2kb] around transcription start site containing the motif TATAAA which matches annotation for TAF TATA	20	0.015	8.07E-09
STEMCELL_HEMATOPOIETIC_UP	1452	Enriched in mouse hematopoietic stem cells, compared to differentiated brain and bone marrow cells	20	0.0138	3.24E-08
GNF2_RAP1B	37	Neighborhood of RAP1B	5	0.1351	1.23E-07
STEMCELL_NEURAL_UP	1838	Enriched in mouse neural stem cells, compared to differentiated brain and bone marrow cells	21	0.0114	3.07E-07
module_2	383	Genes in Module_2	10	0.0261	4.34E-07
CTTTGA_V$LEF1_Q2	1270	Genes with promoter regions [-2kb,2kb] around transcription start site containing the motif CTTTGA which matches annotation for LEF1: lymphoid enhancer-binding factor 1	17	0.0134	5.48E-07
SIGNAL_TRANSDUCTION	1637	Genes annotated by the GO term GO:0007165. The cascade of processes by which a signal interacts with a receptor,	19	0.0116	9.33E-07

		causing a change in the level or activity of a second messenger or other downstream target, and ultimately effecting a change in the functioning of the cell.			
module_385	28	Genes in module 385	4	0.1429	1.91E06
V$MYCMAX_O1	261	Genes with promoter regions [-2kb,2kb] around transcription start site containing the motif NNACCACGTGGTNN which matches annotation for MYC: v-myc myelocytomatosis viral oncogene homolog (avian) MAX: MYC associated factor X	8	0.0307	1.98E06
GGGCGGR_V$SP1_Q6	3053	Genes with promoter regions [-2kb,2kb] around transcription start site containing the motif GGGCGGR which matches annotation for SP1: Sp1 transcription factor	26	0.0085	2.59E-06
AACTTT_UNKNOWN	1963	Genes with promoter regions [-2kb,2kb] around transcription start site containing motif AACTTT. Motif does not match any known transcription factor	20	0.0102	3.29E-06
V$AP1_C	281	Genes with	8	0.0285	3.38E-06

		promoter regions [-2kb,2kb] around transcription start site containing the motif NTGASTCAG which matches annotation for JUN: jun oncogene			
MEMBRANE_PART	1673	Genes annotated by the GO term GO:0044425. Any constituent part of a membrane, a double layer of lipid molecules that encloses all cells, and, in eukaryotes, many organelles; may be a single or double lipid bilayer; also includes associated proteins.	18	0.0108	5.09E-06
STEMCELL_EMBRYONIC_UP	1344	Enriched in mouse embryonic stem cells, compared to differentiated brain and bone marrow cells	16	0.0119	5.14E-06
INTRINSIC_TO_MEMBRANE	1350	Genes annotated by the GO term GO:0031224. Located in a membrane such that some covalently attached portion of the gene product, for example part of a peptide sequence or some other covalently attached moiety such as a GPI anchor, spans or is embedded in one or	16	0.0119	5.43E-06

		both leaflets of the membrane.			
CELL_SURFACE	79	Genes annotated by the GO term GO:0009986. The external part of the cell wall and/or plasma membrane.	5	0.0633	5.58E-06
UVC_XPCS_8HR_DN	408	Down-regulated at 8 hours following treatment of XPB/CS fibroblasts with 3 J/m^2 UVC	9	0.0221	6.35E-06
NOTCH_SIGNALING_PATHWAY	12	Genes annotated by the GO term GO:0007219. The series of molecular signals initiated by binding of an extracellular ligand to a Notch receptor on the surface of the target cell.	3	0.25	6.86E-06
LEI_MYB_REGULATED_GENES	325	Myb-regulated genes	8	0.0246	9.62E-06
MORF_DDBl	246	Neighborhood of DDBl	7	0.0285	1.40E-05

[0083] *Epithelial plasticity and stem cell-like behavior*. It is well appreciated that cells induced to undergo EMT activate stem cell pathways. Work presented here shows that AT3 cells that transitioned towards a more epithelial state, i.e. were involved in MET, also activated expression of stem cell-like markers. This finding suggested a broader relationship between plasticity and stem cell-like character or stemness, which was modeled using a Gibbs free energy diagram (Figure 9). Figure 9 shows a model comparing stem cell-like character and epithelial-mesenchymal phenotype. The x-axis represents the spectrum of epithelial to mesenchymal phenotypes and the y-axis represents the stem cell-like character of the cells. The left arrow represents an EMT and the right arrow represents an MET. The model posits that as cells transition back and forth along the epithelial and mesenchymal x-axis they course through states of varying stemness, and this property peaks at intermediate states between epithelial and

mesenchymal phenotypes. The number of different states and the exact height of the barriers between states are speculative and are not meant to be taken as proportional. Two phenotypic transitions are shown, the first is a partial EMT (left arrow) and the second is a partial MET (right arrow). Both of these transitions result in states with higher stem cell-like character. It should be noted that the model also predicts that some EMTs, and equally some METs, will result in a decrease in stemness and indeed this has been observed when the highly aggressive human DKAT basal-type breast cancer cell line is induced to undergo EMT (N. D'Amato and V. Seewaldt, personal communication). The model also suggests a link between stemness, plasticity, and metastatic propensity, perhaps explained by activation of certain oncogenic pathways (e.g., PI3 kinase/Akt) and developmental pathways.

[0084] The model also predicts that cells with maximal stem-cell character, which by definition will be highly malignant, should display both epithelial and mesenchymal traits, because they inhabit intermediate states in the epithelialmesenchymal axis. The highly malignant rat adenocarcinoma AT3-T cells are in this type of state. Importantly, in humans with metastatic breast and prostate carcinomas many CTCs also exist in these intermediate states. These cells correlate with disease progression and are believed to be highly aggressive. A population of cells enriched in CTCs expressed RNAs encoding mesenchymal markers; however, the data did not indicate whether or not epithelial and mesenchymal markers were co-expressed in the same cell. Another clinical example of cells in intermediate states is found in sarcomatoid renal cell carcinomas, which have been shown to co-express epithelial markers, such as epithelial membrane antigen, and mesenchymal ones, like vimentin. These tumors, though rare (1-8% of renal tumors) are highly aggressive and difficult to treat. A similar situation may be found in carcinosarcomas of both the prostate and breast, highly aggressive, rare tumors with mixed epithelial and mesenchymal components but of clonal origin. It is not completely clear whether or not single cells in these tumor co-express epithelial and mesenchymal markers and are thus truly in intermediate states.

[0085] Finally, the model suggests that as sarcomas undergo MET they will activate stem cell-like pathways and become more aggressive. Indeed, there are many descriptions of sarcomas with mixed epithelial and mesenchymal components in close proximity as seen in some synovial- and osteo- sarcomas. New genetically-defined mouse models of soft tissue sarcoma should shed light on the existence and importance of cells intermediate cell states in progression of these tumors.

Example 7. Phenotypic plasticity among human circulating tumor cells

[0086] The experiments described above indicated that Dunning rat prostate adenocarcinoma cells that inhabit an intermediate phenotypic state are tumorogenic, metastatic, and possess stem cell-like antigens and cellular programs. To investigate whether or not similar transitional cells could play a role in human cancer, cancer cells isolated from blood of men with metastatic castrate resistant progressive prostate cancer (CRPC) or women with progressive metastatic breast cancer (mBC) were examined. Circulating tumor cells (CTCs) represent an ideal source of tissue to investigate evidence of this plasticity *in vivo*, given that these cells are likely to be in circulation prior to and during metastatic colonization. CTCs have both independent prognostic and predictive significance in multiple epithelial malignancies, including breast and prostate cancer. These cells can be collected, isolated, and analyzed for a variety of biomarkers relevant to cancer biology.

[0087] It was tested whether there was a high likelihood of finding transitional cells within a population of CTCs captured by FDA-approved EpCAM (Epithelial Cell Adhesion Molecule)-targeted ferromagnetic antibodies. These cells were interrogated for expression of CD45 (expressed in many leukocytes; Figure 10A), cytokeratin (CK; an epithelial marker), and vimentin (a mesenchymal marker) by immunofluorescence. CTCs were defined as CD45-negative and CK-positive nucleated intact cells (Figure 10B) and transitional CTCs were so defined if they additionally co-expressed vimentin (Figure 10C-D). Figure 10 shows that CTCs from patients with prostate adenocarcinoma stained positive for epithelial and mesenchymal markers. Triple staining was performed using anti-CD45 antibody labeled with Alexa 647, anti-cytokeratin (CK) antibody labeled with Alexa 555, and anti-vimentin antibody labeled with Alexa 488. Nuclei were labeled with DAPI. Figure 10A shows an example of a leukocyte from a human peripheral blood mononuclear cell sample: CD45 (+), CK (-), and vimentin (+). Additionally, CD45 (+), CK (-), and vimentin (-) cells were observed. Figure 10B shows an example of a CD45 (-), CK (+), and vimentin (-) cell from a patient with metastatic breast cancer. Such cells were counted as vimentin (-) CTCs in Table 6. Figure 10C shows an example of a CD45 (-), CK (+), vimentin (+) from a patient with metastatic breast cancer. Such cells were counted as vimentin (+) CTCs in Table 6. Figure 10D shows an example of a CD45 (-), CK (+), vimentin (+) from a patient with metastatic progressive castrate-resistant prostate cancer. Such cells were counted as vimentin (+) CTCs in Table 6.

[0088] Transitional CTCs co-expressed vimentin and CK in many of the patients with elevated CTC counts (≥5 CTCs/7.5 mL by standard testing) (Table 6, Figure 10). In fact,

among nine patients with progressive metastatic CRPC and eight patients with progressive mBC, it was found that approximately 75% (range 0-100%, 85.5% in CRPC, 54% in mBC) of the CTCs stained for both CK and vimentin (Figure 10C-D), indicating a transitional phenotype. These data indicated that circulating tumor cells in patients with metastatic breast and prostate cancer co-express epithelial (EpCAM and cytokeratin) and mesenchymal (vimentin) markers, and thus exist in a transitional phenotypic state, similar to that observed in our preclinical models.

Table 6. Circulating tumor cell (CTC) counts and vimentin expression in patients with metastatic castration resistant prostate or metastatic breast cancer.

Subject Number	CTC Count (Cellsearch)*	Ratio: vimentin (+) CTCs/Total CTC Count
Castrate-Resistance Metastatic Prostates Cancer		
1	5	4/6
2	41	11/11
3	45	6/10
4	626	5/8
5	110	17/21
6	182	5/6
7	17	13/16
8	19	33/34
9	34	12/12
Total		106/124 (85.5%)
Metastatic Breast Cancer		
1	21	0/6
2	7	2/2
3	8	4/4
4	21	1/2
5	12	2/2
6	188	21/22
7	138	8/20
8	377	6/23
Total		44/81 (54.3%)
Overall Total	--	150/205 (73.1%)

*Column 2 represents the CTC count as determined by the standard Cellsearch EpCAM based method for each subject, while column 3 represents the number and proportion of CTCs counted manually that were found to express cytokeratin and co-express vimentin, expressed as a ratio and percentage.

[0089] *Plasticity and CTCs*. The identification of plasticity among CTCs in a significant subset of patient samples offers several important clinical opportunities. Expression of plasticity may have prognostic or predictive value in patients with metastatic cancers,

especially mBC where a significant range of values were shown for plasticity. Thus, the subset of patients with very high plasticity may have a more aggressive natural history and exhibit greater resistance to systemic treatments. In terms of diagnosis and utility as predictive biomarkers the data suggested that in addition to cells expressing both epithelial and mesenchymal markers there may be an unknown number of CTCs that have moved further towards the mesenchymal pole and are EpCAM negative. These cells will be missed by the FDA approved CellSearch® System and also by the Adna Test (AdnaGen AG) system and current microfluidic technologies, which enrich for CTCs by immunoabsorbtion of cells expressing MUC1 or EpCAM. Indeed, recent studies in breast cancer have suggested that "normal" type breast cancer cell lines that overexpress both EMT and stem cell antigens (CD44+, CD24-) may lack EpCAM and are thus not detectable by currently approved CTC detection systems. Therefore it is possible that the number of CTCs in patients with metastatic cancer is much higher than currently appreciated. Identification of this additional subset of CTC can provide greater prognostic value than CTC counts as currently determined, as well as earlier detection of CTCs and the metastatic potential in patients with earlier stage disease.

[0090] Furthermore, CTCs in intermediate states, which comprise the 50-75% of cells isolated herein from patients with metastatic breast and prostate cancer as well as those cells that may go undetected because they have undergone a more complete EMT, represent a therapeutic problem. It has been well documented that EMT alters drug sensitivity of lung cancer cells and it has been challenging to direct therapy to cancer cells with stem cell-like properties, perhaps because of their recalcitrance to undergo apoptosis.

[0091] While recent studies suggest both a screening method and actual compounds (e.g., salinomycin) that can selectively target cancer stem cells, these aggressive cells still represent a formidable therapeutic challenge. Thus, molecules comprising a binding agent that has binding specificity to an EMT biomarker described herein and linked to an anti-cancer agent provide additional therapeutic options.

Example 8. CTCs from patients with metastatic breast and prostate cancer express vimentin and N-cadherin

[0092] Eligible men had progressive metastatic CRPC (progression despite testosterone < 50 ng/dL) and were about to begin a new systemic therapy. Eligible women had progressive metastatic breast cancer (mBC) and were about to begin a new systemic therapy. Baseline characteristics of patients (n = 29) are presented in Table 7.

Table 7. Baseline characteristics of patients (n = 29)

	Metastatic Prostate (n = 17)	Metastatic Breast (n=12)
DEMOGRAPHICS		
Age, median	69 (59-82)	61.5 (48-81)
Race, Ethnicity		
White, non-hispanic	76 %	58%
Other, non-hispanic	23 %	42%
BASELINE DISEASE HISTORY		
Gleason Score, median	7 (7-9)	---
ER/PR, %	---	75% / 67%
Baseline median PSA, Range	396.4 (14-13,419.5)	---
Baseline Pain Score (0-10), median	1 (0-7)	0 (0-6)
Karnofsky Performance Status, median	90 (70-100)	90 (70-100) (n=6)
# of Prior Hormonal Therapies	2 (0-5)	2 (0-4)
Prior Chemotherapy	47%	83%
Baseline CTC Count, median	40 (4-828)	13 (0-1062)
METASTATIC SITES		
Lymph Node	65%	50%
Liver	24%	50%
Lung	47%	42%
Bone	94%	75%

[0093] CTCs were drawn into standard FDA-approved Cellsave tubes and processed within 48 hours using the CellSearch® methodology using EpCAM-based ferromagnetic capture. A CTC was defined as an intact nucleated (DAPI+) cell that expressed pan-CK and lacked expression of the leukocyte antigen CD45, and was enumerated using standard methods. A second Cellsearch® tube was collected and processed using EpCAM capture, and isolated cells were stained for CK (IgG1, AbD Serotec) labeled with Alexa 555, CD45 (IgG1, AbCam) labeled with Alexa 647, and either vimentin (IgG1, BD Biosciences) or N-Cadherin (IgG1, DAKO) using immunoflouresent labeling with Alexa 488. The proportion of CTCs staining positive for an EMT antigen was calculated from the total number of CTCs manually scored from the second tube. Positive controls using American Red Cross-derived PBMCs (CD45), PC3 prostate cancer cells (vimentin, N-cadherin), and T47D breast cancer cells (CK) were used for each marker. Negative controls using mock antibody were used to optimize the staining/scoring of each antigen.

[0094] Prevalence of vimentin and CK co-expression in CTCs, and prevalence of N-cadherin and CK co-expression in CTCs are presented in Tables 8 and 9, respectively. Vimentin co-

expression was detected in 17/20 (85%) patients with mCRPC or mBC and 78% of all CTCs. N-Cadherin co-expression was detected in 8/9 (89%) patients and 81% of CTCs. Immunofluorescent images of CTCs from patients with mCRPC and mBC are shown in Figure 11 (A, a leukocyte; B, vimentin negative CTC (CRPC); C, vimentin positive CTC (BC); and D) vimentin positive CTC (CRPC)). Immunofluorescent images of CTCs from patients with mCRPC and mBC are shown in Figure 12 (A, leukocyte; B, Ncad positive CTC (BC); C, Ncad negative CTC (BC); and D, two NCad positive CTCs (arrows) and 1 Ncad negative CTC (CRPC)). Immunofluorescent images of CTCs from patients with mCRPC and mBC are shown in Figure 13 (A, Phase/DAPI; B, CD45/DAPI; C, CK/DAPI; D, Vimentin/DAPI positivity in a man with mCRPC; E, Phase/DAPI; F, CD45/DAPI; G, CK/DAPI; and H, Vimentin/DAPI negativity in a second man with mCRPC).

[0095] The data showed the co-expression of cytokeratin with the EMT antigens vimentin and N-cadherin in CTCs from men with metatastic CRPC and women with metastatic breast cancer. A majority of CTCs examined co-expressed CK and EMT proteins by immunofluorescent labeling. The majority of patients in this study had CTCs that co-expressed vimentin or N-cadherin suggesting potential epithelial plasticity during metastasis. The data suggests that CTCs can lack epithelial markers and provide methods for assessing patients with breast and prostate cancer as well as for the optimal detection of circulating tumor cells in other common malignancies.

Table 8.

Subject Number		CTC Count (Cellsearch)	Ratio of: Vimentin (+) CTCs / Total Manual CTC Count
castrate-resistant metastatic prostate cancer	1	5	4/6
	2	4	2/2
	3	54	11/11
	4	45	6/10
	5	626	5/8
	6	110	17/21
	7	182	5/6
	8	17*	13/16
	9	19	33/34
	10	34	12/12
Total		1127	108/126 (86%)
metastatic breast cancer	1	13	0/6
	2	85	2/2
	3	8	4/4
	4	21	1/2
	5	12	2/2
	6	188	21/22

Subject Number		CTC Count (Cellsearch)	Ratio of: Vimentin (+) CTCs / Total Manual CTC Count
	7	324**	29/33
	8	377	6/23
	9	0	0/0
	10	3	0/3
Total		884	65/97 (67%)
Overall Total		--	173/223 (78%)

Table 9.

Subject Number		CTC Count (Cellsearch)	Ratio of: N-Cadherin (+) CTCs / Total Manual CTC Count
castrate-resistant metastatic prostate cancer	1	45	13/19
	2	12	5/7
	3	10	8/8
	4	5	8/9
	5	12	4/4
	6	221	11/13
	7	828	81/96
Total		1132	130/156 (83%)
metastatic breast cancer	1	1062	9/13
	2	2	0/3
Total		1064	9/16 (56%)
Overall Total		--	139/172 (81%)

*Count from 3 months prior to baseline (no intervening therapy)
**Count from time point #2

[0096] In a second trial to test for the existence of transitional CTCs, blood was collected from 31 men with mCRPC and 16 women with mBC (see baseline characteristics for the patients in Table 10 and Table 11). CTCs were processed using the CellSearch® EpCAM-based immunocapture method and profiled for expression of CD45 (PTPRC) (a leukocyte marker), cytokeratins (CK) (epithelial markers), vimentin (VIM) and N-cadherin (CDH2) (mesenchymal markers), and CD133 (a stem cell marker) by immunofluorescence (IF) (Table 2). Leukocytes were defined as nucleated (DAPI positive), CD45-positive and CK-negative cells, whereas CTCs were defined as nucleated (DAPI positive), CD45-negative and CK-positive cells. Among CTCs we identified transitional cells as those that additionally expressed vimentin or N-cadherin.

Table 10. Baseline demographic and clinical characteristics of the men with metastatic CPRC.

DEMOGRAPHICS	n = 31
Age, years (range)	71 (59-89)
Race, Ethnicity	
White, non-Hispanic	71 %
Black, non-Hispanic	29 %
BASELINE DISEASE HISTORY	
Median Gleason Score (range)	8 (5-10)
Median Baseline PSA[1] (ng/dl, range)	267.5 (14.0-13,419.5)
Median Baseline Pain (range)[2]	1 (0-7)
Median Karnofsky Performance Status (range)	90 (60-100)
Median Number of Prior Hormonal Therapies (range)	3 (0-5)
Prior Chemotherapy	65 %
Prior Bisphosphonates	71 %
SITES OF METASTATIC DISEASE	
Visceral (lung + liver)	35%
Lymph Node Only	0%
Bone metastatic:	
Bone Metastatic With Lymph Nodes (no visceral metastases)	39%
Bone Metastatic Without Lymph Nodes (no visceral metastases)	26%

[1] PSA: prostate specific antigen.
[2] Pain is scored as a linear analog scale (0-10 range).

