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Methodology article

Gene expression analysis on small numbers of invasive cells collected by chemotaxis from primary mammary tumors of the mouse

Weigang Wang*[1], Jeffrey B Wyckoff[1], Yarong Wang[1], Erwin P Bottinger[2], Jeffrey E Segall[1] and John S Condeelis[1]

Address: [1]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, New York 10461, USA and [2]Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461, USA

Email: Weigang Wang* - wgwang@aecom.yu.edu; Jeffrey B Wyckoff - jwyckoff@aecom.yu.edu; Yarong Wang - yarwang@aecom.yu.edu; Erwin P Bottinger - bottinge@aecom.yu.edu; Jeffrey E Segall - segall@aecom.yu.edu; John S Condeelis - condeeli@aecom.yu.edu

* Corresponding author

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Abstract

Background: cDNA microarrays have the potential to identify the genes involved in invasion and metastasis. However, when used with whole tumor tissue, the results average the expression patterns of different cell types. We have combined chemotaxis-based cell collection of the invasive subpopulation of cells within the primary tumor with array-based gene expression analysis to identify the genes necessary for the process of carcinoma cell invasion.

Results: Invasive cells were collected from live primary tumors using microneedles containing chemotactic growth factors to mimic chemotactic signals thought to be present in the primary tumor. When used with mammary tumors of rats and mice, carcinoma cells and macrophages constitute the invasive cell population. Microbeads conjugated with monoclonal anti-CD11b (Mac-1α) antibodies were used to separate macrophages from carcinoma cells. We utilized PCR-based cDNA amplification from small number of cells and compared it to the quality and complexity of conventionally generated cDNA to determine if amplified cDNA could be used with fidelity for array analysis of this cell population. These techniques showed a very high level of correlation indicating that the PCR based amplification technique yields a cDNA population that resembles, with high fidelity, the original template population present in the small number of cells used to prepare the cDNA for use with the chip.

Conclusions: The specific collection of invasive cells from a primary tumor and the analysis of gene expression in these cells are is now possible. By further comparing the gene expression patterns of cells collected by invasion into microneedles with that of carcinoma cells obtained from the whole primary tumor, the blood, and whole metastatic tumors, genes that contribute to the invasive process in carcinoma cells may be identified.

Background

In spite of advances in screening and adjuvant therapy, breast cancer continues to be a major health problem. Once cancer cells have spread and formed metastases, breast cancers are largely incurable even with state-of-the-art medicine. Understanding how cancer cells spread to

other parts of the body can provide important insights and will ultimately translate into improved diagnostic, prognostic and therapeutic approaches that allow control of cancer metastasis. Recently, emphasis has been on the development of molecular arrays to identify new genes and proteins that contribute to specific steps in metastasis [1,2]. Large-scale nucleic acid arrays have become very useful tools for investigators exploring differences in gene expression between cell types, stages of differentiation, and cellular responses to stimuli [3]. Such approaches are crucial in the analysis of cancer as a genetic disease and in the identification of key genes that might be used in diagnosis and therapy. So far, most gene expression studies have been done using whole tumor tissue. However, human primary tumors show extensive variation in all properties ranging from growth and morphology of the tumor, and formation and growth of metastases, and the application of tissue homogenates results inevitably in averaging of the expression of different cell types. The expression profile of tumor cells essential for invasion may be masked or even lost due to the contributions of surrounding cells. It is important to develop a technology to separate pure populations of invasive cancer cells for gene expression studies. The use of Laser Capture Microdissection as a front end for array-based gene discovery is such an approach. However, some of the cell behaviors that are believed to be essential for metastasis, such as adhesion and motility, cannot be used as criteria in the selection of cells for analysis from fixed material because the behavior and history of individual cells cannot be inferred from fixed material. Methods for the collection of cells from living tumors in which key cell behaviors can be observed and used as the criteria for cell collection need to be developed. An important approach in determining the cellular mechanisms that contribute to metastasis is to collect live cells from the primary tumor based on properties believed necessary for successful metastasis. We have shown previously that one of the properties correlated with metastasis is chemotaxis to blood vessels [4]. This cell behavior allows cells to orient and move toward blood vessels facilitating their intravasation. We have developed a method to selectively collect invasive cells from live primary tumors in intact rats using a microneedle containing a chemoattractant to mimic chemotactic signals from blood vessels and/or surrounding tissue [5].