Table 11. Baseline characteristics of mBC patients.

DEMOGRAPHICS	n = 16
Median age (range)	61 (48-81)
Race, Ethnicity	
White, non-Hispanic	44%
Black, non-Hispanic	50%
Asian, non-hispanic	6%
BASELINE DISEASE HISTORY	

ER and/or PR positive disease	56%
HER2 positive disease (HER2 3+)	0%
Median Karnofsky Performance Status (range)	90 (70-90)
Median Number of Prior EndocrineTherapies (range)	1 (0-4)
Median Number of Prior Chemotherapies	2 (0-7)
SITES OF METASTATIC DISEASE	
Visceral (lung or liver)	75%
Lymph Node Only	0%
Lymph Node, soft tissue, or contralateral breast only	13%
Bone metastases only:	
Bone Metastatic With Lymph Nodes (no visceral metastases)	0%
Bone Metastatic Without Lymph Nodes (no visceral metastases)	13%

[0097] Among ten men with mCRPC, CTCs co-expressed vimentin and CK in 10/10 (100%) patients, and by this criterion 108/126 (86%) of enumerated CTCs were transitional (Table 12, Figure 14). Biopsies of bony metastases performed within one week of CTC collection in two of these patients revealed no vimentin expression in the CK positive tumor foci, but strong vimentin expression in the surrounding bone stroma, which lacks CK expression. These same patients had CTCs taken at the same time as the CT-guided tumor biopsy that commonly expressed co-expressed CK and vimentin. These findings are consistent with invasion and metastasis by transitional CTCs that subsequently undergo MET; alternatively, vimentin expression may be heterogeneously expressed in metastases, similar to CTC expression.

Table 12. Circulating tumor cell (CTC) and transitional CTCs in patients with metastatic CRPC.

Subject Number	CTC Count (Cellsearch)[i]	Ratio: Vimentin (+) CTCs / Total Manual CTC Count[ii]
1	5	4/6
2	4	2/2
3	54	11/11
4	45	6/10
5	626	5/8
6	110	17/21
7	182	5/6
8	17	13/16
9	19	33/34
10	34	12/12
Total	1127	108/126 (86%)
Subject Number	CTC Count (Cellsearch)	Ratio: N-Cadherin (+) CTCs/ Total Manual CTC Count
11	45	13/19
12	12	5/7
13	10	8/8
14	5	7/8
15	12	3/4
16	220	11/13
17	828	81/96
18	26	6/11
19	12	18/22
20	42	15/18
Total	1224	167/206 (81%)
Subject Number	CTC Count (Cellsearch)	Ratio: CD133 (+) CTCs/ Total Manual CTC Count
21	485	38/38
22	16	6/11
23	91	15/21
24	6	0/0
25	36	29/29
26	27	9/9
27	43	10/15
28	2	0/0
29	23	12/14
30	38	23/26
31	30	12/17
Total	797	154/180 (86%)

i The middle column represents the CTC Count from the FDA-approved Cellsearch® enumeration of CTCs for each subject.

ii Right column represents the ratio of vimentin (co-expression of vimentin ranged from 60-100% of cells in a given individual and did not correlate with CTC count (R^2=0.11)), N-cadherin (Co-expression of N-cadherin ranged from 55-100% of cells in a given individual,

and did not correlate with CTC count (R^2=-0.09)), or CD133 (CD133 co-expression ranged from 55-100% of evaluable cells in a given individual and did not correlate with CTC number (R^2=0.04)) expressing CTCs among the total number of CTCs that were manually enumerated. A CTC was defined as an intact DAPI positive (nucleated) cell that lacked CD45 expression and expressed cytokeratin.

Table 13. CTCs and transitional CTCs in patients with mBC.

Subject Number	CTC Count (Cellsearch)i	Ratio: Vimentin (+) CTCs / Total Manual CTC Countii
1	21	0/6
2	7	2/2
3	8	4/4
4	21	1/2
5	12	2/2
6	188	21/22
7	324	29/33
8	377	6/23
9	0	0/0
10	3	0/3
Total	961	65/97 (67%)
Subject Number	**CTC Count (Cellsearch)**	**Ratio: N-Cadherin (+) CTCs/ Total Manual CTC Count**
11	1062	9/13
12	2	0/3
13	147	52/59
14	6	2/5
15	33	15/15
16	2	0/0
Total	1252	78/95 (82%)

[0098] Among the next cohort of 10 men with mCRPC, CTCs co-expressed N-cadherin and CK in 10/10 (100%) patients, and by this criterion 167/206 (81%) of CTCs were identified as transitional (Table 12, Figure 15). Among 10 women with mBC, nine had detectable CTCs and of these, we found evidence of vimentin co-expression in seven (78%) patients, and 55/88 CTCs overall (63%) co-expressed vimentin (Table 13, Figure 14). Among another six women with detectable CTCs and mBC, four had evidence of CK and N-cadherin co-expression, and overall 78/95 CTCs (82%) had N-cadherin expression, with significant heterogeneity in expression in a given individual (Table 13, Figure 15). These data indicate that many CTCs in patients with mBC and mCRPC co-express epithelial (EpCAM and cytokeratin) and mesenchymal (vimentin, N-cadherin) markers, and thus exist in a transitional phenotypic state, similar to that observed in our preclinical models.

[0099] Given the expression of the stem cell associated antigen CD133 in transitional AT3-T cells, CD133 expression in CTCs from men with mCRPC was evaluated. CD133 was expressed in 11/11 (100%) men with CTCs, and in 154/180 (86%) of CTCs from these men (Table 12, Figure 16). These data suggest that CTCs from patients with common epithelial malignancies inhabit transitional states characterized by co-expression of epithelial and mesenchymal markers as well as CD133, biomarkers that have been associated with stem-like properties, invasiveness, and chemoresistance.

SEQUENCES

SEQ ID NO: 1

N-cadherin (also known as cadherin-2, cdh2)

From Mus musculus

Gene No. 12558, Accession No. AB008811

nucleotide (mRNA), 4321 bp

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4321 g

SEQ ID NO: 2

N-cadherin (also known as cadherin-2, cdh2)

From Mus musculus

Gene No. 12558, Accession No. AB008811

polypeptide, translation of SEQ ID NO: 1

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SEQ ID NO: 3

O-cadherin (also known as cadherin-11, cdh11, or ob-cadherin)

From Xenopus laevis

Gene No. 100337621, Accession No. AF002983

nucleotide (RNA), 3237 bp

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2461 ccttatgact ccattcagat ctatggatac gaagggagag gttctgtggc tggctctctt
2521 agttcattag agtcagcctc tacagattca gatttggact atgattatct acaaaactgg
2581 ggacctcgat ttaagaaact agcaaattta tatgggtcca aagacacttg tgaagatgat
2641 tcttaacaaa taagttctga atttggcctt atgaactgca taatgtactg aaatatccag
2701 agtaaacatt aacaggtatt tttttaaagg aaaacatgaa aaaggcttct ttaaccttcc
2761 aaggtttaca aacaggattc cttccaaaac aagaactgtt aaatggtggt ggatactgtg
2821 aaaaccctat ggcctgtgta gaagttgtgt attcattttt ttttttgttt tttgtttttt
2881 ttccaagaaa ccacttgtaa aatgcagcct atttaaggga atggaaatgc aggaaaaacg
```

```
2941 caacaaaaaa ggggaatctt tacagtatta aacataacca tcaaatcttc tcaaacaaag
3001 cttccacaca aaaaaaaaaa aagataacag ttttgagctg taatttcgcc ttaaactatg
3061 gacactttat atgtagtgca tttttaaact tgaaaaaaat atatatataa tatccagcca
3121 gcttcaatcc atataatgta tgtacagtaa aatgtacaat tattctgtct cttgagcatc
3181 agacttgtta ctgctgattc ttgtaaatct tttttgctta taatcccctc gtgccga
```

SEQ ID NO: 4

O-cadherin (also known as cadherin-11, cdh11, or ob-cadherin)

From Xenopus laevis

Gene No. 100337621, Accession No. AF002983

polypeptide, translation of SEQ ID NO: 3

```
MKKDFCLHGLLLCLGIAYCSHATSLRKNNKLRQSFHGHHEKGKE
GQVLHRSKRGWVWNQFFVIEEYTGPDPVLVGRLHSDVDSGDWKIKYILSGEGAGTIFV
IDDKSGNIHATKTLDREERAQYTLMAQAVDRETNKPLEPPSEFIVKVQDINDNPPEFL
HENYHANVPEMSNVGTSVIQVTASDADDPTYGNSAKLVYSILEGQPYFSVEAQSGIIR
TALPNMDREAKEEYHVVIQAKDMGGHMGGLSGTTKVTITLTDVNDNPPKFPQSAYPMS
VSEAAVPGEEVGRIKAKDPDIGENGLIKYRILEGDGAEMFEITADYVTQEGVVKLKKV
VDYETKKFYSMKVEAVNVHIDPRFLSRGPFKDTATVKISVEDFDEPPIFLERSYILEV
YENAPSDTVVGRVHAKDPDAANSPIRYSIDRHTDLDRFFSINPEDGVIKTTKGLDREE
SPWHNISVIATEVHNRIHETRVPVAIKVLDKNDNAPEFAKPYEAFVCENAPINQEFLT
ITAVDKDDTANGLRFLFSFPPEIVHPNPNFTIIDKRDNTASIRVGRGVFSRQKQDLYL
VPIVISDGGSPPMSSTNTLSVRICSCNSDGSQLSCNAEPQSLNAGLSTGALIAILACI
VILLVIVVLFVTLRREKKEPLIVFEEEDIRENIITYDDEGGGEEDTEAFDIATLQNPD
GINGFMPRKDIKPEFQYNPRDIGIRPAPNSVDVDDFINTRIHEADNDPAAPPYDSIQI
YGYEGRGSVAGSLSSLESASTDSDLDYDYLQNWGPRFKKLANLYGSKDTCEDDS
```

SEQ ID NO: 5

CD133 (also known as PROM-1, prominin-1), isoform 2

From Mus musculus

Gene No. 19126, Accession No. BC028286

nucleotide (mRNA), 3701 bp

```
   1 gtccaatcag tgcgctcaga ctcagagccc taggctcctg ctctttaaat taccgagcct
  61 tgtggagacc ccggcacctg gccttaagct cagccctgag gatggtactt tgagtgaatg
 121 accaccttgg agaccgttct tctgtttccc ttgttaccag ccaggaggca gaagagtcca
 181 ccggtccagg aaagacccat ttcccttgag tttccagaaa gtacctcatg cttgagagat
 241 caggccaaca actatggctc tcgtcttcag tgccctgctg ttactggggc tgtgtggaaa
 301 gatctcttca gaaggtcagc ctgcattcca taacactcct ggggctatga attatgaatt
 361 gcctaccacc aaatatgaga cccaagatac cttcaatgct gggattgttg gccctctcta
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421 caaaatggtg cacatcttcc tcaacgtggt ccagccgaat gacttccctc tagatttgat
481 caaaaaactc atacagaaca agaactttga catctcagtt gattccaagg agccagaaat
541 catagtcttg gctctgaaga ttgccctcta tgagatcgga gtccttatct gcgccatcct
601 gggactgctg ttcattatcc tcatgcctct ggtgggctgc ttcttttgta tgtgccgttg
661 ctgcaacaaa tgcggcggag agatgcacca gcggcagaag cagaatgcgc catgcaggag
721 gaagtgcttg ggcctctccc tcctggtgat ttgtctgctc atgagccttg gcattatata
781 tggctttgtg gctaaccagc agaccaggac tcggatcaaa gggacccaga aactggcaaa
841 gagcaatttc agagactttc aaacactcct gactgaaaca ccaaagcaaa ttgactatgt
901 agtggagcag tacaccaaca ccaagaacaa ggcattctca gacctggatg gcatcggctc
961 cgtgctggga ggcagaataa aggaccaact aaaacccaaa gtaactcctg tcctcgaaga
1021 gattaaggcc atggcgacag ccatcaaaca gaccaaggat gccctgcaga acatgagcag
1081 cagcctgaaa agtctccaag atgcagccac ccagctcaat accaacctga gctctgtgag
1141 aaacagcatc gagaattcgc tcagcagcag tgactgtacc tcagatccag ccagcaagat
1201 ctgcgatagc atcagaccaa gcctaagcag tctggggagc agcctcaatt caagtcagct
1261 cccatcagtg gatagagaac tcaacactgt tactgaagtc gacaaaactg atctggagag
1321 cctcgtcaaa agggggtata cgacaattga tgaaataccc aatacaatac aaaaccaaac
1381 tgtggatgtc atcaaagacg tcaaaaatac cttggactcc attagctcca acattaagga
1441 catgagccaa agtattccta ttgaggatat gctgttacag gtctcccatt accttaataa
1501 cagcaacaga tacttaaacc aggagctgcc caagctggaa gaatatgact cgtactggtg
1561 gctgggtggc ttgattgtct gctttctgct gactctcatt gtgaccttct ttttcctggg
1621 cttgctgtgt ggtgtgtttg gctatgacaa gcatgccacc ccaactagaa gaggctgtgt
1681 gtccaacact ggaggcatct tcctcatggc tggggttgga ttcggcttcc ttttttgctg
1741 gatattgatg atccttgtgg ttcttacgtt tgttgttggt gcaaatgtgg aaaagttgct
1801 ctgcgaacct tatgaaaaca agaaattatt acaggttttg gacactccct atctgctcaa
1861 ggaacaatgg caattttatc tttctggcat gctattcaat aacccagaca ttaacatgac
1921 ctttgagcaa gtctacaggg attgcaaaag aggtcgaggt atatatgctg cttttcagct
1981 tgagaatgtc gtcaacgtca gtgatcattt caacattgac cagatttctg aaaacataaa
2041 tacggagttg gaaaacctga atgtgaacat tgatagcatt gaactgttgg ataacacagg
2101 aaggaagagc ctcgaggact ttgcacattc tgggatagat acaatcgatt attccacata
2161 cttgaaggag actgagaaat cccctactga agtgaatctg ctgacatttg cctctaccct
2221 ggaagcaaaa gcaaaccagt tgcctgaagg aaagctgaaa caggccttct tactggatgt
2281 acagaatata agagccatcc accagcatct cctccctcct gtgcagcaat cactgaaatt
2341 tgtgagggtg aggaatacgt taagacaaag tgtctggacc ctccagcaaa caagcaacaa
2401 gttgccggag aaagtgaaga agatccttgc ctctttggac tctgttcagc atttcctcac
2461 caataacgtt tccctcatcg ttatcgggga aacgaagaag tttgggaaaa caatactagg
2521 ctactttgaa cattatctgc actgggtctt ttatgccatc acagagaaga tgacatcctg
2581 caaacccatg gccaccgcga tggactctgc tgttaatggc attctgtgtg gctatgttgc
2641 ggaccctctg aatttgttct ggttcggcat agggaaagcc acggtgctct tacttccggc
2701 tgtaatcatt gctatcaagc tggccaagta ctatcgcagg atggattcag aggatgtata
2761 cgacgacccg tctcgatact gacaactgga gttgaagctg cttgaacaac aagatagtca

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2821 acatggaaag catcacagat tttggatagt ttctgagtct tctagaacgt tccaagtgca
2881 gaagaaacct ggtggagact caggcgggca ctaggaacat ggcatcagtg gtcttagggt
2941 agcactttgt caggaatgaa cagtcatcat ggttataatc cacatatcca ttgcaactca
3001 tgaatgattc tctcctgttt tgtttttaac ttttcttttt acactgattt tctatttaga
3061 cactaaaaca tataggggtg cttattcccc ctggatacat ttacctgtga accagctatt
3121 ccggtgtcat agctgggtac ctaacttact tccatatgtg aagtgtgcta aacacaaacc
3181 agtttacaga agagatgtat tttgtgtata gtaaactgta tatataccct tttaccacag
3241 tcagtttttt aaacaaatga atactctaga tttttcttct aaatgaggtt actgttgggg
3301 tggttgtgac ctagtgatgc tgtagaaagg agtctgcatt cactaaaagt gtgtcaacct
3361 agagcaggca atgcccttcc ttgtggattt ctgtctgctc gttttggagc tacctgcggt
3421 ttagaaatag aattcaagaa caatcacgga gtttcccact tgatgccact gccaaagtca
3481 gaacaaggga tcttgagaga aggaactgtc gctcagctgg gagcggaatc attatcgcaa
3541 tcacaggtcc tggttcacag tttagtggca ctctctggtt tgtaagaatg ggcattacgt
3601 tcagtgtcat ctggtcatct gtgatgtgtg tcatcagcct gtcctgatgt tgagatttaa
3661 aataaagcat gaatgaacag aaaaaaaaaa aaaaaaaaaa a
```

SEQ ID NO: 6

CD133 (also known as PROM-1, prominin-1), isoform 2

From Mus musculus

Gene No. 19126, Accession No. BC028286

polypeptide, translation of SEQ ID NO: 5

```
MALVFSALLLLGLCGKISSEGQPAFHNTPGAMNYELPTTKYETQ
DTFNAGIVGPLYKMVHIFLNVVQPNDFPLDLIKKLIQNKNFDISVDSKEPEIIVLALK
IALYEIGVLICAILGLLFIILMPLVGCFFCMCRCCNKCGGEMHQRQKQNAPCRRKCLG
LSLLVICLLMSLGIIYGFVANQQTRTRIKGTQKLAKSNFRDFQTLLTETPKQIDYVVE
QYTNTKNKAFSDLDGIGSVLGGRIKDQLKPKVTPVLEEIKAMATAIKQTKDALQNMSS
SLKSLQDAATQLNTNLSSVRNSIENSLSSSDCTSDPASKICDSIRPSLSSLGSSLNSS
QLPSVDRELNTVTEVDKTDLESLVKRGYTTIDEIPNTIQNQTVDVIKDVKNTLDSISS
NIKDMSQSIPIEDMLLQVSHYLNNSNRYLNQELPKLEEYDSYWWLGGLIVCFLLTLIV
TFFFLGLLCGVFGYDKHATPTRRGCVSNTGGIFLMAGVGFGFLFCWILMILVVLTFVV
GANVEKLLCEPYENKKLLQVLDTPYLLKEQWQFYLSGMLFNNPDINMTFEQVYRDCKR
GRGIYAAFQLENVVNVSDHFNIDQISENINTELENLNVNIDSIELLDNTGRKSLEDFA
HSGIDTIDYSTYLKETEKSPTEVNLLTFASTLEAKANQLPEGKLKQAFLLDVQNIRAI
HQHLLPPVQQSLKFVRVRNTLRQSVWTLQQTSNKLPEKVKKILASLDSVQHFLTNNVS
LIVIGETKKFGKTILGYFEHYLHWVFYAITEKMTSCKPMATAMDSAVNGILCGYVADP
LNLFWFGIGKATVLLLPAVIIAIKLAKYYRRMDSEDVYDDPSRY
```

SEQ ID NO: 7

FGFR2 IIIc

From Mus musculus

Gene No. 14183, Accession No. M86441

nucleotide (mRNA), 3306 bp

```
   1 gaattcccgc gcggccgcca gagctccggc ccgggggctg cctgtgtgtt cctggcccgg
  61 cgtggcgact gctctccggg ctggcggggg ccgggcgtga gcccgggcct cagcgttcct
 121 gagcgctgcg agtgttcact actcgccagc aaagtttgga gtaggcaacg caagctccag
 181 tcctttcttc tgctgctgcc cagatccgag agcagctccg gtgtatgtct agctgttctg
 241 cgatcccggc gcgcgtgaag cctcggaacc ttggcgccgg ctgctaccca aggaatcgtt
 301 ctctttttgg agttttcctc cgagatcatc gcctgctcca tcccgatcca ctctgggctc
 361 cggcgcagca ccgagcgcag aggagcgctg ccattcaagt ggcagccaca gcagcagcag
 421 cagcagcagt gggagcagga acagcagtaa caacagcaac agcagcacag ccgcctcaga
 481 gctttgctcc tgagcccctg tgggctgaag gcattgcagg tagcccatgg tctcagaaga
 541 agtgtgcaga tgggattacc gtccacgtgg agatatggaa gaggaccagg gattggcact
 601 gtgaccatgg tcagctgggg gcgcttcatc tgcctggtct tggtcaccat ggcaaccttg
 661 tccctggccc ggccctcctt cagtttagtt gaggatacca ctttagaacc agaagagcca
 721 ccaaccaaat accaaatctc ccaaccagaa gcgtacgtgg ttgcccccgg ggaatcgcta
 781 gagttgcagt gcatgttgaa agatgccgcc gtgatcagtt ggactaagga tggggtgcac
 841 ttggggccca acaataggac agtgcttatt ggggagtatc tccagataaa aggtgccaca
 901 cctagagact ccggcctcta tgcttgtact gcagctagga cggtagacag tgaaacttgg
 961 atcttcatgg tgaatgtcac agatgccatc tcatctggag atgatgagga cgacacagat
1021 agctccgaag acgttgtcag tgagaacagg agcaaccaga gagcaccgta ctggaccaac
1081 accgagaaga tggagaagcg gctccacgct tgtcctgccg ccaacactgt gaagttccgc
1141 tgtccggctg gggggaatcc aacgtccaca atgaggtggt taaaaaacgg gaaggagttt
1201 aagcaggagc atcgcattgg aggctataag gtacgaaacc agcactggag ccttattatg
1261 gaaagtgtgg tcccgtcaga caaaggcaac tacacctgcc tggtggagaa tgaatacggg
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1381 caagctggac tgcctgcaaa tgcctccacg gtggtcggag gggatgtgga gtttgtctgc
1441 aaggtttaca gcgatgccca gccccacatc cagtggatca agcacgtgga aaagaacggc
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1621 gaatatacgt gcttggcggg taattctatc gggatatcct ttcactctgc atggttgaca
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1861 accaagcgca tccccctgcg gagacaggta acagtttcgg ccgagtccag ctcctccatg
1921 aactccaaca ccccgctggt gaggataaca acgcgtctgt cctcaacagc ggacaccccg
1981 atgctagcag gggtctccga gtatgagttg ccagaggatc caaagtggga attccccaga
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2041 gataagctga cgctgggcaa acccctgggg gaaggttgct tcgggcaagt agtcatggct
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2281 cctctctacg tcatagttga atatgcatcg aaaggcaacc tccgggaata cctccgagcc
2341 cggaggccac ctggcatgga gtactcctat gacattaacc gtgtccccga ggagcagatg
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2641 gtttacactc atcagagcga tgtctggtcc ttcggggtgt taatgtggga gatctttact
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2761 ggacacagga tggacaagcc caccaactgc accaatgaac tgtacatgat gatgagggat
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2881 cgaattctga ctctcacaac caatgaggaa tacttggatc tcacccagcc tctcgaacag
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3061 gttaaaacat gagtgaatgt gtcttcctgt ccccaaacag gacagcacca ggaacctact
3121 tacactgagc agagaggctg tgctccagag cctgtgacac gcctccactt gtatatatgg
3181 atcagaggag taaatagtgg gaagcatatt tgtcacgtgt gtaaagattt atacagttgg
3241 aacatgtact acaggaagga gactgttctg atagtgacag ccgccaccat gccacctttg
3301 accaca
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SEQ ID NO: 8

FGFR2 IIIc

From Mus musculus

Gene No. 14183, Accession No. M86441

polypeptide, translation of SEQ ID NO: 7

```
MVSWGRFICLVLVTMATLSLARPSFSLVEDTTLEPEEPPTKYQI
SQPEAYVVAPGESLELQCMLKDAAVISWTKDGVHLGPNNRTVLIGEYLQIKGATPRDS
GLYACTAARTVDSETWIFMVNVTDAISSGDDEDDTDSSEDVVSENRSNQRAPYWTNTE
KMEKRLHACPAANTVKFRCPAGGNPTSTMRWLKNGKEFKQEHRIGGYKVRNQHWSLIM
ESVVPSDKGNYTCLVENEYGSINHTYHLDVVERSPHRPILQAGLPANASTVVGGDVEF
VCKVYSDAQPHIQWIKHVEKNGSKNGPDGLPYLKVLKAAGVNTTDKEIEVLYIRNVTF
EDAGEYTCLAGNSIGISFHSAWLTVLPAPVREKEITASPDYLEIAIYCIGVFLIACMV
VTVIFCRMKTTTKKPDFSSQPAVHKLTKRIPLRRQVTVSAESSSSMNSNTPLVRITTR
LSSTADTPMLAGVSEYELPEDPKWEFPRDKLTLGKPLGEGCFGQVVMAEAVGIDKDKP
KEAVTVAVKMLKDDATEKDLSDLVSEMEMMKMIGKHKNIINLLGACTQDGPLYVIVEY
ASKGNLREYLRARRPPGMEYSYDINRVPEEQMTFKDLVSCTYQLARGMEYLASQKCIH
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RDLAARNVLVTENNVMKIADFGLARDINNIDYYKKTTNGRLPVKWMAPEALFDRVYTH
QSDVWSFGVLMWEIFTLGGSPYPGIPVEELFKLLKEGHRMDKPTNCTNELYMMMRDCW
HAVPSQRPTFKQLVEDLDRILTLTTNEEYLDLTQPLEQYSPSYPDTSSSCSSGDDSVF
SPDPMPYEPCLPQYPHINGSVKT

SEQ ID NO: 9

FGFR2 IIIb

From Mus musculus

Gene No. 14183, Accession No. M63503

nucleotide (mRNA), 3037 bp

```
   1 ggcgagggga gagagccggg agaggcgagc ggcggcgcgg caggcgcgga acgggcgcac
  61 ggacgatcga acgcgcggcc gccagagctc cggcgcgggg gctgcctgtg tgttcctggc
 121 ccggcgtggc gactgctctc cgggctggcg ggggccgggc gtgagcccgg gcctcagcgt
 181 tcctgagcgc tgcgagtgtt cactactcgc cagcaaagtt tggagtaggc aacgccaagc
 241 tccagtcctt tcttctgctg ctgcccagat ccgagagcag ctccggtgtc atgtcctagc
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 421 ctgggctccg gcgcagaccg agcgcagagg agcgctgcca ttcaagtggc agccacagca
 481 gcagcagcag cagcagtggg agcaggaaca gcagtaacaa cagcaacagc agcacagccg
 541 cctcagagct ttggctcctg agccccctgt gggctgaagg cattgcaggt agcccatggt
 601 ctcagaagaa gtgtgcagat gggattaccg tccacgtgga gatatggaag aggaccaggg
 661 attggcactg tgaccatggt cagctggggg cgcttcatct gcctggtctt ggtcaccatg
 721 gcaaccttgt ccctggcccg gccctccttc agtttagttg aggataccac tttagaacca
 781 gaaggagcac cgtactggac caacaccgag aagatggaga agcggctcca cgctgtccct
 841 gccgccaaca ctgtgaagtt ccgctgtccg gctgggggga atccaacgcc cacaatgagg
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1741 gaggatccaa agtgggaatt ccccagagat aagctgacgc tgggcaaacc cctgggggaa
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1861 gaggcggtca ccgtggcagt gaagatgttg aaagatgatg ccacagagaa ggacctgtct
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2521 aatgaactgt acatgatgat gagggattgc tggcatgctg taccctcaca gagacccaca
2581 ttcaagcagt tggtcgaaga cttggatcga attctgactc tcacaaccaa tgaggaatac
2641 ttggatctca cccagcctct cgaacagtat tctcctagtt accccgacac aaggagctct
2701 tgttcttcag gggacgattc tgtgttttct ccagacccca tgccttatga accctgtctg
2761 cctcagtatc cacacataaa cggcagtgtt aaaacatgag tgaatgtgtc ttcctgtccc
2821 caaacaggac agcaccagga acctacttac actgagcaga gaggctgtct cagagcctgt
2881 gacacgcctc cacttgtata tatggatcag aggagtaaat agtgggaagc atattgtcac
2941 gtgtgtaaag atttatacag ttcggaaaca tgttacctaa ccaggaaagg aagactgttt
3001 tcctgataag tggacagccg caagccacca tgccacc
```

SEQ ID NO: 10

FGFR2 IIIb

From Mus musculus

Gene No. 14183, Accession No. M63503

polypeptide, translation of SEQ ID NO: 9

```
MVSWGRFICLVLVTMATLSLARPSFSLVEDTTLEPEGAPYWTNT
EKMEKRLHAVPAANTVKFRCPAGGNPTPTMRWLKNGKEFKQEHRIGGYKVRNQHWSLI
MESVVPSDKGNYTCLVENEYGSINHTYHLDVVERSPHRPILQAGLPANASTVVGGDVE
FVCKVYSDAQPHIQWIKHVEKNGSKYGPDGLPYLKVLKHSGINSSNAEVLALFNVTEM
DAGEYICKVSNYIGQANQSAWLTVLPKQQAPVREKEITASPDYLEIAIYCIGVFLIAC
MVVTVIFCRMKTTTKKPDFSSQPAVHKLTKRIPLRRQVTVSAESSSSMNSNTPLVRIT
TRLSSTADTPMLAGVSEYELPEDPKWEFPRDKLTLGKPLGEGCFGQVVMAEAVGIDKD
KPKEAVTVAVKMLKDDATEKDLSDLVSEMEMMKMIGKHKNIINLLGACTQDGPLYVIV
EYASKGNLREYLRARRPPGMEYSYDINRVPEEQMTFKDLVSCTYQLARGMEYLASQKC
IHRDLAARNVLVTENNVMKIADFGLARDINNIDYYKKTTNGRLPVKWMAPEALFDRVY
THQSDVWSFGVLMWEIFTLGGSPYPGIPVEELFKLLKEGHRMDKPTNCTNELYMMMRD
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CWHAVPSQRPTFKQLVEDLDRILTLTTNEEYLDLTQPLEQYSPSYPDTRSSCSSGDDS
VFSPDPMPYEPCLPQYPHINGSVKT

SEQ ID NO: 11

E-cadherin (also known as cadherin-1, cdh1)

From Xenopus (Silurana) tropicalis

Gene No. 779546, Accession No. XM_002935997

nucleotide (mRNA), 3344 bp

```
   1 agagcaggga agtacagcgc tgcgctacaa gaactgagca aacgagcaga aaagtacaca
  61 ttcctgatcc ttcggtcttt ccaaaagtcc ccaatggggt cacacaggcc atggttactt
 121 ggtgctgtgg tgctgctggc actccttcag gtacagggag gactggcaga atggacacag
 181 tgtcaaatgg gattttccaa ggaaaggtac agcttttcgg tacctaagaa cttggagaca
 241 gacaaagcac tgggtagagt gatctttaac agctgtgagg gaccagtgag aattcagttt
 301 gcctctaaag atcctaattt tgaaattcac aaagatggca cagtttatgt taagaatcct
 361 accaagatga aagacaacag aaaaacattc cgtgtcctgg cttgggagaa tcaaggtcat
 421 gtatactcta ccagtgtaac cttgaaaggg gaagggcatc accataagca ggacatttct
 481 tctgtgaaac attcccacca cccaaaatct gagactggtt taaaaagaca aaaaagagac
 541 tgggtgattc caccaatcgt aacatctgag aatgaaaagg gcccatttcc caaacggctt
 601 gtgcagatca agtccagtaa tgcaaaggaa atcaaggttt tttacagtat cacaggccag
 661 ggtgccgata cccctccaga aggagtgttc actattggac gggaggatgg atggctaaat
 721 gtgacacgac ctttggacag agaagccatt gatagttaca ctctttttc tcatgctgtg
 781 tcagtaaatg ggcaaaatgt ggaagatccc atggaaatcc aaattaaagt acaagatcag
 841 aatgataatg acccagtttt cacacaggag gtctttgaag gctatgtgcc tgaagggtct
 901 aagccaggta cgcccgtcat gactgtatct gcaacagatg ccgatgatgc tatagacatg
 961 tacaatggtg tgattactta ctccattctc aaccaagacc ctaaagagcc caacaatcaa
1021 atgttcacta ttgattccca gtctgggttg atcagcgtag ttacaactgg attagacaga
1081 gagaaaatac cagtgtacac actgactatt caagctgcag atggagaatt tgggaaagat
1141 cgcacaacaa ctgcaaaagc tgtgatcatt gtgacagaca ccaatgataa ccctcctgtg
1201 tttaacccaa cgcaatacat tgcagaggtt cctgaaaatg aagttggata tgaggttgca
1261 cgtcttacgg taacagatgc agatattgaa gggtcagatg cctggaatgc tgtgtacaag
1321 atcattaaag gaaatgaggc tggctttttc agcatccaaa cagatattga caacattggg
1381 ctactgaaaa cagtgaaggg tctggactat gagctgaaga agcagtatat tctgtcagtc
1441 attgtgacaa acaaagctaa cttttctgtt ccactacaaa cttcaactgc aacggtcact
1501 gtaactgtca cagatgtgaa tgaggcccca gtatttgtac cagtgttgaa agacgtgtct
1561 gtgccagagg atctgcccag tggccaagtt gttgctacct ataccgcaca ggatccagac
1621 aaggaacaga accagaaaat aagttacttc attggaaatg acccagcagg gtgggtgtct
1681 gtgaacagag ataatgggat tgtcactgga aatggaaact tggatcggga atcaaagttt
1741 gtgctaaaca acacctacaa agtcataatc ttggccgctg acagtggcac tccttctgcc
1801 actgggactg gaacccttgt gcttaatctc attgatgtta atgataatgg cccatttttg
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1861 gatccccaac aaaatagttt ctgccagaag gatccaggct ttcgtgtatt taatatcatt
1921 gacaaagatc tttaccctaa cacataccca tatacagtag acctgactgg tgaatccaat
1981 gaaaactgga ctgctacagt gacagaacag agtttacttg agctgagacc taaaaaggaa
2041 ctggatattg gacgatacga agttttgatc tcattgagag acaatcaggg actgacagat
2101 gtgacaaagc tacagattac aatctgtcaa tgtaatggtg accaaatgca atgtgaggaa
2161 aaggctgctc aagcaggagg tttggggata tcagccatag ttggaatcct tggagggatc
2221 ctagcgcttc ttttattgtt gttgctgctc ttactgtttg tacgacgaaa gaaagtggta
2281 aaagaacctt tattaccacc agaagatgag actcgggaca atgtattttt ctatgatgaa
2341 gaaggcggtg gtgaggaaga ccaggatttt gatctaagcc agcttcaccg tggtctagat
2401 gctcgtccag atataatccg taatgatgtc gttccagttt tagctgctcc ccagtatcga
2461 ccccgtcctg ccaatccaga tgaaattgga aatttcattg atgagaactt gcatgcagct
2521 gacaatgacc ccactgctcc tccatacgac tcgctccttg tgttcgatta cgaaggcagt
2581 ggctctgagg ccgcatcact cagctctctt aactcttcca actctgattt agatcaggat
2641 tacagtgctt tgaataactg gggacctcgt ttcaccaaac tggcagaaat gtatggagga
2701 gatgaggatt agaatgtgca ctgcaatacc attttgattc taaacagtaa actaaaaacc
2761 ataattgtgt atgcagtctt tggaattcac tttgttttct cctgctctta aaacagagat
2821 aaggactgct caaaagttac tcctcctgct tttgtaaaat cgttcaaaaa tattttatgt
2881 atatgtatat atgaaaaaat cgtatttttt gtactatttg tgttcttata tccctgcaat
2941 ttgtaataca agaggatctt tatctgctta attataaata taaaatgccc gatatgattc
3001 actatgattt taatgtgttg agaaatcttt ttttaaaaag gtttccagac acctgacgct
3061 tggaagggaa ttccataaaa atataattga attgggggga gattgtgttt tgccatggtc
3121 tgatatacat tttcatatat atacatatga tcattcacag agtacagtca acatttggaa
3181 tttgatgagc ttgctggtcg aactgaaaaa aaaatgtatt atagctgggg taaaaattaa
3241 tgtatgagct aaatggggca caattttgat atctctgcat ttgtatttta cttggcatgt
3301 atacttttgt aataaaataa agatatacat taatatacaa cata
```

SEQ ID NO: 12

E-cadherin (also known as cadherin-1, cdh1)

From Xenopus (Silurana) tropicalis

Gene No. 779546, Accession No. XM_002935997

polypeptide (translation of SEQ ID NO: 11), 872 amino acids

```
MGSHRPWLLGAVVLLALLQVQGGLAEWTQCQMGFSKERYSFSVP
KNLETDKALGRVIFNSCEGPVRIQFASKDPNFEIHKDGTVYVKNPTKMKDNRKTFRVL
AWENQGHVYSTSVTLKGEGHHHKQDISSVKHSHHPKSETGLKRQKRDWVIPPIVTSEN
EKGPFPKRLVQIKSSNAKEIKVFYSITGQGADTPPEGVFTIGREDGWLNVTRPLDREA
IDSYTLFSHAVSVNGQNVEDPMEIQIKVQDQNDNDPVFTQEVFEGYVPEGSKPGTPVM
TVSATDADDAIDMYNGVITYSILNQDPKEPNNQMFTIDSQSGLISVVTTGLDREKIPV
YTLTIQAADGEFGKDRTTTAKAVIIVTDTNDNPPVFNPTQYIAEVPENEVGYEVARLT
VTDADIEGSDAWNAVYKIIKGNEAGFFSIQTDIDNIGLLKTVKGLDYELKKQYILSVI
```

VTNKANFSVPLQTSTATVTVTVTDVNEAPVFVPVLKDVSVPEDLPSGQVVATYTAQDP
DKEQNQKISYFIGNDPAGWVSVNRDNGIVTGNGNLDRESKFVLNNTYKVIILAADSGT
PSATGTGTLVLNLIDVNDNGPFLDPQQNSFCQKDPGFRVFNIIDKDLYPNTYPYTVDL
TGESNENWTATVTEQSLLELRPKKELDIGRYEVLISLRDNQGLTDVTKLQITICQCNG
DQMQCEEKAAQAGGLGISAIVGILGGILALLLLLLLLLLFVRRKKVVKEPLLPPEDET
RDNVFFYDEEGGGEEDQDFDLSQLHRGLDARPDIIRNDVVPVLAAPQYRPRPANPDEI
GNFIDENLHAADNDPTAPPYDSLLVFDYEGSGSEAASLSSLNSSNSDLDQDYSALNNW
GPRFTKLAEMYGGDED