For the study of the invasive subpopulation of cells within the primary tumor, the combination of chemotaxis-based cell collection in microneedles with array-based gene expression analysis has the potential to identify the genes necessary for the individual steps of invasion at the cellular level, and for the rational interpretation of gene expression patterns in metastatic tumors. One drawback to the array technique is the need to isolate and purify microgram amounts of total RNA [6] to generate the appropriate amounts of probe needed for conventional microarrays. However, the number of cells collected from mammary tumors with microneedles in vivo is currently limited to fewer than 1000 per needle. This number of cells typically contains 20–50 ng of total RNA [7,8], well below the amounts needed for conventional array protocols. In order to combine the collection of invasive cells from live animals with cDNA arrays technology, we have experimented with a number of methods that have been used to amplify the starting RNA in other applications [9]. We have found that the SMART PCR cDNA amplification method (ClonTech Laboratories) can be used in gene expression profiling experiments to produce cDNA libraries from total RNA that are representative of the starting mRNA. This has the potential to allow the routine analysis of differential gene expression in very small tissue samples [10–12]. Key to this alternative approach is the reproducible and representative synthesis of cDNA probes that retain faithfully the complexity of the mRNA population present in the original sample [9]. In this study we compared two approaches for synthesizing cDNA probes from total RNA for use with subsequent hybridization to high-density cDNA microarrays: 1) the conventional approach of reverse transcription (RT) of 100 μg of total RNA from cultures of carcinoma cells and 2) amplification of ~30 ng of total RNA from 1000 carcinoma cells using the PCR based cDNA amplification system. The results reported here demonstrate that, for situations with limited RNA, the RT-PCR based probe synthesis method retains the original mRNA message profile, and is suitable for gene expression profiling of invasive cells collected in microneedles.

The efficiency of separation of the pure population of invasive carcinoma cells and the quality of RNA isolated from microneedles were also tested in this study.

Results

Separation of carcinoma cells and macrophages for use in microarray analysis

As shown by our group elsewhere [13], the invasive cells that enter microneedles in the primary tumor are a mixture of macrophages and carcinoma cells. This conclusion is based on both cell type-specific antibody staining and real time PCR using cell type-specific primers. In order to analyze the gene expression pattern of only a single cell type at a time, we have investigated the efficiency of separating these two cell types using standard techniques. As shown in Figure 1A and 1B based on cell type-specific antibody staining, the use of MAC 1 antibody-coated magnetic beads can be used to selectively remove the macrophage sub-population of cells from the carcinoma cells collected in a single microneedle. The separation allows removal 80% of the macrophage contribution. The ability to separate the macrophages is further demonstrated by