SEQ ID NO: 13

Vimentin

From homo sapiens

Accession No. BC000163

nucleotide (mRNA), 1862 bp

```
   1 gtccccgcgc cagagacgca gccgcgctcc caccacccac acccaccgcg ccctcgttcg
  61 cctcttctcc gggagccagt ccgcgccacc gccgccgccc aggccatcgc caccctccgc
 121 agccatgtcc accaggtccg tgtcctcgtc ctcctaccgc aggatgttcg gcggcccggg
 181 caccgcgagc cggccgagct ccagccggag ctacgtgact acgtccaccc gcacctacag
 241 cctgggcagc gcgctgcgcc ccagcaccag ccgcagcctc tacgcctcgt ccccgggcgg
 301 cgtgtatgcc acgcgctcct ctgccgtgcg cctgcggagc agcgtgcccg gggtgcggct
 361 cctgcaggac tcggtggact tctcgctggc cgacgccatc aacaccgagt tcaagaacac
 421 ccgcaccaac gagaaggtgg agctgcagga gctgaatgac cgcttcgcca actacatcga
 481 caaggtgcgc ttcctggagc agcagaataa gatcctgctg gccgagctcg agcagctcaa
 541 gggccaaggc aagtcgcgcc tgggggacct ctacgaggag gagatgcggg agctgcgccg
 601 gcaggtggac cagctaacca acgacaaagc ccgcgtcgag gtggagcgcg acaacctggc
 661 cgaggacatc atgcgcctcc gggagaaatt gcaggaggag atgcttcaga gagaggaagc
 721 cgaaaacacc ctgcaatctt tcagacagga tgttgacaat gcgtctctgg cacgtcttga
 781 ccttgaacgc aaagtggaat ctttgcaaga agagattgcc tttttgaaga aactccacga
 841 agaggaaatc caggagctgc aggctcagat tcaggaacag catgtccaaa tcgatgtgga
 901 tgtttccaag cctgacctca cggctgccct gcgtgacgta cgtcagcaat atgaaagtgt
 961 ggctgccaag aacctgcagg aggcagaaga atggtacaaa tccaagtttg ctgacctctc
1021 tgaggctgcc aaccggaaca atgacgccct gcgccaggca aagcaggagt ccactgagta
1081 ccggagacag gtgcagtccc tcacctgtga agtggatgcc cttaaaggaa ccaatgagtc
1141 cctggaacgc cagatgcgtg aaatggaaga gaactttgcc gttgaagctg ctaactacca
1201 agacactatt ggccgcctgc aggatgagat tcagaatatg aaggaggaaa tggctcgtca
1261 ccttcgtgaa taccaagacc tgctcaatgt taagatggcc cttgacattg agattgccac
1321 ctacaggaag ctgctggaag gcgaggagag caggatttct ctgcctcttc caaacttttc
1381 ctccctgaac ctgagggaaa ctaatctgga ttcactccct ctggttgata cccactcaaa
1441 aaggacactt ctgattaaga cggttgaaac tagagatgga caggttatca acgaaacttc
```

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1501 tcagcatcac gatgaccttg aataaaaatt gcacacactc agtgcagcaa tatattacca
1561 gcaagaataa aaaagaaatc catatcttaa agaaacagct ttcaagtgcc tttctgcagt
1621 ttttcaggag cgcaagatag atttggaata ggaataagct ctagttctta acaaccgaca
1681 ctcctacaag atttagaaaa aagtttacaa cataatctag tttacagaaa aatcttgtgc
1741 tagaatactt tttaaaaggt attttgaata ccattaaaac tgctttttt tttccagcaa
1801 gtatccaacc aacttggttc tgcttcaata aatctttgga aaaactcaaa aaaaaaaaaa
1861 aa
```

SEQ ID NO: 14

Vimentin

From homo sapiens

Accession No. BC000163

polypeptide (translation of SEQ ID NO: 13), 466 amino acids

```
MSTRSVSSSSYRRMFGGPGTASRPSSSRSYVTTSTRTYSLGSAL
RPSTSRSLYASSPGGVYATRSSAVRLRSSVPGVRLLQDSVDFSLADAINTEFKNTRTN
EKVELQELNDRFANYIDKVRFLEQQNKILLAELEQLKGQGKSRLGDLYEEEMRELRRQ
VDQLTNDKARVEVERDNLAEDIMRLREKLQEEMLQREEAENTLQSFRQDVDNASLARL
DLERKVESLQEEIAFLKKLHEEEIQELQAQIQEQHVQIDVDVSKPDLTAALRDVRQQY
ESVAAKNLQEAEEWYKSKFADLSEAANRNNDALRQAKQESTEYRRQVQSLTCEVDALK
GTNESLERQMREMEENFAVEAANYQDTIGRLQDEIQNMKEEMARHLREYQDLLNVKMA
LDIEIATYRKLLEGEESRISLPLPNFSSLNLRETNLDSLPLVDTHSKRTLLIKTVETR
DGQVINETSQHHDDLE
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SEQ ID NO: 15

N-cadherin

From homo sapiens

CCDS ID No. CCDS11891.1

nucleotide, 2721 bp

```
ATGTGCCGGATAGCGGGAGCGCTGCGGACCCTGCTGCCGCTGCTGGCGGCCCTGCTTCAGGCGTCTGTAG
AGGCTTCTGGTGAAATCGCATTATGCAAGACTGGATTTCCTGAAGATGTTTACAGTGCAGTCTTATCGAA
GGATGTGCATGAAGGACAGCCTCTTCTCAATGTGAAGTTTAGCAACTGCAATGGAAAAAGAAAAGTACAA
TATGAGAGCAGTGAGCCTGCAGATTTTAAGGTGGATGAAGATGGCATGGTGTATGCCGTGAGAAGCTTTC
CACTCTCTTCTGAGCATGCCAAGTTCCTGATATATGCCCAAGACAAAGAGACCCAGGAAAAGTGGCAAGT
GGCAGTAAAATTGAGCCTGAAGCCAACCTTAACTGAGGAGTCAGTGAAGGAGTCAGCAGAAGTTGAAGAA
ATAGTGTTCCCAAGACAATTCAGTAAGCACAGTGGCCACCTACAAAGGCAGAAGAGAGACTGGGTCATCC
CTCCAATCAACTTGCCAGAAAACTCCAGGGGACCTTTTCCTCAAGAGCTTGTCAGGATCAGGTCTGATAG
AGATAAAAACCTTTCACTGCGGTACAGTGTAACTGGGCCAGGAGCTGACCAGCCTCCAACTGGTATCTTC
ATTATCAACCCCATCTCGGGTCAGCTGTCGGTGACAAAGCCCCTGGATCGCGAGCAGATAGCCCGGTTTC
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ATTTGAGGGCACATGCAGTAGATATTAATGGAAATCAAGTGGAGAACCCCATTGACATTGTCATCAATGT
TATTGACATGAATGACAACAGACCTGAGTTCTTACACCAGGTTTGGAATGGGACAGTTCCTGAGGGATCA
AAGCCTGGAACATATGTGATGACCGTAACAGCAATTGATGCTGACGATCCCAATGCCCTCAATGGGATGT
TGAGGTACAGAATCGTGTCTCAGGCTCCAAGCACCCCTTCACCCAACATGTTTACAATCAACAATGAGAC
TGGTGACATCATCACAGTGGCAGCTGGACTTGATCGAGAAAAAGTGCAACAGTATACGTTAATAATTCAA
GCTACAGACATGGAAGGCAATCCCACATATGGCCTTTCAAACACAGCCACGGCCGTCATCACAGTGACAG
ATGTCAATGACAATCCTCCAGAGTTTACTGCCATGACGTTTTATGGTGAAGTTCCTGAGAACAGGGTAGA
CATCATAGTAGCTAATCTAACTGTGACCGATAAGGATCAACCCCATACACCAGCCTGGAACGCAGTGTAC
AGAATCAGTGGCGGAGATCCTACTGGACGGTTCGCCATCCAGACCGACCCAAACAGCAACGACGGGTTAG
TCACCGTGGTCAAACCAATCGACTTTGAAACAAATAGGATGTTTGTCCTTACTGTTGCTGCAGAAAATCA
AGTGCCATTAGCCAAGGGAATTCAGCACCCCCCTCAGTCAACTGCAACCGTGTCTGTTACAGTTATTGAC
GTAAATGAAAACCCTTATTTTGCCCCCAATCCTAAGATCATTCGCCAAGAAGAAGGGCTTCATGCCGGTA
CCATGTTGACAACATTCACTGCTCAGGACCCAGATCGATATATGCAGCAAAATATTAGATACACTAAATT
ATCTGATCCTGCCAATTGGCTAAAAATAGATCCTGTGAATGGACAAATAACTACAATTGCTGTTTTGGAC
CGAGAATCACCAAATGTGAAAAACAATATATATAATGCTACTTTCCTTGCTTCTGACAATGGAATTCCTC
CTATGAGTGGAACAGGAACGCTGCAGATCTATTTACTTGATATTAATGACAATGCCCCTCAAGTGTTACC
TCAAGAGGCAGAGACTTGCGAAACTCCAGACCCCAATTCAATTAATATTACAGCACTTGATTATGACATT
GATCCAAATGCTGGACCATTTGCTTTTGATCTTCCTTTATCTCCAGTGACTATTAAGAGAAATTGGACCA
TCACTCGGCTTAATGGTGATTTTGCTCAGCTTAATTTAAAGATAAAATTTCTTGAAGCTGGTATCTATGA
AGTTCCCATCATAATCACAGATTCGGGTAATCCTCCCAAATCAAATATTTCCATCCTGCGTGTGAAGGTT
TGCCAGTGTGACTCCAACGGGGACTGCACAGATGTGGACAGGATTGTGGGTGCGGGGCTTGGCACCGGTG
CCATCATTGCCATCCTGCTCTGCATCATCATCCTGCTTATCCTTGTGCTGATGTTTGTGGTATGGATGAA
ACGCCGGGATAAAGAACGCCAGGCCAAACAACTTTTAATTGATCCAGAAGATGATGTAAGAGATAATATT
TTAAAATATGATGAAGAAGGTGGAGGAGAAGAAGACCAGGACTATGACTTGAGCCAGCTGCAGCAGCCTG
ACACTGTGGAGCCTGATGCCATCAAGCCTGTGGGAATCCGACGAATGGATGAAAGACCCATCCACGCCGA
GCCCCAGTATCCGGTCCGATCTGCAGCCCCACACCCTGGAGACATTGGGGACTTCATTAATGAGGGCCTT
AAAGCGGCTGACAATGACCCCACAGCTCCACCATATGACTCCCTGTTAGTGTTTGACTATGAAGGCAGTG
GCTCCACTGCTGGGTCCTTGAGCTCCCTTAATTCCTCAAGTAGTGGTGGTGAGCAGGACTATGATTACCT
GAACGACTGGGGGCCACGGTTCAAGAAACTTGCTGACATGTATGGTGGAGGTGATGACTGA

SEQ ID NO: 16

N-cadherin

From homo sapiens

CCDS ID No. CCDS11891.1

polypeptide (translation of SEQ ID NO: 15), 906 amino acids

MCRIAGALRTLLPLLAALLQASVEASGEIALCKTGFPEDVYSAVLSKDVHEGQPLLNVKFSNCNGKRKVQ
YESSEPADFKVDEDGMVYAVRSFPLSSEHAKFLIYAQDKETQEKWQVAVKLSLKPTLTEESVKESAEVEE
IVFPRQFSKHSGHLQRQKRDWVIPPINLPENSRGPFPQELVRIRSDRDKNLSLRYSVTGPGADQPPTGIF
IINPISGQLSVTKPLDREQIARFHLRAHAVDINGNQVENPIDIVINVIDMNDNRPEFLHQVWNGTVPEGS

KPGTYVMTVTAIDADDPNALNGMLRYRIVSQAPSTPSPNMFTINNETGDIITVAAGLDREKVQQYTLIIQ
ATDMEGNPTYGLSNTATAVITVTDVNDNPPEFTAMTFYGEVPENRVDIIVANLTVTDKDQPHTPAWNAVY
RISGGDPTGRFAIQTDPNSNDGLVTVVKPIDFETNRMFVLTVAAENQVPLAKGIQHPPQSTATVSVTVID
VNENPYFAPNPKIIRQEEGLHAGTMLTTFTAQDPDRYMQQNIRYTKLSDPANWLKIDPVNGQITTIAVLD
RESPNVKNNIYNATFLASDNGIPPMSGTGTLQIYLLDINDNAPQVLPQEAETCETPDPNSINITALDYDI
DPNAGPFAFDLPLSPVTIKRNWTITRLNGDFAQLNLKIKFLEAGIYEVPIIITDSGNPPKSNISILRVKV
CQCDSNGDCTDVDRIVGAGLGTGAIIAILLCIIILLILVLMFVVWMKRRDKERQAKQLLIDPEDDVRDNI
LKYDEEGGGEEDQDYDLSQLQQPDTVEPDAIKPVGIRRMDERPIHAEPQYPVRSAAPHPGDIGDFINEGL
KAADNDPTAPPYDSLLVFDYEGSGSTAGSLSSLNSSSSGGEQDYDYLNDWGPRFKKLADMYGGGDD

SEQ ID NO: 17

O-cadherin (also known as ob-cadherin)

From homo sapiens

CCDS ID No. CCDS10803.1

nucleotide, 2391 bp

ATGAAGGAGAACTACTGTTTACAAGCCGCCCTGGTGTGCCTGGGCATGCTGTGCCACAGCCATGCCTTTG
CCCCAGAGCGGCGGGGGCACCTGCGGCCCTCCTTCCATGGGCACCATGAGAAGGGCAAGGAGGGGCAGGT
GCTACAGCGCTCCAAGCGTGGCTGGGTCTGGAACCAGTTCTTCGTGATAGAGGAGTACACCGGGCCTGAC
CCCGTGCTTGTGGGCAGGCTTCATTCAGATATTGACTCTGGTGATGGGAACATTAAATACATTCTCTCAG
GGGAAGGAGCTGGAACCATTTTTGTGATTGATGACAAATCAGGGAACATTCATGCCACCAAGACGTTGGA
TCGAGAAGAGAGAGCCCAGTACACGTTGATGGCTCAGGCGGTGGACAGGGACACCAATCGGCCACTGGAG
CCACCGTCGGAATTCATTGTCAAGGTCCAGGACATTAATGACAACCCTCCGGAGTTCCTGCACGAGACCT
ATCATGCCAACGTGCCTGAGAGGTCCAATGTGGGAACGTCAGTAATCCAGGTGACAGCTTCAGATGCAGA
TGACCCCACTTATGGAAATAGCGCCAAGTTAGTGTACAGTATCCTCGAAGGACAACCCTATTTTTCGGTG
GAAGCACAGACAGGTATCATCAGAACAGCCCTACCCAACATGGACAGGGAGGCCAAGGAGGAGTACCACG
TGGTGATCCAGGCCAAGGACATGGGTGGACATATGGGCGGACTCTCAGGGACAACCAAAGTGACGATCAC
ACTGACCGATGTCAATGACAACCCACCAAAGTTTCCGCAGAGCGTATACCAGATGTCTGTGTCAGAAGCA
GCCGTCCCTGGGGAGGAAGTAGGAAGAGTGAAAGCTAAAGATCCAGACATTGGAGAAAATGGCTTAGTCA
CATACAATATTGTTGATGGAGATGGTATGGAATCGTTTGAAATCACAACGGACTATGAAACACAGGAGGG
GGTGATAAAGCTGAAAAAGCCTGTAGATTTTGAAACCAAAAGAGCCTATAGCTTGAAGGTAGAGGCAGCC
AACGTGCACATCGACCCGAAGTTTATCAGCAATGGCCCTTTCAAGGACACTGTGACCGTCAAGATCTCAG
TAGAAGATGCTGATGAGCCCCCTATGTTCTTGGCCCCAAGTTACATCCACGAAGTCCAAGAAAATGCAGC
TGCTGGCACCGTGGTTGGGAGAGTGCATGCCAAAGACCCTGATGCTGCCAACAGCCCGATAAGGTATTCC
ATCGATCGTCACACTGACCTCGACAGATTTTTCACTATTAATCCAGAGGATGGTTTTATTAAAACTACAA
AACCTCTGGATAGAGAGGAAACAGCCTGGCTCAACATCACTGTCTTTGCAGCAGAAATCCACAATCGGCA
TCAGGAAGCCAAAGTCCCAGTGGCCATTAGGGTCCTTGATGTCAACGATAATGCTCCCAAGTTTGCTGCC
CCTTATGAAGGTTTCATCTGTGAGAGTGATCAGACCAAGCCACTTTCCAACCAGCCAATTGTTACAATTA
GTGCAGATGACAAGGATGACACGGCCAATGGACCAAGATTTATCTTCAGCCTACCCCCTGAAATCATTCA
CAATCCAAATTTCACAGTCAGAGACAACCGAGATAACACAGCAGGCGTGTACGCCCGGCGTGGAGGGTTC

AGTCGGCAGAAGCAGGACTTGTACCTTCTGCCCATAGTGATCAGCGATGGCGGCATCCCGCCCATGAGTA
GCACCAACACCCTCACCATCAAAGTCTGCGGGTGCGACGTGAACGGGGCACTGCTCTCCTGCAACGCAGA
GGCCTACATTCTGAACGCCGGCCTGAGCACAGGCGCCCTGATCGCCATCCTCGCCTGCATCGTCATTCTC
CTGGTCATTGTAGTATTGTTTGTGACCCTGAGAAGGCAAAAGAAAGAACCACTCATTGTCTTTGAGGAAG
AAGATGTCCGTGAGAACATCATTACTTATGATGATGAAGGGGGTGGGGAAGAAGACACAGAAGCCTTTGA
TATTGCCACCCTCCAGAATCCTGATGGTATCAATGGATTTATCCCCCGCAAAGACATCAAACCTGAGTAT
CAGTACATGCCTAGACCTGGGCTCCGGCCAGCGCCCAACAGCGTGGATGTCGATGACTTCATCAACACGA
GAATACAGGAGGCAGACAATGACCCCACGGCTCCTCCTTATGACTCCATTCAAATCTACGGTTATGAAGG
CAGGGGCTCAGTGGCCGGGTCCCTGAGCTCCCTAGAGTCGGCCACCACAGATTCAGACTTGGACTATGAT