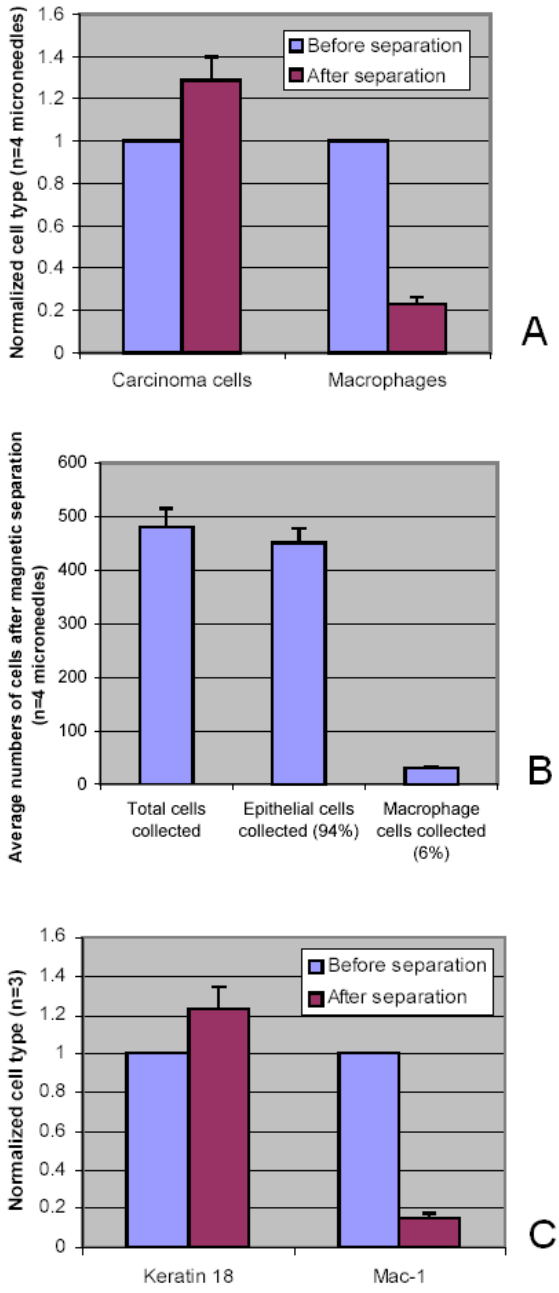

Figure 1

1A and 1B: Macrophages can be separated from carcinoma cells after microneedle collection. Cells collected from a *MMTV-PyMT* primary tumor in a microneedle filled with matrigel and 25 nM EGF were magnetically separated using Mac-1 coated iron beads. Approximately 80% of the macrophages were removed from the sample, leaving only a 6% contamination of the carcinoma cell population by macrophages after a single separation step.

Figure 1C: Quantitative real-time PCR shows a decrease in the amount of Mac-1 mRNA in cells separated after collection from the primary tumor. Cells were collected and separated as described in Materials and Methods. After RNA purification from cells before and after separation, real-time PCR was run for the Mac-1 (macrophage marker) and keratin 18 (carcinoma cell marker) primers. Normalization was done using β-actin as reference gene. A decrease in expression of Mac-1 is seen in the cell-separated sample compared to that of the sample containing both cell types. This further confirms our ability to reduce the contaminating cells from our collected sample, so as to have a pure carcinoma cell population for gene discovery. The level of Keratin 18 expression shows a slight increase after separation.

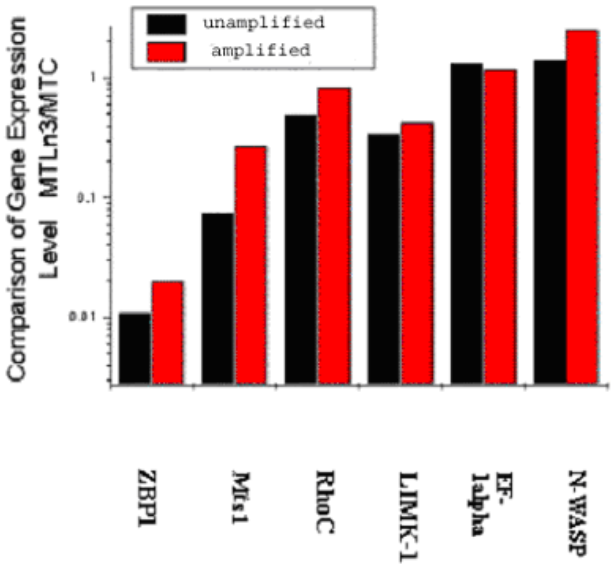

Figure 2
Relative abundance of genes is maintained during cDNA amplification as verified by Quantitative real-time PCR. cDNA from 1000 MTLn3 (a metastatic cell line) or 1000 MTC cells (non-metastatic cell line) was diluted (1:10) after 20 cycles of SMART PCR amplification, and the expression of selected genes was compared with cDNA from 3 μg unamplified total RNA from MTLn3 or MTC cells by real-time PCR.

comparing quantitative real time PCR using cell type-specific primers to the results of real time PCR on samples after removal of the macrophages by the use of magnetic beads (Fig 1C). Again, real time PCR demonstrates the extensive removal of the macrophage-specific marker, yielding a purer carcinoma cell population for further analysis.

Characterization of cDNA amplification techniques

We have adopted a SMART (Switch Mechanism At the 5' end of RNA Transcript) amplification method [14,15] to amplify cDNA from our needle collection cells. Key to this alternative approach is the reproducible and representative synthesis of cDNA probes that retain faithfully the complexity of the mRNA population present in the original sample [9]. In this study we compared two approaches for synthesizing cDNA probes from total RNA for use with subsequent hybridization to high-density cDNA microarrays: 1) the conventional approach of reverse transcription (RT) of 100 μg of total RNA from cultures of carcinoma cells and 2) amplification of ~30 ng of total RNA from 1000 carcinoma cells using the PCR based cDNA amplification system.

Selection of the number of cycles for the exponential PCR amplification of the cDNA is the crucial point of the technique. 10 μl aliquots from the amplification reactions after 17, 20, 23 cycles were analyzed in a 1% agarose gel. Overcycled reactions can be identified by the disappearance of the distinct bands corresponding cDNAs and the increased smear molecular weight. In this study, preparative amplifications were subsequently performed at 20 cycles for cells from culture and microneedle.

As shown in Figure 2, comparison of the relative expression level of six genes in a metastatic cell line (MTLn3) and the non-metastatic cell line (MTC) was done using real time PCR. The amplified RNA shows the similar relative level of expression as unamplified RNA indicating that the PCR based cDNA amplification technique does not give rise to a misrepresentation of the original template complexity of RNA isolated from small numbers of cells.

By comparing the distribution of ratios of gene expression in a metastatic cell line (MTLn3) and the non-metastatic cell line (MTC), it was possible to establish the relatedness of the expression patterns of the two cell types on a 9000 gene chip. As shown in Figure 3, the correlation of the expression ratios allows one to establish the reproducibility and relatedness of the preparations of cDNA. In Figure 3A, RNA isolated from ten million cells of each cell line was used to prepare the cDNA probe for use in the array. As shown in Fig 3A, comparison of two separate chips probed with unamplified sample shows a correlation coefficient of greater than 0.91. We then evaluated amplification of RNA from 1000 cells, followed by hybridization to two microarrays (Figure 3B). The amplified RNA samples showed a correlation coefficient in excess of 0.93 indicating that this technique generates a highly reproducible cDNA product. Finally, the average ratio of expression patterns for each gene in 3A and 3B are plotted in Figure 3C and show a correlation in excess of 0.9. This indicates that the PCR based amplification technique yields a cDNA population that resembles with high fidelity the original template population present in the small number of cells used.

This conclusion is supported by the data in Figure 4, in which the level of expression of a select number of over-expressed genes identified initially in unamplified RNA was also found to be over-expressed in chips probed with amplified cDNA. All of these results indicate that the PCR based cDNA amplification technique is eminently suitable for use with small numbers of cells as those obtained using needle collection.