TATCTACAGAACTGGGGACCTCGTTTTAAGAAACTAGCAGATTTGTATGGTTCCAAAGACACTTTTGATG
ACGATTCTTAA

SEQ ID NO: 18

O-cadherin (also known as ob-cadherin)

From homo sapiens

CCDS ID No. CCDS10803.1

polypeptide (translation of SEQ ID NO: 17), 796 amino acids

MKENYCLQAALVCLGMLCHSHAFAPERRGHLRPSFHGHHEKGKEGQVLQRSKRGWVWNQFFVIEEYTGPD
PVLVGRLHSDIDSGDGNIKYILSGEGAGTIFVIDDKSGNIHATKTLDREERAQYTLMAQAVDRDTNRPLE
PPSEFIVKVQDINDNPPEFLHETYHANVPERSNVGTSVIQVTASDADDPTYGNSAKLVYSILEGQPYFSV
EAQTGIIRTALPNMDREAKEEYHVVIQAKDMGGHMGGLSGTTKVTITLTDVNDNPPKFPQSVYQMSVSEA
AVPGEEVGRVKAKDPDIGENGLVTYNIVDGDGMESFEITTDYETQEGVIKLKKPVDFETKRAYSLKVEAA
NVHIDPKFISNGPFKDTVTVKISVEDADEPPMFLAPSYIHEVQENAAAGTVVGRVHAKDPDAANSPIRYS
IDRHTDLDRFFTINPEDGFIKTTKPLDREETAWLNITVFAAEIHNRHQEAKVPVAIRVLDVNDNAPKFAA
PYEGFICESDQTKPLSNQPIVTISADDKDDTANGPRFIFSLPPEIIHNPNFTVRDNRDNTAGVYARRGGF
SRQKQDLYLLPIVISDGGIPPMSSTNTLTIKVCGCDVNGALLSCNAEAYILNAGLSTGALIAILACIVIL
LVIVVLFVTLRRQKKEPLIVFEEEDVRENIITYDDEGGGEEDTEAFDIATLQNPDGINGFIPRKDIKPEY
QYMPRPGLRPAPNSVDVDDFINTRIQEADNDPTAPPYDSIQIYGYEGRGSVAGSLSSLESATTDSDLDYD
YLQNWGPRFKKLADLYGSKDTFDDDS

SEQ ID NO: 19

CD133 (also known as PROM1)

From homo sapiens

CCDS ID No. CCDS47029.1

nucleotide, 2598 bp

ATGGCCCTCGTACTCGGCTCCCTGTTGCTGCTGGGGCTGTGCGGGAACTCCTTTTCAGGAGGGCAGCCTT
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TAAAGCTGGACCCATTGGCATTCTCTTTGAACTAGTGCATATCTTTCTCTATGTGGTACAGCCGCGTGAT

TTCCCAGAAGATACTTTGAGAAAATTCTTACAGAAGGCATATGAATCCAAAATTGATTATGACAAGCCAG
AAACTGTAATCTTAGGTCTAAAGATTGTCTACTATGAAGCAGGGATTATTCTATGCTGTGTCCTGGGGCT
GCTGTTTATTATTCTGATGCCTCTGGTGGGGTATTTCTTTTGTATGTGTCGTTGCTGTAACAAATGTGGT
GGAGAAATGCACCAGCGACAGAAGGAAAATGGGCCCTTCCTGAGGAAATGCTTTGCAATCTCCCTGTTGG
TGATTTGTATAATAATAAGCATTGGCATCTTCTATGGTTTTGTGGCAAATCACCAGGTAAGAACCCGGAT
CAAAAGGAGTCGGAAACTGGCAGATAGCAATTTCAAGGACTTGCGAACTCTCTTGAATGAAACTCCAGAG
CAAATCAAATATATATTGGCCCAGTACAACACTACCAAGGACAAGGCGTTCACAGATCTGAACAGTATCA
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CACCAACAAAGTACACAGCTTAGCAGCAGTCTGACCAGCGTGAAAACTAGCCTGCGGTCATCTCTCAATG
ACCCTCTGTGCTTGGTGCATCCATCAAGTGAAACCTGCAACAGCATCAGATTGTCTCTAAGCCAGCTGAA
TAGCAACCCTGAACTGAGGCAGCTTCCACCCGTGGATGCAGAACTTGACAACGTTAATAACGTTCTTAGG
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TCAGCGTCTTCCTATTCAGGATATACTCTCAGCATTCTCTGTTTATGTTAATAACACTGAAAGTTACATC
CACAGAAATTTACCTACATTGGAAGAGTATGATTCATACTGGTGGCTGGGTGGCCTGGTCATCTGCTCTC
TGCTGACCCTCATCGTGATTTTTTACTACCTGGGCTTACTGTGTGGCGTGTGCGGCTATGACAGGCATGC
CACCCCGACCACCCGAGGCTGTGTCTCCAACACCGGAGGCGTCTTCCTCATGGTTGGAGTTGGATTAAGT
TTCCTCTTTTGCTGGATATTGATGATCATTGTGGTTCTTACCTTTGTCTTTGGTGCAAATGTGGAAAAAC
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GTACTATCGTCGAATGGATTCGGAGGACGTGTACGATGATGTTGAAACTATACCCATGAAAAATATGGAA
AATGGTAATAATGGTTATCATAAAGATCATGTATATGGTATTCACAATCCTGTTATGACAAGCCCATCAC
AACATTGA

SEQ ID NO: 20

CD133 (also known as PROM1)

From homo sapiens

CCDS ID No. CCDS47029.1

poplypeptide (translation of SEQ ID NO: 19), 865 amino acids

MALVLGSLLLLGLCGNSFSGGQPSSTDAPKAWNYELPATNYETQDSHKAGPIGILFELVHIFLYVVQPRD
FPEDTLRKFLQKAYESKIDYDKPETVILGLKIVYYEAGIILCCVLGLLFIILMPLVGYFFCMCRCCNKCG
GEMHQRQKENGPFLRKCFAISLLVICIIISIGIFYGFVANHQVRTRIKRSRKLADSNFKDLRTLLNETPE
QIKYILAQYNTTKDKAFTDLNSINSVLGGGILDRLRPNIIPVLDEIKSMATAIKETKEALENMNSTLKSL
HQQSTQLSSSLTSVKTSLRSSLNDPLCLVHPSSETCNSIRLSLSQLNSNPELRQLPPVDAELDNVNNVLR
TDLDGLVQQGYQSLNDIPDRVQRQTTTVVAGIKRVLNSIGSDIDNVTQRLPIQDILSAFSVYVNNTESYI
HRNLPTLEEYDSYWWLGGLVICSLLTLIVIFYYLGLLCGVCGYDRHATPTTRGCVSNTGGVFLMVGVGLS
FLFCWILMIIVVLTFVFGANVEKLICEPYTSKELFRVLDTPYLLNEDWEYYLSGKLFNKSKMKLTFEQVY
SDCKKNRGTYGTLHLQNSFNISEHLNINEHTGSISSELESLKVNLNIFLLGAAGRKNLQDFAACGIDRMN
YDSYLAQTGKSPAGVNLLSFAYDLEAKANSLPPGNLRNSLKRDAQTIKTIHQQRVLPIEQSLSTLYQSVK
ILQRTGNGLLERVTRILASLDFAQNFITNNTSSVIIEETKKYGRTIIGYFEHYLQWIEFSISEKVASCKP
VATALDTAVDVFLCSYIIDPLNLFWFGIGKATVFLLPALIFAVKLAKYYRRMDSEDVYDDVETIPMKNME
NGNNGYHKDHVYGIHNPVMTSPSQH

SEQ ID NO: 21

FGFR2, isoform 1

From homo sapiens

CCDS ID No. CCDS31298.1

nucleotide, 2466 bp

ATGGTCAGCTGGGGTCGTTTCATCTGCCTGGTCGTGGTCACCATGGCAACCTTGTCCCTGGCCCGGCCCT
CCTTCAGTTTAGTTGAGGATACCACATTAGAGCCAGAAGAGCCACCAACCAAATACCAAATCTCTCAACC
AGAAGTGTACGTGGCTGCGCCAGGGGAGTCGCTAGAGGTGCGCTGCCTGTTGAAAGATGCCGCCGTGATC
AGTTGGACTAAGGATGGGGTGCACTTGGGGCCCAACAATAGGACAGTGCTTATTGGGGAGTACTTGCAGA
TAAAGGGCGCCACGCCTAGAGACTCCGGCCTCTATGCTTGTACTGCCAGTAGGACTGTAGACAGTGAAAC
TTGGTACTTCATGGTGAATGTCACAGATGCCATCTCATCCGGAGATGATGAGGATGACACCGATGGTGCG
GAAGATTTTGTCAGTGAGAACAGTAACAACAAGAGAGCACCATACTGGACCAACACAGAAAAGATGGAAA
AGCGGCTCCATGCTGTGCCTGCGGCCAACACTGTCAAGTTTCGCTGCCCAGCCGGGGGGAACCCAATGCC
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AACCAGCACTGGAGCCTCATTATGGAAAGTGTGGTCCCATCTGACAAGGGAAATTATACCTGTGTAGTGG
AGAATGAATACGGGTCCATCAATCACACGTACCACCTGGATGTTGTGGAGCGATCGCCTCACCGGCCCAT
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TACAGTGATGCCCAGCCCCACATCCAGTGGATCAAGCACGTGGAAAAGAACGGCAGTAAATACGGGCCCG
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CTATATTCGGAATGTAACTTTTGAGGACGCTGGGGAATATACGTGCTTGGCGGGTAATTCTATTGGGATA
TCCTTTCACTCTGCATGGTTGACAGTTCTGCCAGCGCCTGGAAGAGAAAAGGAGATTACAGCTTCCCCAG
ACTACCTGGAGATAGCCATTTACTGCATAGGGGTCTTCTTAATCGCCTGTATGGTGGTAACAGTCATCCT
GTGCCGAATGAAGAACACGACCAAGAAGCCAGACTTCAGCAGCCAGCCGGCTGTGCACAAGCTGACCAAA
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TGGTGAGGATAACAACACGCCTCTCTTCAACGGCAGACACCCCCATGCTGGCAGGGGTCTCCGAGTATGA
ACTTCCAGAGGACCCAAAATGGGAGTTTCCAAGAGATAAGCTGACACTGGGCAAGCCCCTGGGAGAAGGT
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TATGTCATAGTTGAGTATGCCTCTAAAGGCAACCTCCGAGAATACCTCCGAGCCCGGAGGCCACCCGGGA
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TAGAGTATACACTCATCAGAGTGATGTCTGGTCCTTCGGGGTGTTAATGTGGGAGATCTTCACTTTAGGG
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GACCTCAGCCAACCTCTCGAACAGTATTCACCTAGTTACCCTGACACAAGAAGTTCTTGTTCTTCAGGAG
ATGATTCTGTTTTTTCTCCAGACCCCATGCCTTACGAACCATGCCTTCCTCAGTATCCACACATAAACGG
CAGTGTTAAAACATGA

SEQ ID NO: 22

FGFR2, isoform 1

From homo sapiens

CCDS ID No. CCDS31298.1

polypeptide (translation of SEQ ID NO: 21), 821 amino acids

MVSWGRFICLVVVTMATLSLARPSFSLVEDTTLEPEEPPTKYQISQPEVYVAAPGESLEVRCLLKDAAVI
SWTKDGVHLGPNNRTVLIGEYLQIKGATPRDSGLYACTASRTVDSETWYFMVNVTDAISSGDDEDDTDGA
EDFVSENSNNKRAPYWTNTEKMEKRLHAVPAANTVKFRCPAGGNPMPTMRWLKNGKEFKQEHRIGGYKVR
NQHWSLIMESVVPSDKGNYTCVVENEYGSINHTYHLDVVERSPHRPILQAGLPANASTVVGGDVEFVCKV
YSDAQPHIQWIKHVEKNGSKYGPDGLPYLKVLKAAGVNTTDKEIEVLYIRNVTFEDAGEYTCLAGNSIGI
SFHSAWLTVLPAPGREKEITASPDYLEIAIYCIGVFLIACMVVTVILCRMKNTTKKPDFSSQPAVHKLTK
RIPLRRQVTVSAESSSSMNSNTPLVRITTRLSSTADTPMLAGVSEYELPEDPKWEFPRDKLTLGKPLGEG