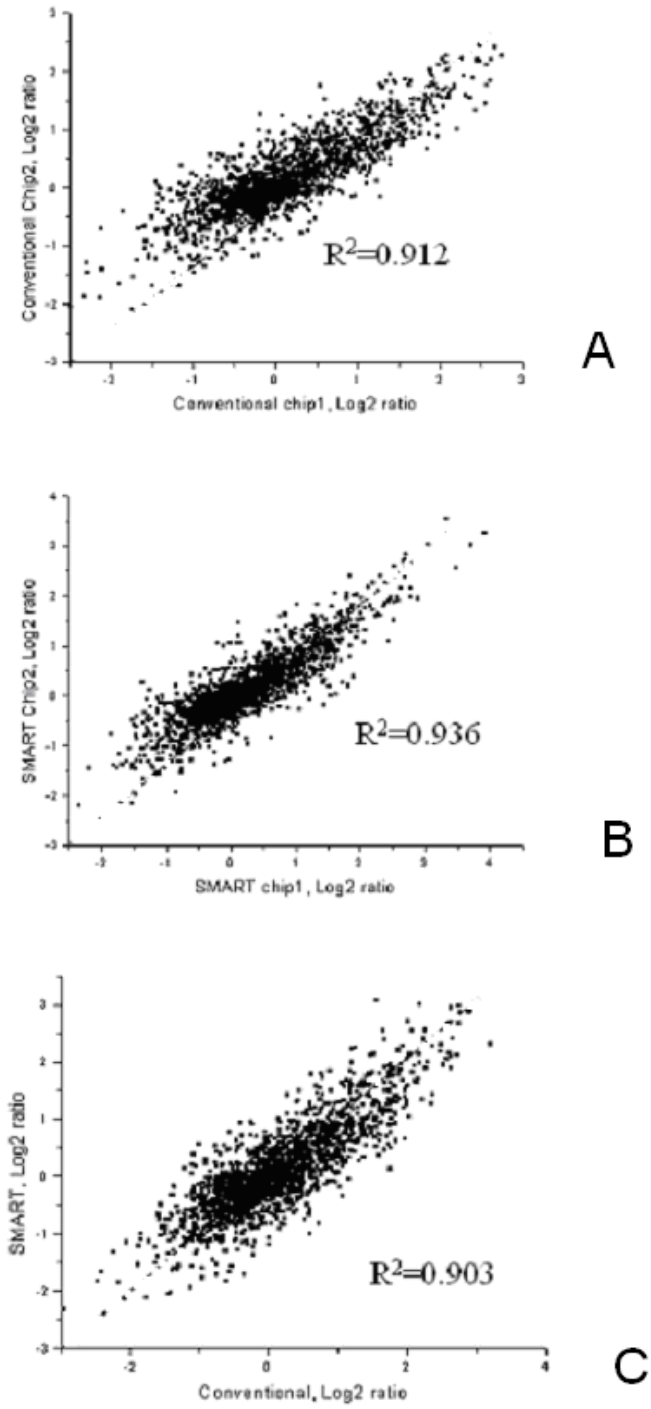

Figure 3

The correlation coefficient demonstrates that amplified cDNA is comparable to that of conventionally isolated RNA from large numbers of cells. A) By comparing the distribution of ratios of gene expression in MTLn3 and MTC, the relatedness of the expression patterns of the two cell types is establish on a 9000 gene chip. Log base 2 ratios from 2 chips were plotted for unamplified probes from MTLn3 and MTC cells. B) Log base 2 ratios from 2 chips using PCR amplified probes of MTLn3 and MTC cells shows a higher correlation coefficient. C) The mean of the log base 2 ratios in A and B shows a correlation coefficient within the accepted range for conventional RNA methods.

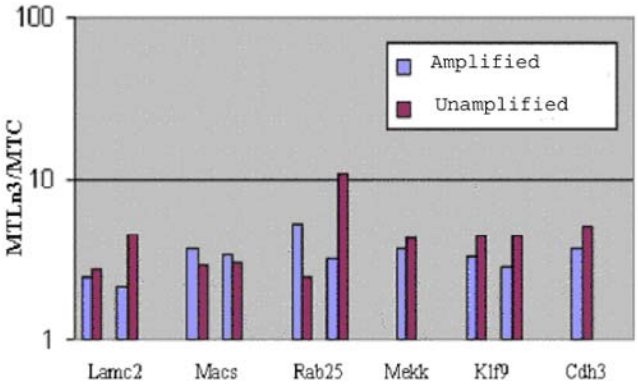

Figure 4
Cancer metastasis genes overexpressed in MTLn3 cells are detected by both probe synthesis techniques. Amplified cDNA probe from MTLn3 cells and MTC cells were hybridized to a 9000 gene cDNA microarray. When compared to a chip hybridized with probes generated from unamplified RNA, the level of expression for selected metastasis genes are seen to show similar levels of expression on the amplified cDNA probe chip.

Use of PCR based cDNA amplification with cells collected in microneedles from the primary tumor

RNA quality is an important issue for gene expression analysis. As shown in Fig 5A, total RNA isolated from microneedle collected cells was checked using the Agilent Bioanalyzer and the RNA 6000 Pico kit. The RNA 6000 Pico kit used here allows the determination of the integrity of very low amount of RNA as well as an estimation of the amount of the isolated RNA. The size distribution and rRNA ratio (28S/18S = 2.7) indicates good intactness of RNA sample. Furthermore, validation of the RNA quality using the relative abundance of the marker template, beta-actin mRNA, demonstrates that the amount of the mRNA template isolated from cells in the collection needle was identical to that of RNA isolated conventionally from cells obtained from the whole primary tumor (Figure 5B).

In order to study the gene expression patterns of invasive cells collected from the primary tumor using microneedles, we amplified RNA from the cells collected in a single microneedle in the primary tumor. Amplification of the RNA from 800 cells collected in a microneedle yielded a pattern of PCR products with a size complexity that was very similar to the size complexity of the PCR product obtained from one thousand cultured carcinoma cells (Fig 6A). Figure 6B demonstrates the use of the cDNA amplified from cells collected in a microneedle from the primary tumor in probing a high-density cDNA microarray. More than 91% of the spots showed a good hybridization signal. Finally, as shown in Figure 6C, the distribution diagram shows a similar distribution of gene expression patterns for amplified samples indicating the cDNA prepared from the small number of cells is of sufficient quality and complexity to be useful in probing the nine thousand genes present in this high-density array.

Discussion

Microneedle collection

In our previous study [5], we reported that needles containing chemoattractants can be used to collect the subpopulation of motile and chemotactic tumor cells from a primary tumor *in vivo* as a population suitable for further analysis. It suggests that needles filled with growth factors and matrigel, when inserted into the primary tumor, can faithfully mimic the environment that supports invasion and intravasation *in vivo*, and that the same cell behaviors that contribute to chemotaxis *in vitro* also contribute to invasion *in vivo*.

An advantage of using the needle collection technique described here for the collection of cells for genomic/proteomic analysis is that the cell behavior can be characterized during the collection process. This can be done by varying the conditions required for cell collection such as the extracellular matrix composition and/or cytokines used as chemoattractants, determining how these changes affect efficiency of cell collection, and then relating these observations to the gene expression and protein composition patterns subsequently obtained from array analysis of the collected cells. Furthermore, cells can also be characterized by intravital imaging during collection to directly visualize the cell-cell and cell-extracellular matrix interactions that contribute to the invasion of the needle under these different conditions [16]. In addition, cells could be cultured and transplanted into other host animals to determine whether they stably retain differential characteristics that contribute to metastatic potential. Finally, by comparing the gene expression patterns of cells collected by invasion into needles with that of cells obtained from the whole primary tumor, the blood, and whole metastatic tumors, genes that contribute to the invasive process uniquely may be identified.

Separation of macrophages from the microneedle collection samples also makes it possible to analyze the gene expression pattern of Tumor Associated Macrophages

The tumor microenvironment contains stromal cells that influence the behavior of the tumor. Of these, there is increasing evidence that macrophages play an important role in modulating the metastatic capacity of the tumor. This includes clinical evidence showing a strong correlation between TAM (Tumor Associated Macrophages) [17,18] and poor prognosis, and genetic studies