CFGQVVMAEAVGIDKDKPKEAVTVAVKMLKDDATEKDLSDLVSEMEMMKMIGKHKNIINLLGACTQDGPL
YVIVEYASKGNLREYLRARRPPGMEYSYDINRVPEEQMTFKDLVSCTYQLARGMEYLASQKCIHRDLAAR
NVLVTENNVMKIADFGLARDINNIDYYKKTTNGRLPVKWMAPEALFDRVYTHQSDVWSFGVLMWEIFTLG
GSPYPGIPVEELFKLLKEGHRMDKPANCTNELYMMMRDCWHAVPSQRPTFKQLVEDLDRILTLTTNEEYL

DLSQPLEQYSPSYPDTRSSCSSGDDSVFSPDPMPYEPCLPQYPHINGSVKT

SEQ ID NO: 23

E-cadherin (also known as CDH1)

From homo sapiens

CCDS ID No. CCDS10869.1

nucleotide, 2649 bp

ATGGGCCCTTGGAGCCGCAGCCTCTCGGCGCTGCTGCTGCTGCTGCAGGTCTCCTCTTGGCTCTGCCAGG
AGCCGGAGCCCTGCCACCCTGGCTTTGACGCCGAGAGCTACACGTTCACGGTGCCCCGGCGCCACCTGGA
GAGAGGCCGCGTCCTGGGCAGAGTGAATTTTGAAGATTGCACCGGTCGACAAAGGACAGCCTATTTTTCC
CTCGACACCCGATTCAAAGTGGGCACAGATGGTGTGATTACAGTCAAAAGGCCTCTACGGTTTCATAACC
CACAGATCCATTTCTTGGTCTACGCCTGGGACTCCACCTACAGAAAGTTTTCCACCAAAGTCACGCTGAA
TACAGTGGGGCACCACCACCGCCCCCCGCCCCATCAGGCCTCCGTTTCTGGAATCCAAGCAGAATTGCTC
ACATTTCCCAACTCCTCTCCTGGCCTCAGAAGACAGAAGAGAGACTGGGTTATTCCTCCCATCAGCTGCC
CAGAAAATGAAAAAGGCCCATTTCCTAAAAACCTGGTTCAGATCAAATCCAACAAAGACAAAGAAGGCAA
GGTTTTCTACAGCATCACTGGCCAAGGAGCTGACACACCCCCTGTTGGTGTCTTTATTATTGAAAGAGAA
ACAGGATGGCTGAAGGTGACAGAGCCTCTGGATAGAGAACGCATTGCCACATACACTCTCTTCTCTCACG
CTGTGTCATCCAACGGGAATGCAGTTGAGGATCCAATGGAGATTTTGATCACGGTAACCGATCAGAATGA
CAACAAGCCCGAATTCACCCAGGAGGTCTTTAAGGGGTCTGTCATGGAAGGTGCTCTTCCAGGAACCTCT
GTGATGGAGGTCACAGCCACAGACGCGGACGATGATGTGAACACCTACAATGCCGCCATCGCTTACACCA
TCCTCAGCCAAGATCCTGAGCTCCCTGACAAAAATATGTTCACCATTAACAGGAACACAGGAGTCATCAG
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CAAGGTGAGGGGTTAAGCACAACAGCAACAGCTGTGATCACAGTCACTGACACCAACGATAATCCTCCGA
TCTTCAATCCCACCACGTACAAGGGTCAGGTGCCTGAGAACGAGGCTAACGTCGTAATCACCACACTGAA
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GGACAATTTGTCGTCACCACAAATCCAGTGAACAACGATGGCATTTTGAAAACAGCAAAGGGCTTGGATT
TTGAGGCCAAGCAGCAGTACATTCTACACGTAGCAGTGACGAATGTGGTACCTTTTGAGGTCTCTCTCAC
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AAGAGAGTGGAAGTGTCCGAGGACTTTGGCGTGGGCCAGGAAATCACATCCTACACTGCCCAGGAGCCAG
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TACACAGCCCTAATCATAGCTACAGACAATGGTTCTCCAGTTGCTACTGGAACAGGGACACTTCTGCTGA
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AAAGCCTCAGGTCATAAACATCATTGATGCAGACCTTCCTCCCAATACATCTCCCTTCACAGCAGAACTA
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CAAAGATGGCCTTAGAGGTGGGTGACTACAAAATCAATCTCAAGCTCATGGATAACCAGAATAAAGACCA
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GTCGAAGCAGGATTGCAAATTCCTGCCATTCTGGGGATTCTTGGAGGAATTCTTGCTTTGCTAATTCTGA
TTCTGCTGCTCTTGCTGTTTCTTCGGAGGAGAGCGGTGGTCAAAGAGCCCTTACTGCCCCCAGAGGATGA

CACCCGGGACAACGTTTATTACTATGATGAAGAAGGAGGCGGAGAAGAGGACCAGGACTTTGACTTGAGC
CAGCTGCACAGGGGCCTGGACGCTCGGCCTGAAGTGACTCGTAACGACGTTGCACCAACCCTCATGAGTG
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GAAGCTGCTAGTCTGAGCTCCCTGAACTCCTCAGAGTCAGACAAAGACCAGGACTATGACTACTTGAACG
AATGGGGCAATCGCTTCAAGAAGCTGGCTGACATGTACGGAGGCGGCGAGGACGACTAG

SEQ ID NO: 24

E-cadherin (also known as CDH1)

From homo sapiens

CCDS ID No. CCDS10869.1

polypeptide (translation of SEQ ID NO: 23), 882 amino acids

MGPWSRSLSALLLLLQVSSWLCQEPEPCHPGFDAESYTFTVPRRHLERGRVLGRVNFEDCTGRQRTAYFS
LDTRFKVGTDGVITVKRPLRFHNPQIHFLVYAWDSTYRKFSTKVTLNTVGHHHRPPPHQASVSGIQAELL
TFPNSSPGLRRQKRDWVIPPISCPENEKGPFPKNLVQIKSNKDKEGKVFYSITGQGADTPPVGVFIIERE
TGWLKVTEPLDRERIATYTLFSHAVSSNGNAVEDPMEILITVTDQNDNKPEFTQEVFKGSVMEGALPGTS
VMEVTATDADDDVNTYNAAIAYTILSQDPELPDKNMFTINRNTGVISVVTTGLDRESFPTYTLVVQAADL
QGEGLSTTATAVITVTDTNDNPPIFNPTTYKGQVPENEANVVITTLKVTDADAPNTPAWEAVYTILNDDG
GQFVVTTNPVNNDGILKTAKGLDFEAKQQYILHVAVTNVVPFEVSLTTSTATVTVDVLDVNEAPIFVPPE
KRVEVSEDFGVGQEITSYTAQEPDTFMEQKITYRIWRDTANWLEINPDTGAISTRAELDREDFEHVKNST
YTALIIATDNGSPVATGTGTLLLILSDVNDNAPIPEPRTIFFCERNPKPQVINIIDADLPPNTSPFTAEL
THGASANWTIQYNDPTQESIILKPKMALEVGDYKINLKLMDNQNKDQVTTLEVSVCDCEGAAGVCRKAQP
VEAGLQIPAILGILGGILALLILILLLLLFLRRRAVVKEPLLPPEDDTRDNVYYYDEEGGGEEDQDFDLS
QLHRGLDARPEVTRNDVAPTLMSVPRYLPRPANPDEIGNFIDENLKAADTDPTAPPYDSLLVFDYEGSGS
EAASLSSLNSSESDKDQDYDYLNEWGNRFKKLADMYGGGEDD

CLAIMS

What is claimed is:

1. A method for detecting a circulating tumor cell (CTC) in a biological sample, the method comprising detecting at least one epithelial mesenchymal transition (EMT) biomarker in the biological sample.

2. The method of claim 1, wherein the sample is a blood sample.

3. The method of any one of the preceding claims, wherein the at least one EMT biomarker is vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms, or CD133.

4. The method of any one of the preceding claims, wherein the method is performed at the time of or prior to cancer metastasis.

5. The method of any one of the preceding claims, wherein the at least one EMT biomarker is detected by flow cytometry, ferromagnetic enrichment, ferromagnetic sorting, or EMT antigen-antibody binding.

6. The method of any one of the preceding claims, comprising detecting at least two EMT biomarkers.

7. A kit for detecting a circulating tumor cell (CTC) in a biological sample, the kit comprising an antibody to at least one EMT biomarker and instructions for use.

8. The kit of claim 7, wherein the antibody is linked to a fluorescent reporter molecule, radionuclide, enzyme, or magnetic bead.

9. The kit of claim 7 or 8, wherein the at least one EMT biomarker is vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms, or CD133.

10. A method of predicting responsiveness of a subject having cancer to a course of treatment, the method comprising:

determining the level or presence of expression of at least one EMT biomarker in a sample from the subject to obtain a gene expression pattern or biomarker profile in CTCs for the subject; and

predicting responsiveness of the subject to the cancer drug based on the gene expression pattern or biomarker pattern obtained.

11. The method of claim 10, wherein the at least one EMT biomarker is vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms, or CD133.

12. A method of targeting delivery of a cancer drug in a subject having cancer comprising administering to the subject the cancer drug linked to an antibody specific for at least one EMT biomarker.

13. The method of claim 12, wherein the at least one EMT biomarker is vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms, or CD133.

14. A method of determining a prognosis cancer prognosis to a subject, the method comprising:

determining the level of expression of at least one EMT biomarker in a sample from the subject to determine the number of CTCs in the subject and to obtain a gene expression pattern for the subject; and