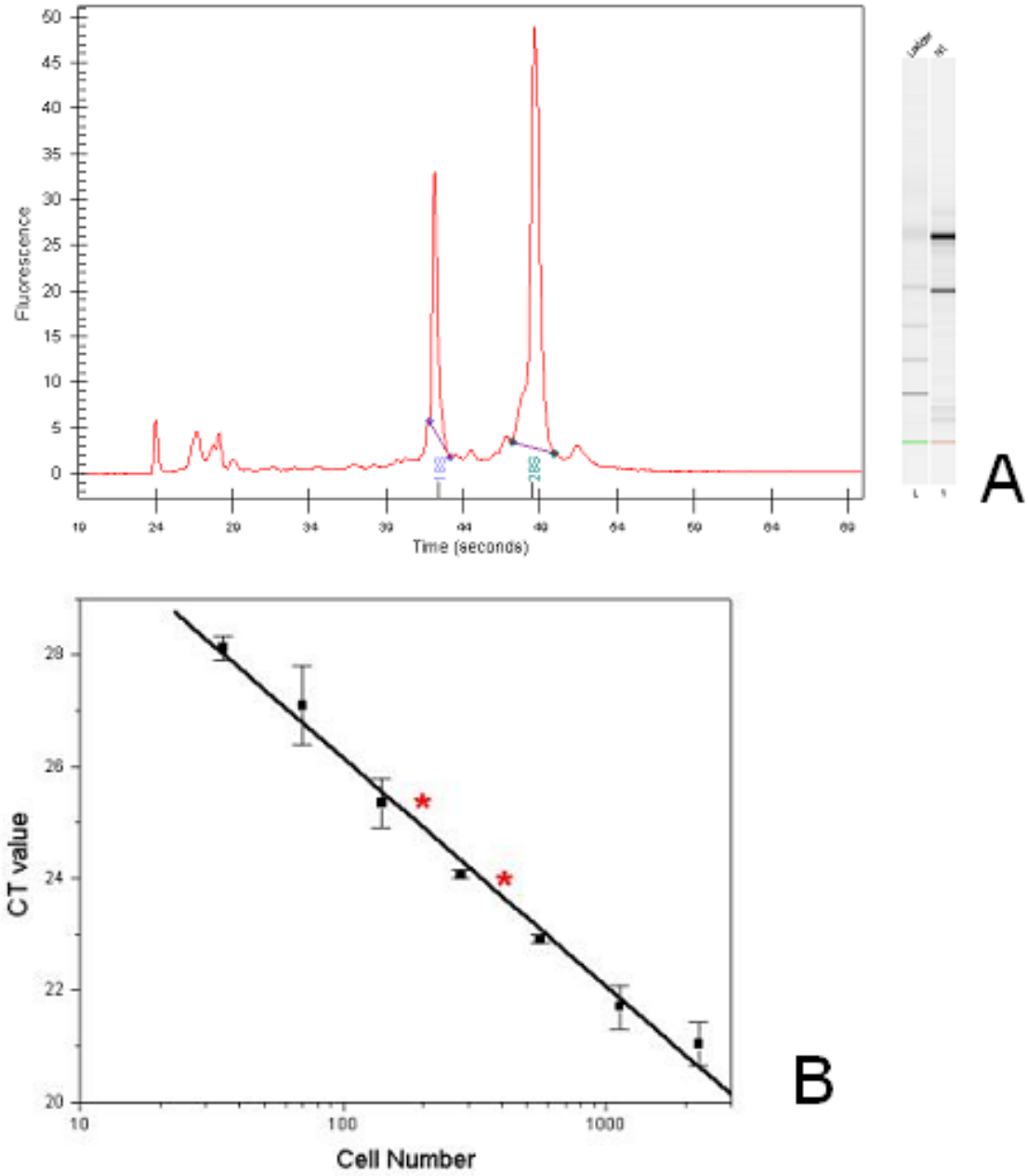

Figure 5

The RNA from 200 and 400 cells is of equal amount and quality of RNA purified by conventional methods. Figure 5A: Total RNA isolated from microneedle collected cells was checked using Agilent Bioanalyzer and RNA 6000 Pico kit. The size distribution and rRNA ratio (28S/18S = 2.7) indicates good intactness of RNA sample. Figure 5B: A standard curve was generated for the CT value of a known quantity of RNA from a specific cell number using the β-actin primers for real-time PCR. The RNA from 200 and 400 cells were amplified by the SMART PCR method and run identically by real-time PCR. The CT values for the amplified RNA fall on the curve showing that the appropriate amount of the house keeping gene is present in the amplified sample. The CT values for the amplified samples are designated by the red asterisks.