providing a prognosis to the subject based on the gene expression pattern obtained.

15. The method of claim 14, wherein the at least one EMT biomarker is vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms, or CD133.

16. The method of claim 14 or 15, wherein the cancer is selected from prostate, colon, and breast cancer, solid tumor malignancies, or sarcomas.

17. A method for monitoring progression of cancer in a subject undergoing therapeutic treatment, the method comprising:

detecting the number of CTCs based on the expression of at least one EMT biomarker in a first and a second sample taken from the subject at a first and a second time; and comparing the first and second levels of expression;

wherein a detected difference in number of CTCs based on the level of expression of the at least one EMT biomarker in the first and second samples indicates a change in the progression of the cancer.

18. The method of claim 17, wherein an increase in the detected level of the at least one EMT biomarker in the second sample relative to the first sample indicates progression of the cancer.

19. The method of claim 17, wherein a decrease in the detected level of the at least one EMT biomarker in the second sample relative to the first sample indicates that the therapeutic treatment is effective.

20. The method of claim 19, wherein the decrease indicates remission of the cancer.

21. The method of claim 17, whereby no difference in the detected level of the at least one EMT biomarker in the second sample relative to the first sample indicates arrest or stability in the progression of the cancer.

22. The method of any of claims 17 - 21, wherein the at least one EMT biomarker is vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms, or CD133.

23. The method of any of claims 17 - 21, wherein the cancer is selected from prostate, colon, and breast cancer.

24. A method for detecting cancer in a subject, the method comprising

detecting the presence of at least one EMT biomarker in a sample from the subject;

comparing the detected amount of the at least one EMT biomarker from the sample to a control sample;

correlating the detected amount of the at least one EMT biomarker from the sample to the presence of CTCs in the sample;

wherein the presence of CTCs in the sample indicates the presence of cancer in the subject.

25. The method of claim 24, wherein the at least one EMT biomarker is vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms, or CD133.

26. The method of claim 24 or 25, wherein the cancer is selected from prostate, colon, and breast cancer.

27. A method of treating cancer in a subject comprising administering to the subject a cancer drug linked to an antibody that specifically binds at least one EMT biomarker.

28. The method of claim 27, wherein the at least one EMT biomarker is vimentin, N-cadherin, O-cadherin, E-cadherin, FGFR2 splice variant isoforms, or CD133.

29. The method of claim 27 or 28, wherein the cancer is selected from prostate, colon, and breast cancer.

ABSTRACT

[00100] Provided are methods for detecting circulating tumor cells (CTCs) in a subject. The methods may include detecting the expression of at least one epithelial mesenchymal transition (EMT) biomarker. Further provided are kits for detecting CTCs. The kits may include antibodies to at least one EMT biomarker. Further provided are methods of predicting the responsiveness of a subject to a cancer drug, methods of targeting delivery of a cancer drug in a subject, methods of providing a cancer prognosis to a subject, and methods for following the progress of cancer in a subject.

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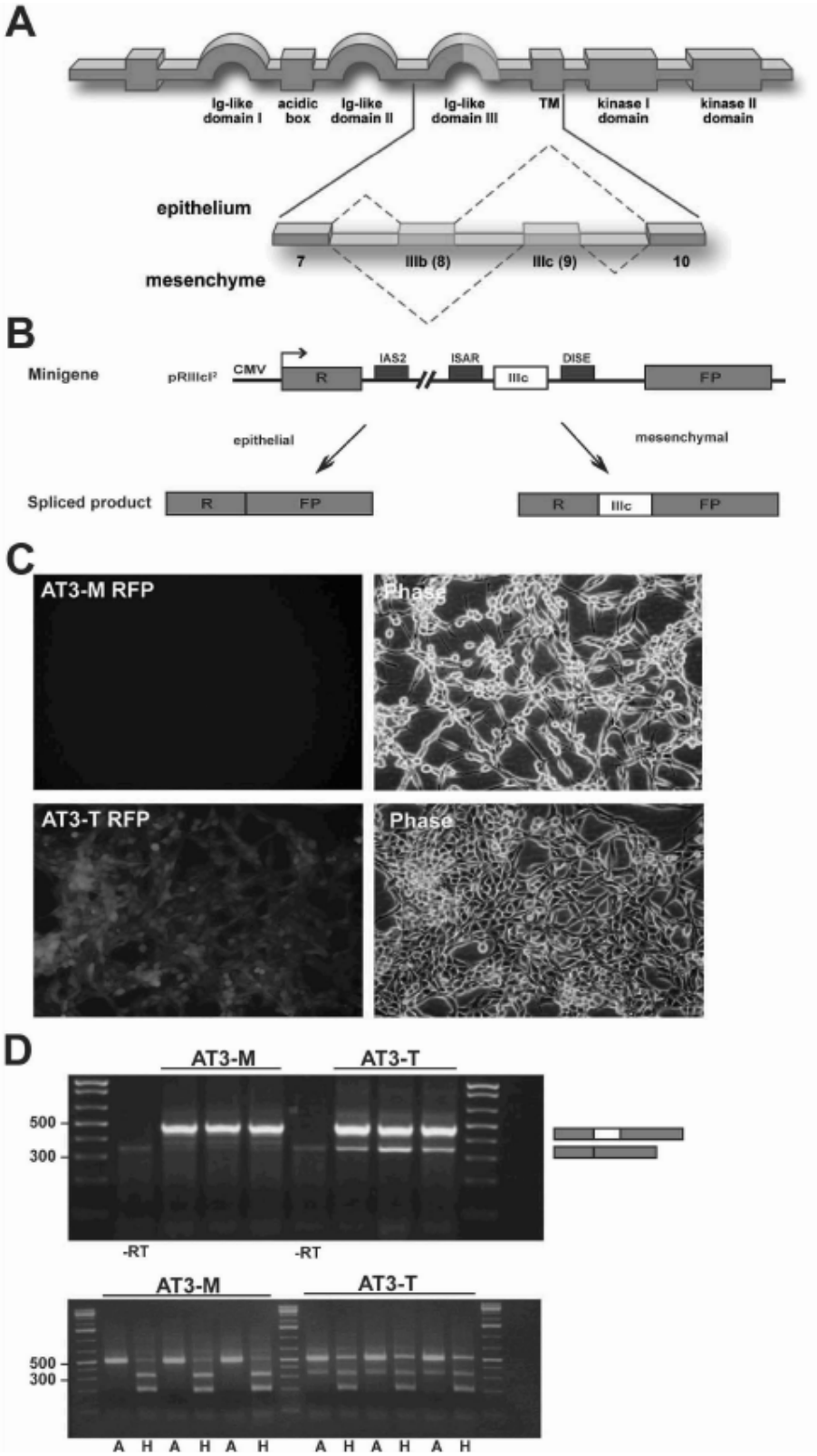
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ISAR
IIIc
DISE
FP
epithelial
mesenchymal
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Phase
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A H A H A H
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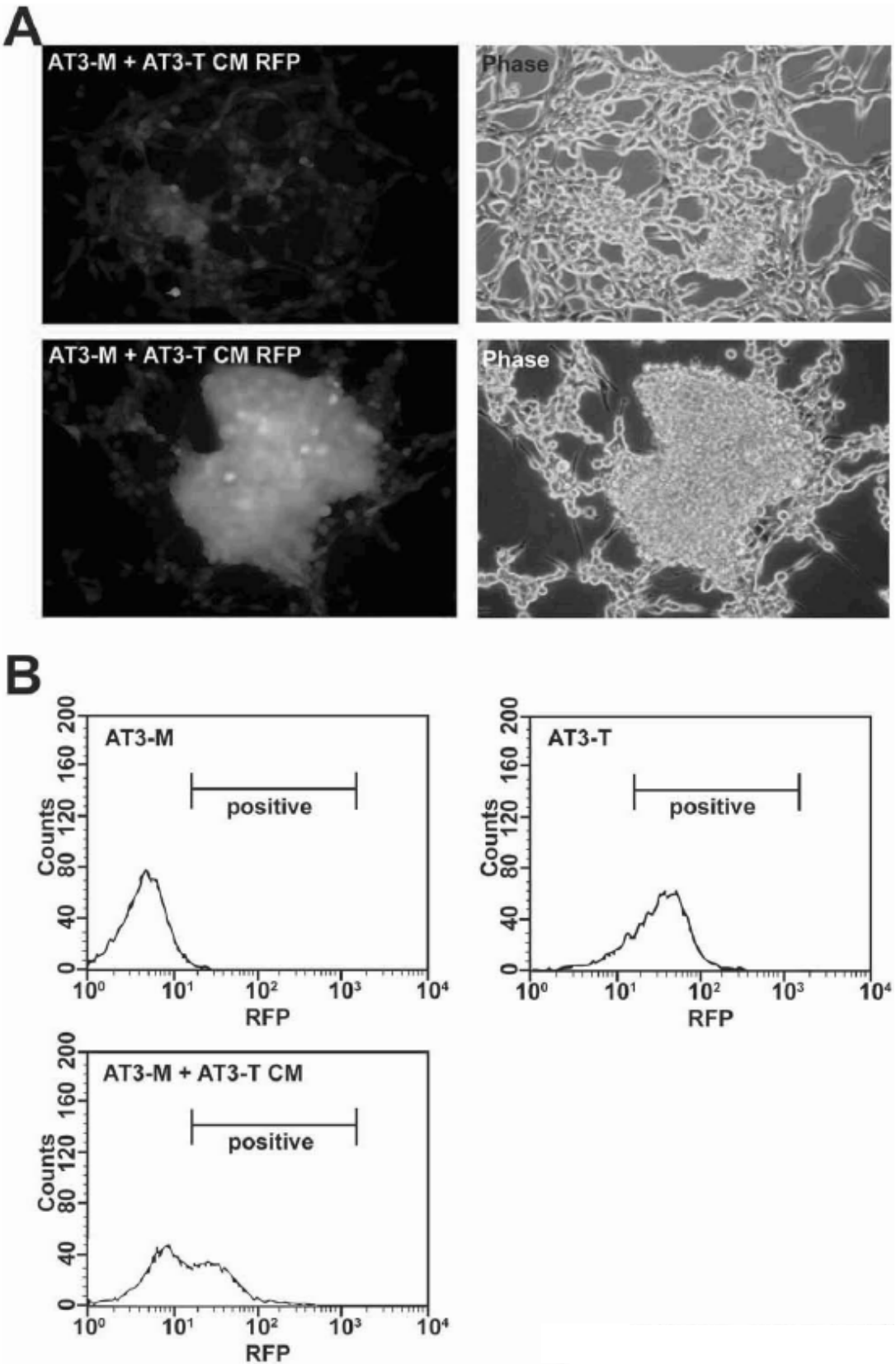
FIGURE 1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Phase
AT3-M + AT3-T CM RFP
Phase
B
AT3-M
positive
Counts
RFP
AT3-T
positive
Counts
RFP
AT3-M + AT3-T CM
positive
Counts
RFP

FIGURE 2

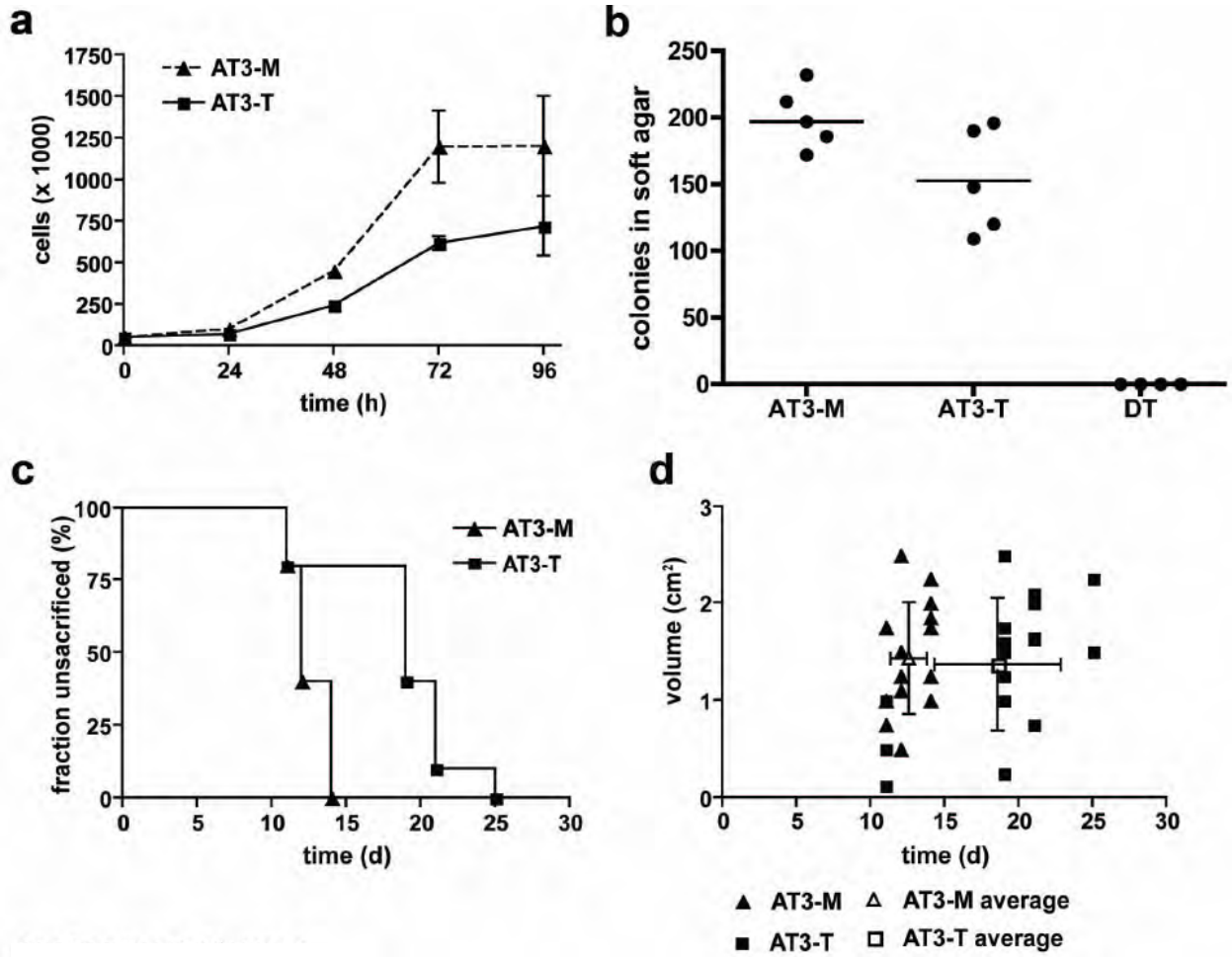
a
cells (x 1000)
AT3-M
AT3-T
time (h)
b
colonies in soft agar
AT3-M
AT3-T
DT
c
fraction unsacrificed (%)
AT3-M
AT3-T
time (d)
d
volume (cm³)
time (d)
AT3-M
AT3-T
AT3-M average
AT3-T average

FIGURE 3

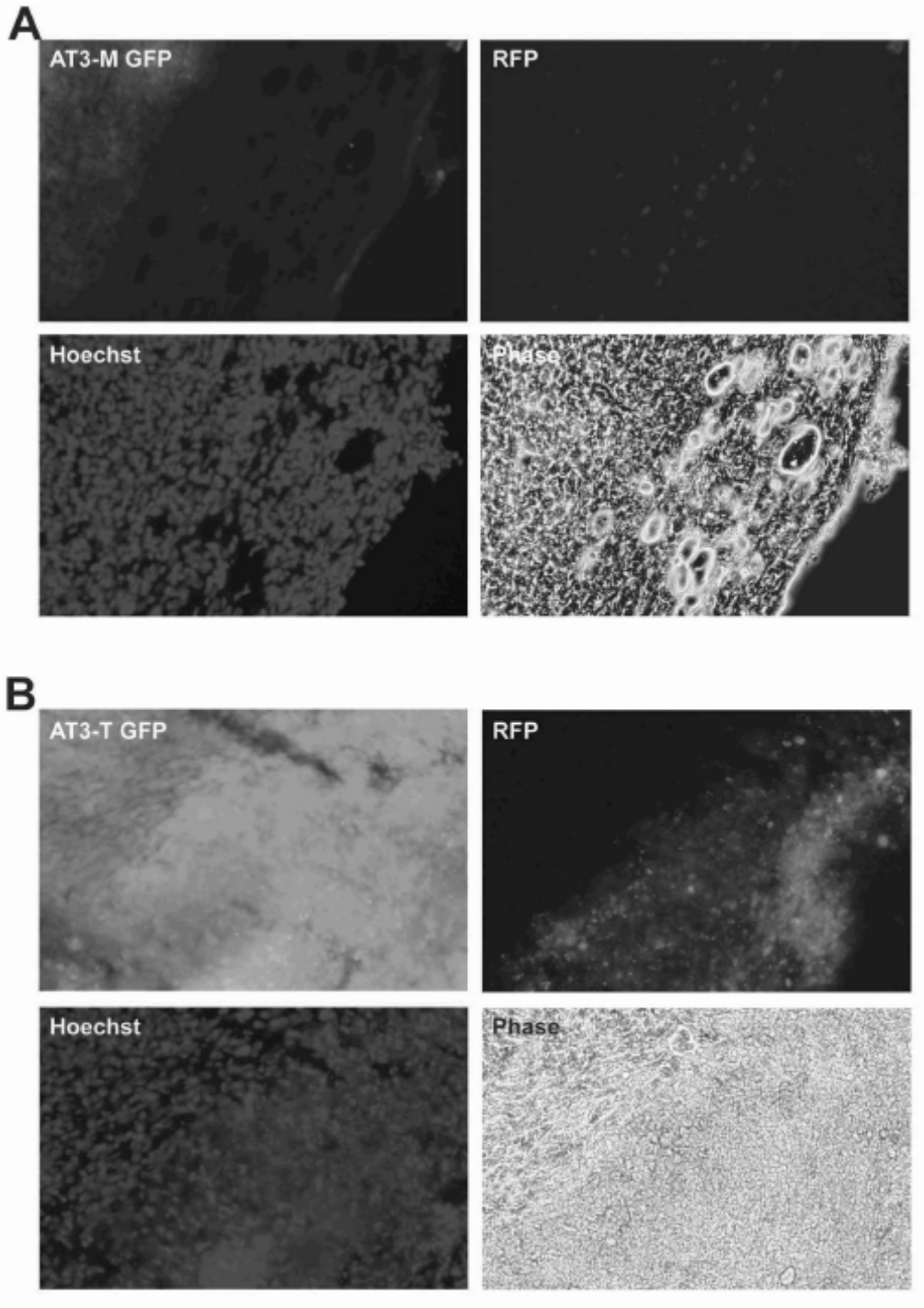

FIGURE 4

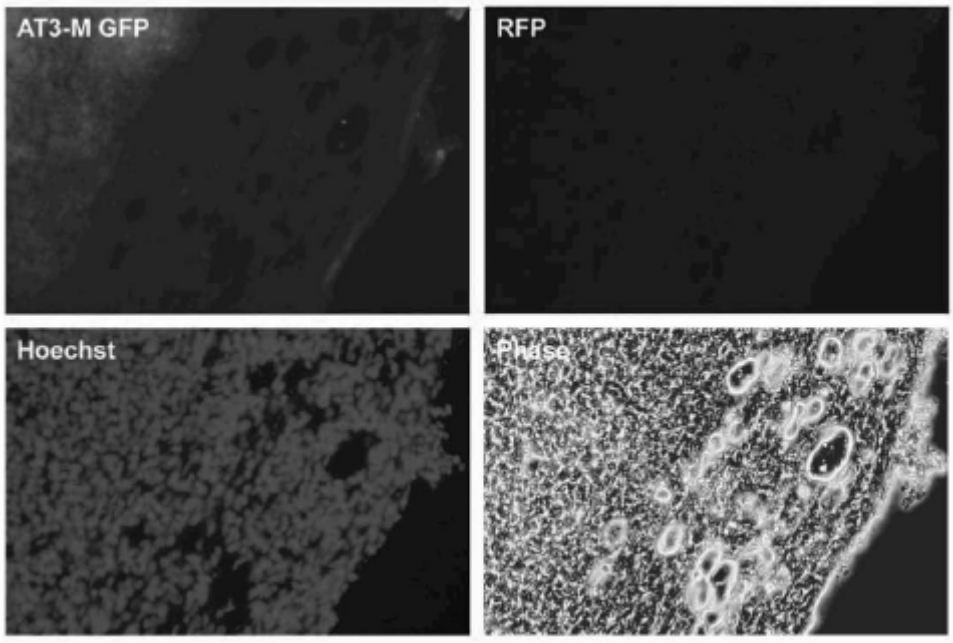

FIGURE 5

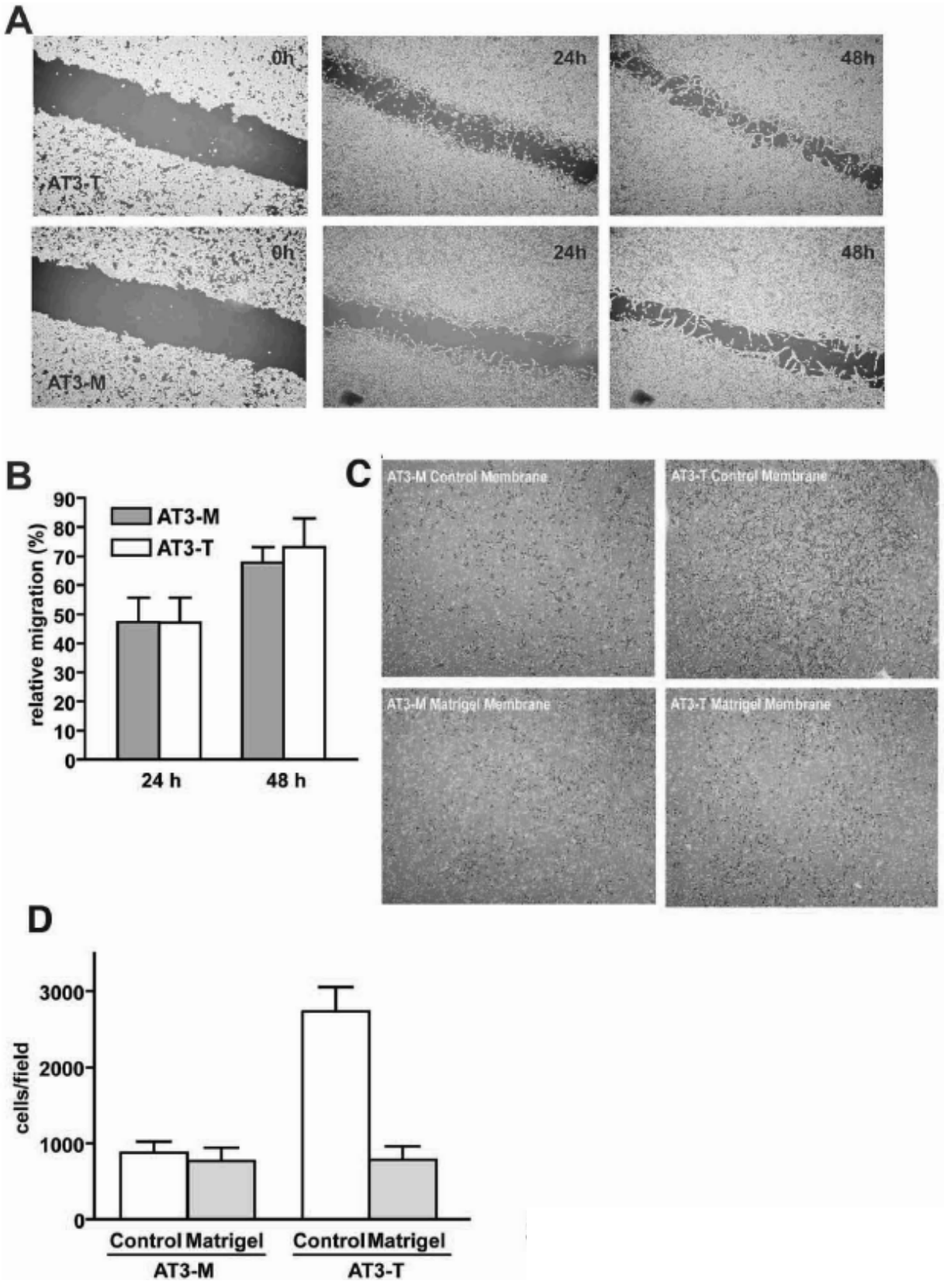
A
0h
24h
48h
AT3-T
0h
24h
48h
AT3-M
B
relative migration (%)
AT3-M
AT3-T
24 h
48 h
C
AT3-M Control Membrane
AT3-T Control Membrane
AT3-M Matrigel Membrane
AT3-T Matrigel Membrane
D
cells/field
Control
Matrigel
AT3-M
Control
Matrigel
AT3-T

FIGURE 6

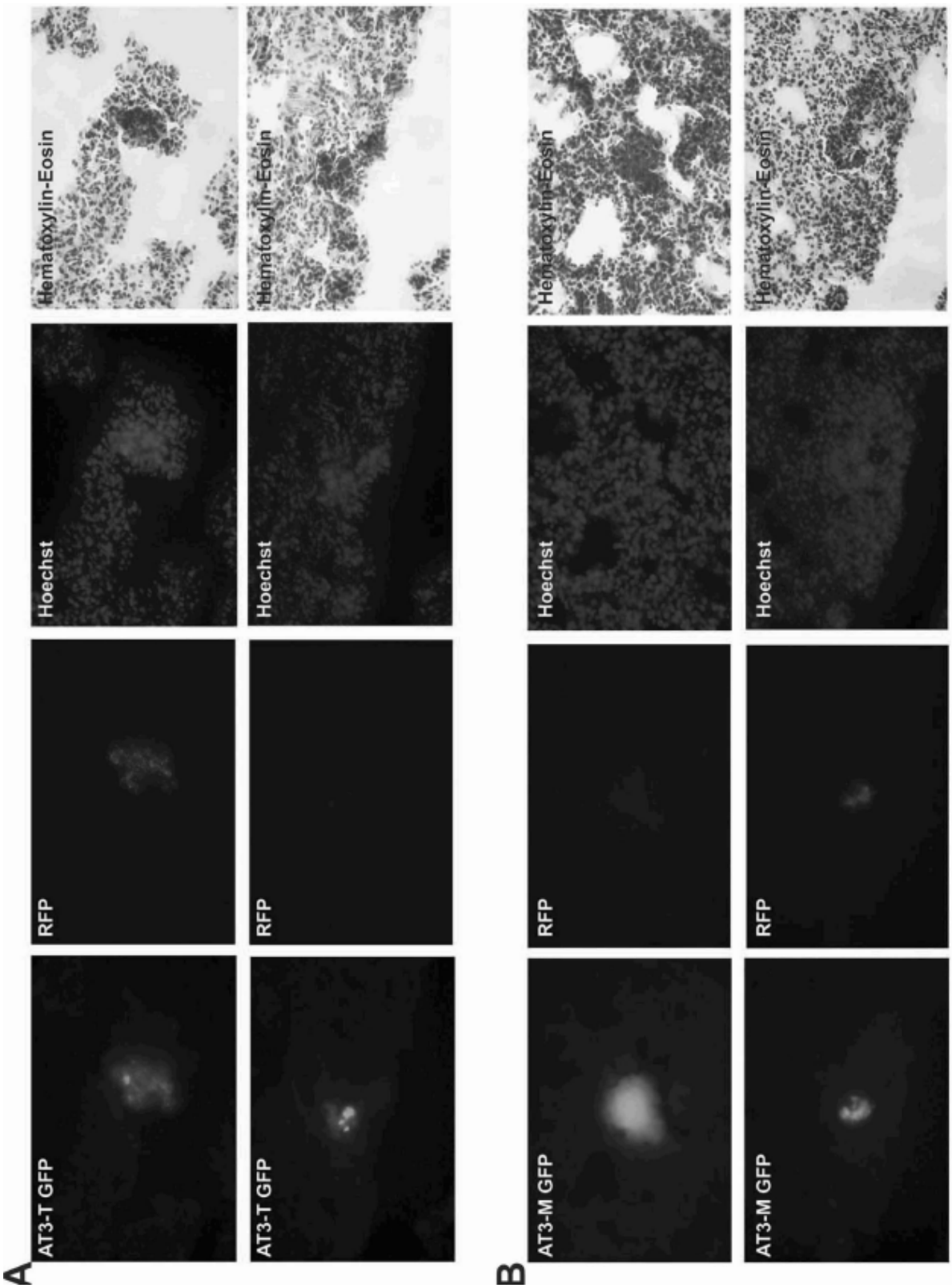
A
AT3-T GFP
RFP
Hoechst
Hematoxylin-Eosin
AT3-T GFP
RFP
Hoechst
Hematoxylin-Eosin
B
AT3-M GFP
RFP
Hoechst
Hematoxylin-Eosin
AT3-M GFP
RFP
Hoechst
Hematoxylin-Eosin

FIGURE 7

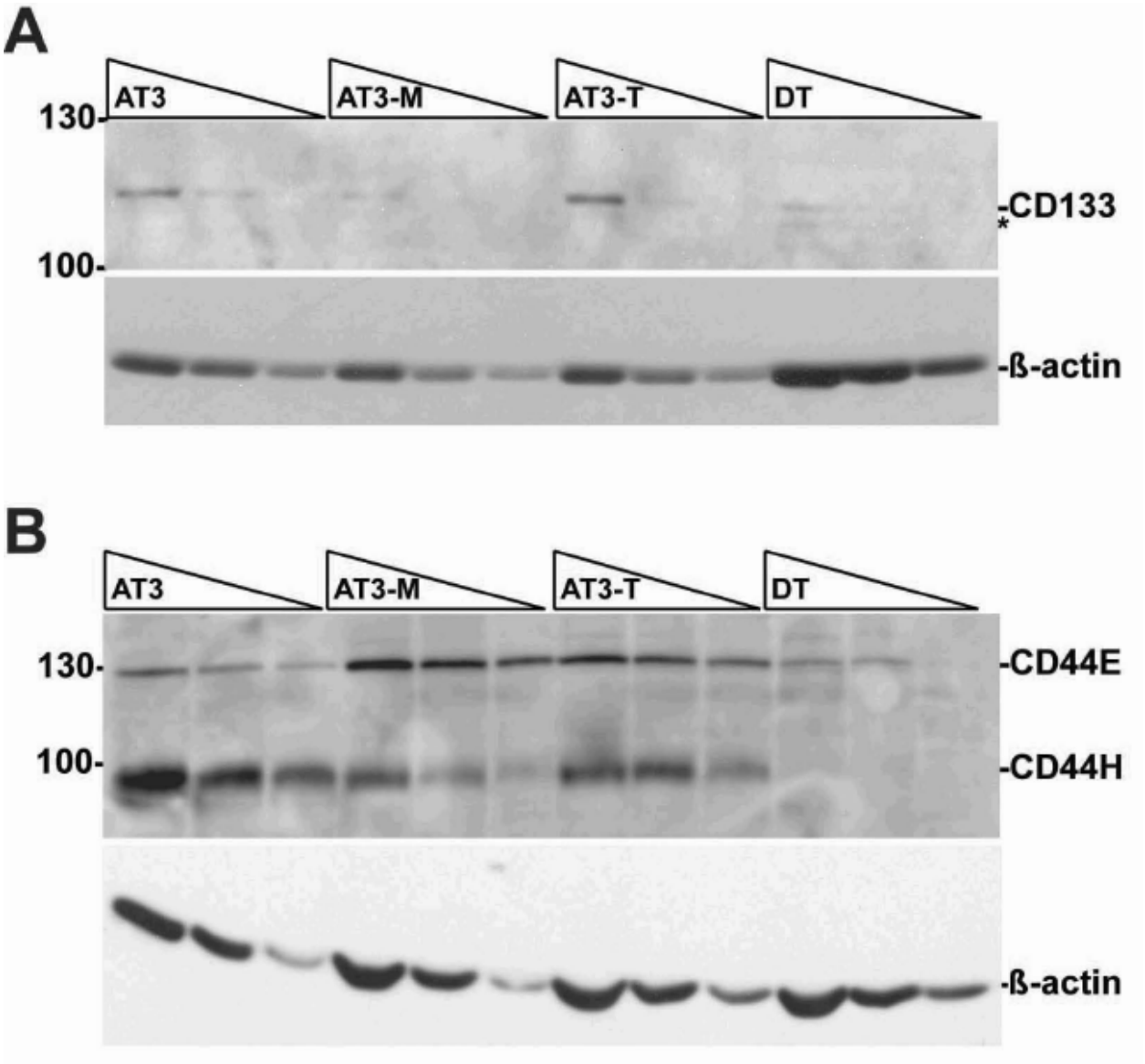
A
AT3
AT3-M
AT3-T
DT
130-
100-
-CD133
*
-ß-actin
B
AT3
AT3-M
AT3-T
DT
130-
100-
-CD44E
-CD44H
-ß-actin

FIGURE 8

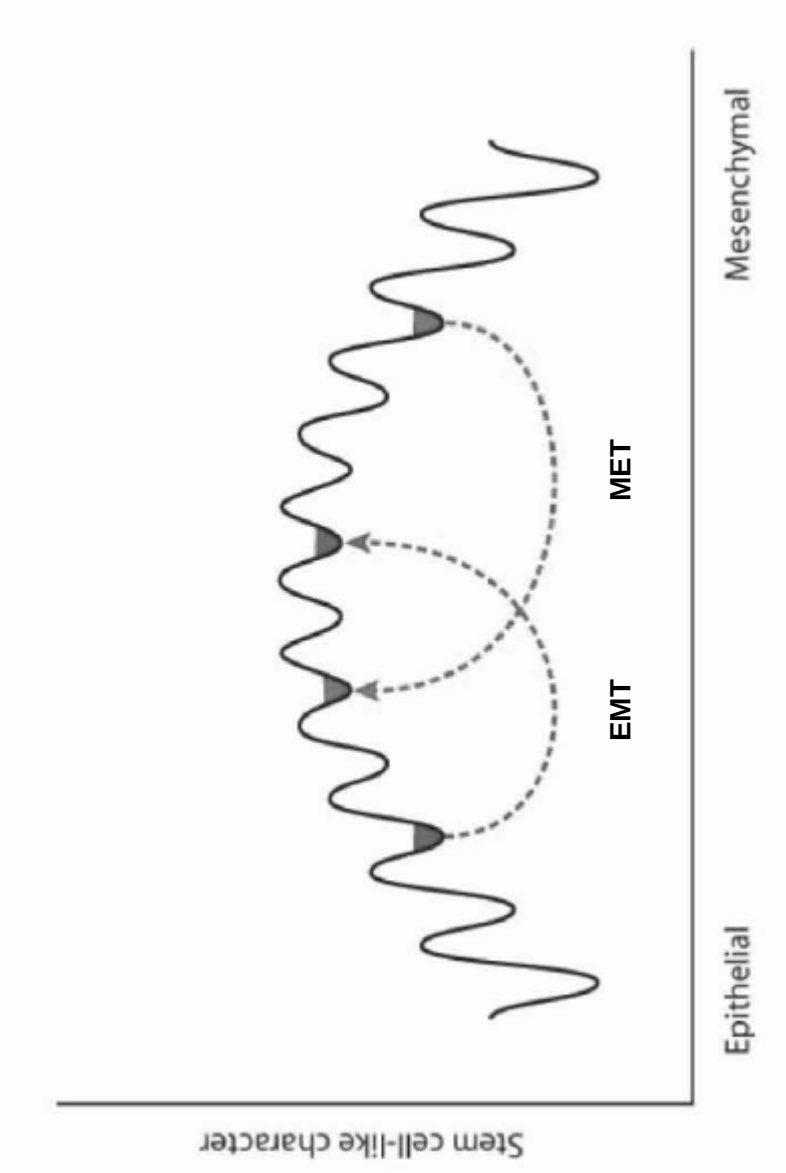

FIGURE 9

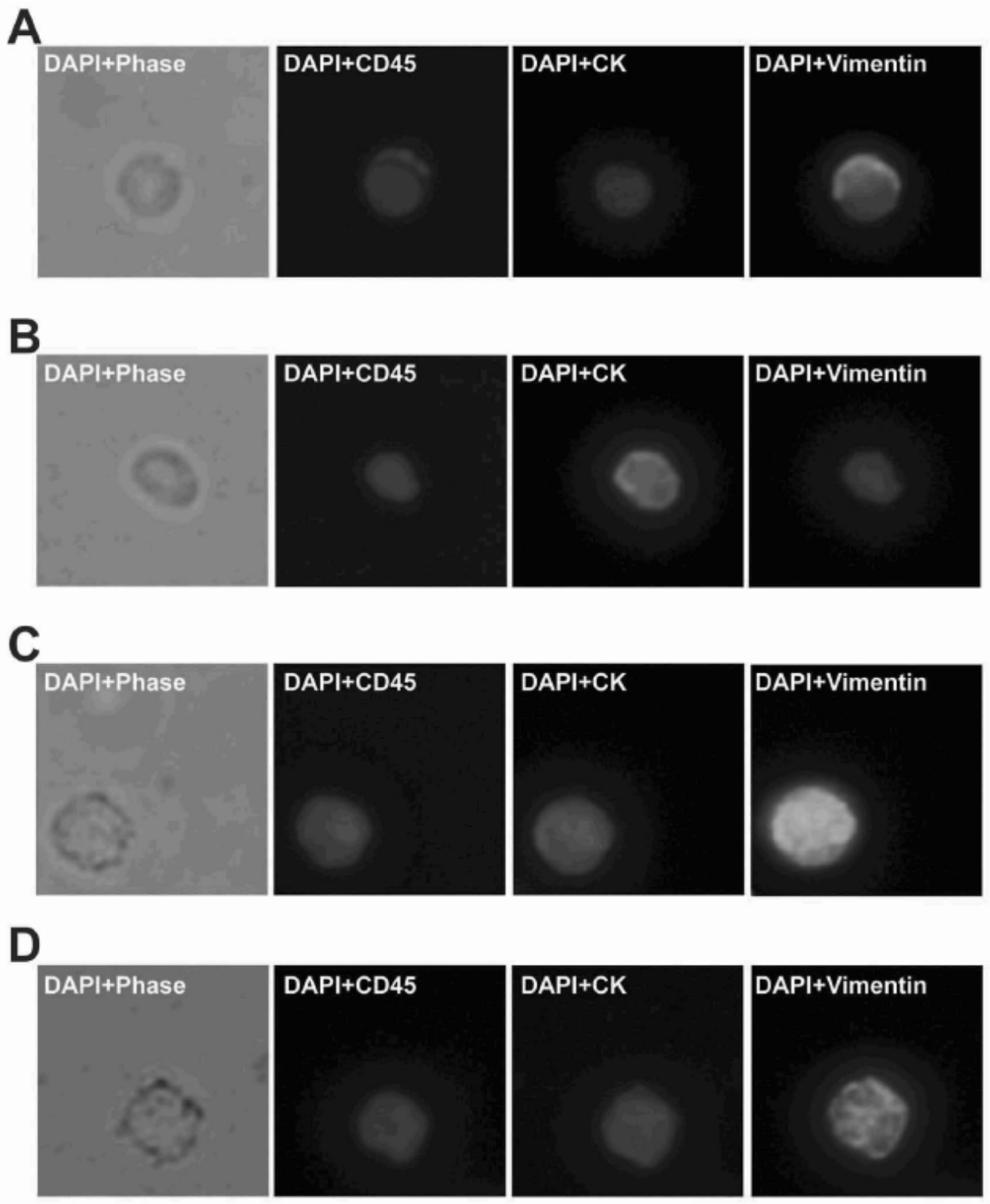
A
DAPI+Phase
DAPI+CD45
DAPI+CK
DAPI+Vimentin
B
DAPI+Phase
DAPI+CD45
DAPI+CK
DAPI+Vimentin
C
DAPI+Phase
DAPI+CD45
DAPI+CK
DAPI+Vimentin
D
DAPI+Phase
DAPI+CD45
DAPI+CK
DAPI+Vimentin

FIGURE 10

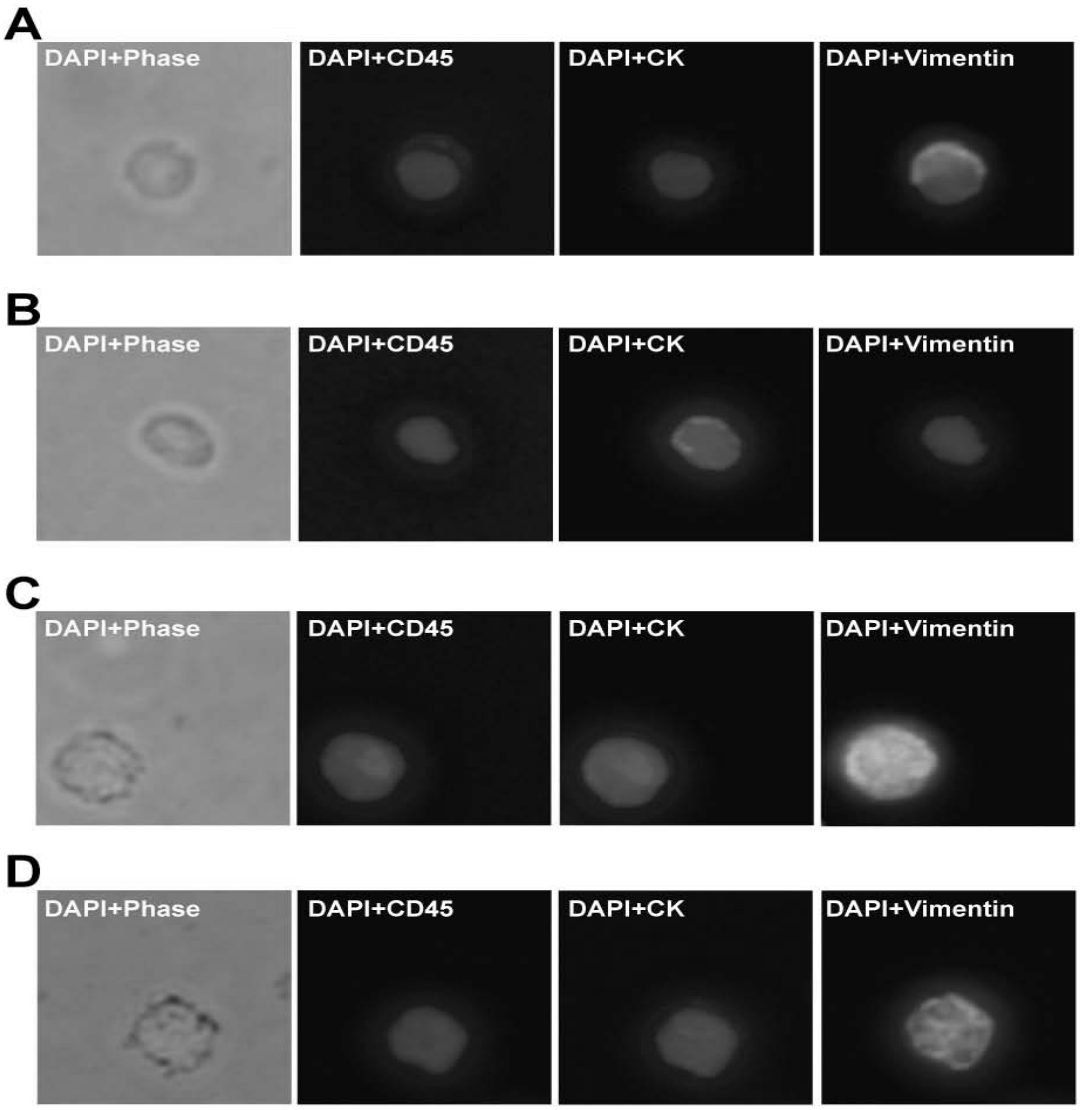
A
DAPI+Phase
DAPI+CD45
DAPI+CK
DAPI+Vimentin
B
DAPI+Phase
DAPI+CD45
DAPI+CK
DAPI+Vimentin
C
DAPI+Phase
DAPI+CD45
DAPI+CK
DAPI+Vimentin
D
DAPI+Phase
DAPI+CD45
DAPI+CK
DAPI+Vimentin

FIGURE 11

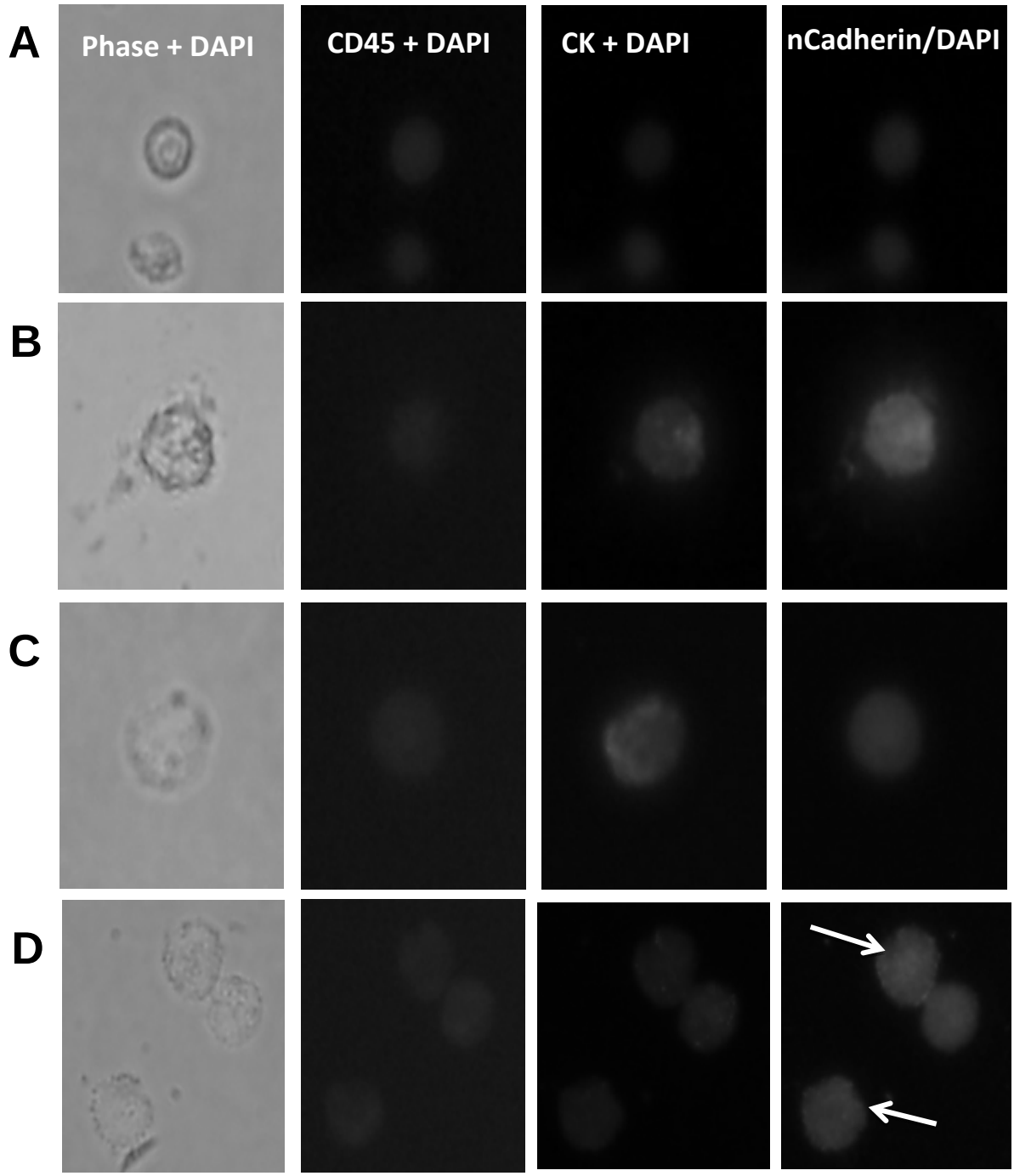

FIGURE 12

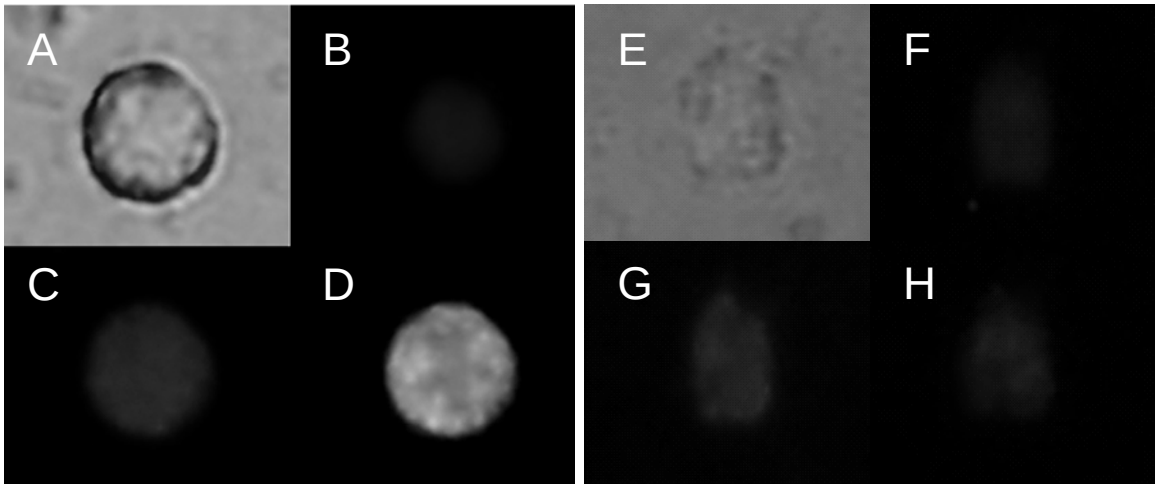

FIGURE 13

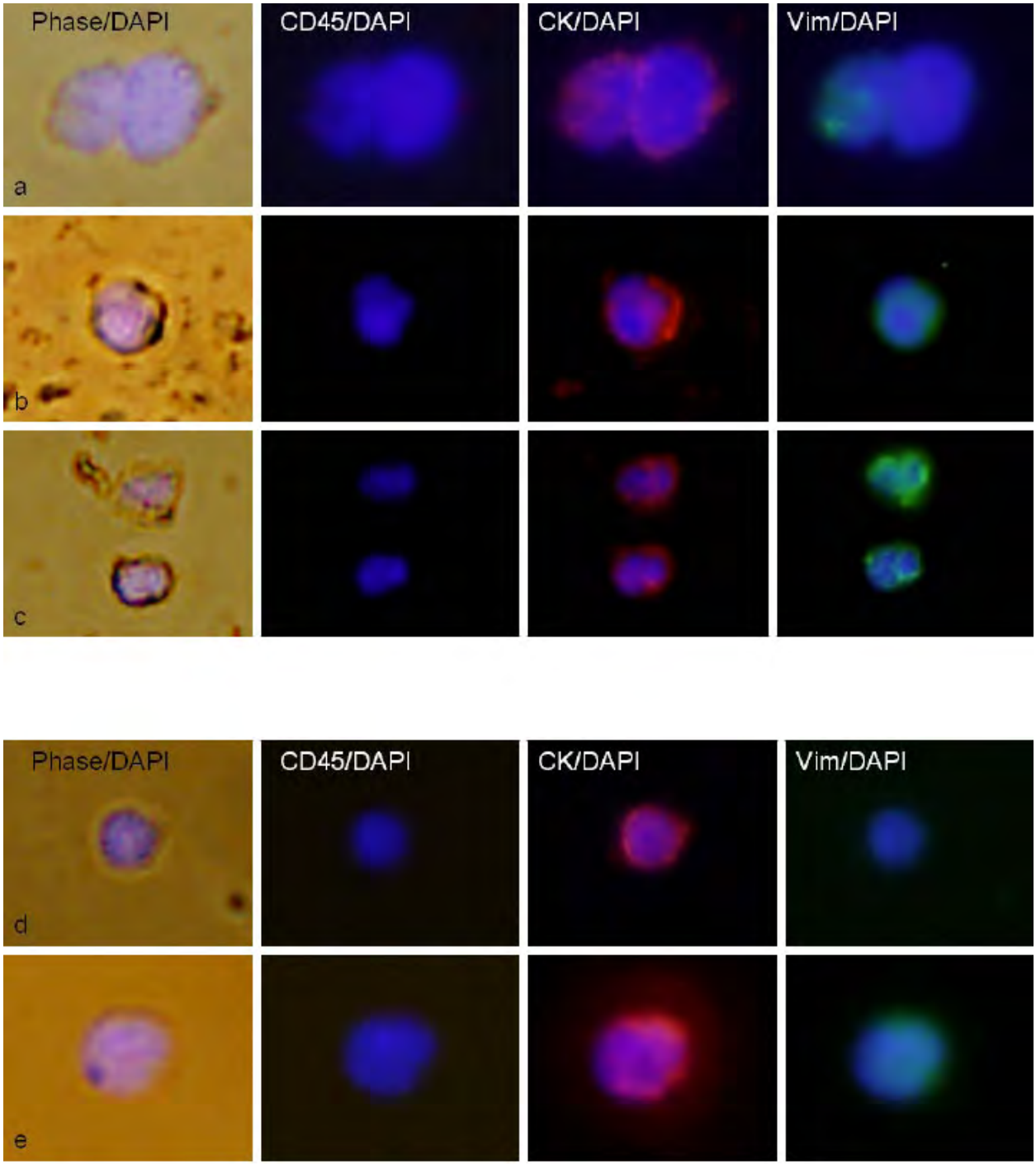
Phase/DAPI
CD45/DAPI
CK/DAPI
Vim/DAPI
a
b
c
Phase/DAPI
CD45/DAPI
CK/DAPI
Vim/DAPI
d
e

FIGURE 14

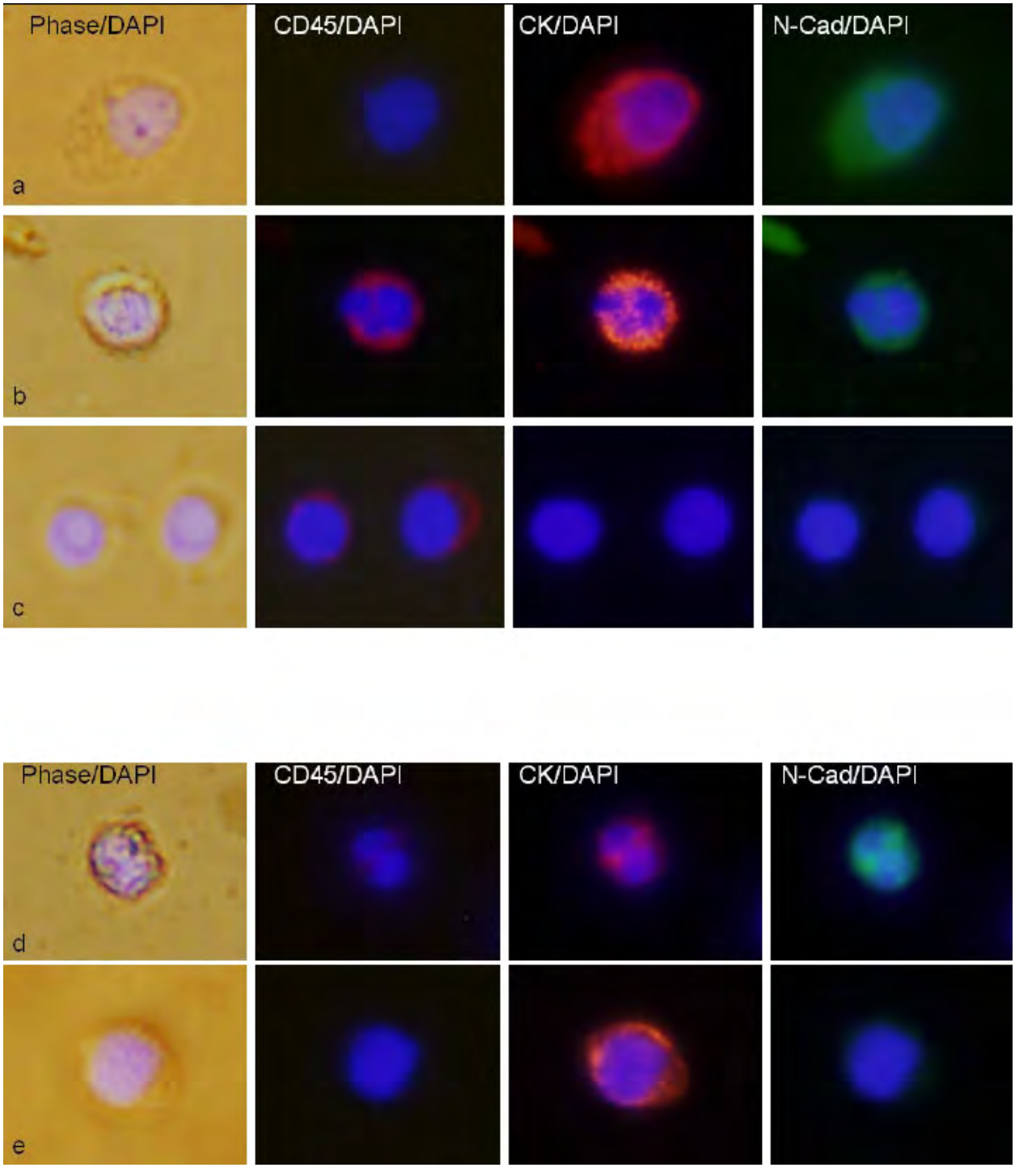
Phase/DAPI
CD45/DAPI
CK/DAPI
N-Cad/DAPI
a
b
c
Phase/DAPI
CD45/DAPI
CK/DAPI
N-Cad/DAPI
d
e

FIGURE 15

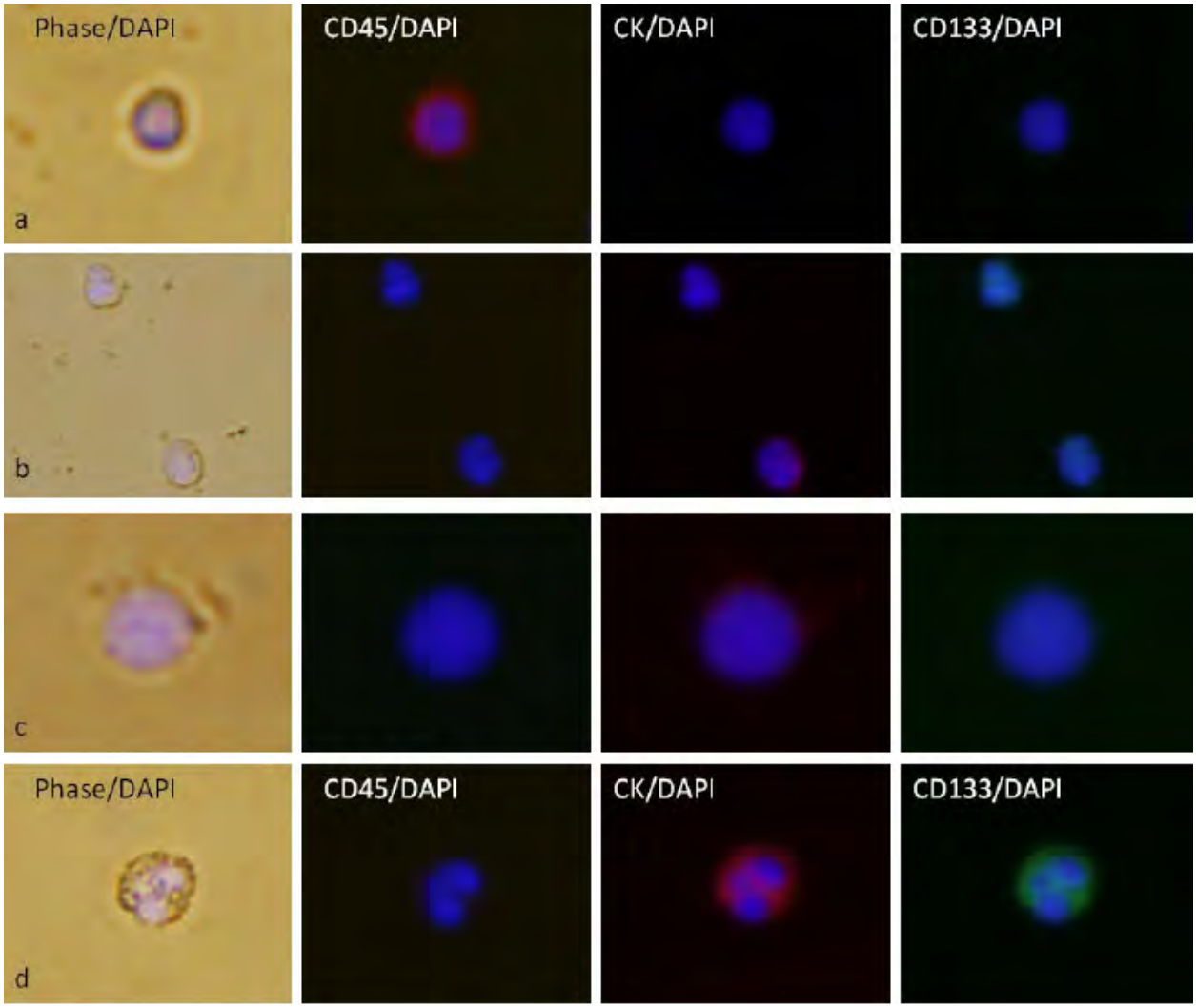
Phase/DAPI
CD45/DAPI
CK/DAPI
CD133/DAPI
a
b
c
Phase/DAPI
CD45/DAPI
CK/DAPI
CD133/DAPI
d

FIGURE 16