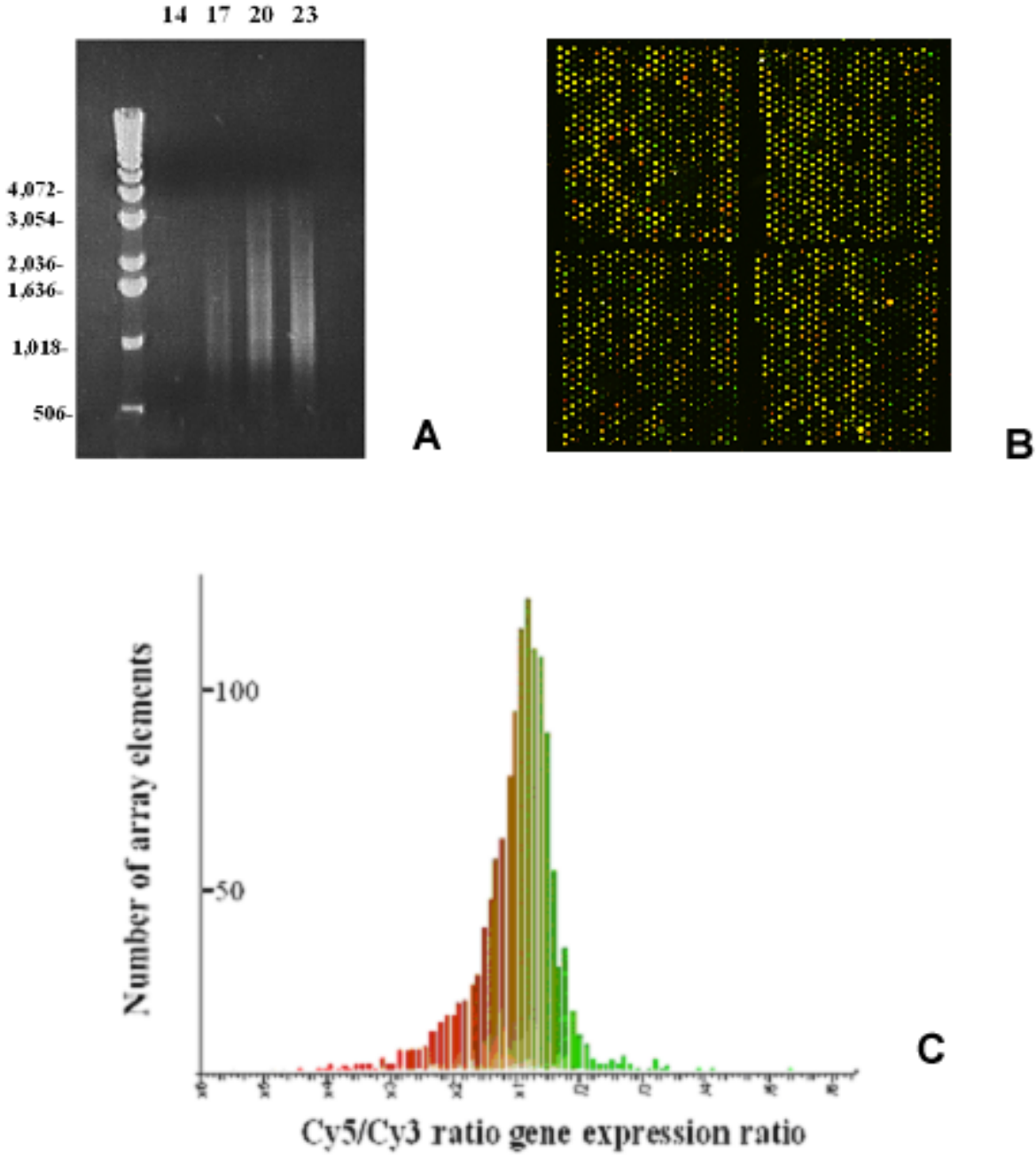

Figure 6
The RNA from microneedle collected cells was extracted and amplification was performed using SMART PCR. Separately, 1μg of Universal Mouse Reference RNA (Stratagene) were used to generate reference cDNA. Fig 6A shows a gel picture of amplified cDNA from microneedle collected cells. The numbers indicate the PCR cycle number. The generated PCR product from the needle sample as well as the Universal Mouse Reference cDNA were labeled with Cy5 or Cy3. The two resulting cDNA probes were then mixed together and hybridized to a microarray slide containing 9700 genes. Fig 6B shows a part of the entire array (4 out of 16 blocks) generated using PCR amplified probe. Fig 6C is a histogram of the distribution of gene expression (CY5/Cy3) ratios for the array elements for hybridizations comparing gene expression.

in mice where removal of macrophages from the tumor bed leads to severely reduced rates of metastasis [19,20]. Macrophages may contribute factors that affect tumor progression by altering the microenvironment including angiogenic and proteolytic factors [19]. These cells are also capable of producing many growth factors, including members of the EGF-family, which directly influence the behavior of tumor cells. In wound healing or at sites of infection, macrophages synthesize chemotactic factors that recruit other blood cells. The unique ability of macrophages to localize to specific sites and perform such tasks suggests that they could also provide chemotactic cues in tumors promoting the egress of the carcinoma cells from the tumor core. The collection of macrophages with carcinoma cells into microneedles in response to EGF is consistent with a role for macrophages in cancer cell invasion.

The methods described here allow for the collection of invasive tumor cells and Tumor Associated Macrophages and their separation into two cell types. These techniques will allow us to analyze the gene expression pattern not only for tumor cells but also for macrophages. It will make possible the identification of paracrine and other microenvironment-dependent interactions that contribute to the invasive process.

RNA quality of microneedle collected cells

Integrity of RNA samples is essential in the context of doing gene expression analysis on the microneedle collected cells. The matrigel in the needle makes it difficult to extract the RNA due to the abundance of proteins and collagen. In order to remove these proteins, which can interfere with the procedure, the standard RNeasy Mini Protocol from animal tissue has been adapted to include a proteinase K digest. After dilution of the lysate, the sample is treated with proteinase K. Debris is pelleted by centrifugation. Ethanol is then added to the cleared lysate and RNA is bound to the RNeasy membrane. This protocol has been used successfully for RNA isolation from microneedle collection samples and ensured high-quality RNA. As shown in our result, total cellular RNA prepared from microneedle collection samples remains intact after isolation, increasing confidence in subsequent molecular analyses.

PCR based cDNA amplification for use in microarray analysis of invasive carcinoma cells

PCR based cDNA amplification results in a cDNA product that resembles the starting template in size hetero-dispersion and complexity. Amplified cDNA is of a quality sufficient for use with high-density cDNA microarrays.

The specific collection of invasive cells from the primary tumor and the analysis of gene expression in these cells is now possible. We have demonstrated that RNA obtained from as few as 400 cells collected in a microneedle from the primary tumor, when amplified as cDNA using the PCR based protocol, can be used for microarray expression analysis. We have further documented that calibration of the number of PCR cycles used in this method allows amplification without loss of either relative mRNA copy abundance or complexity of the amplified product. This technology will allow the characterization of gene expression patterns of invasive tumor cells within the primary tumor during invasion and in response to varying genetic backgrounds. It will also make possible the identification of paracrine and other microenvironment-dependent interactions that contribute to the invasive process.

Conclusions

The specific collection of invasive cells from the primary tumor and the analysis of gene expression in these cells are now possible. By further comparing the gene expression patterns of cells collected by invasion into microneedles with that of carcinoma cells obtained from the whole primary tumor, the blood, and whole metastatic tumors, genes that contribute to the invasive process in carcinoma cells may be identified.

Methods

Mice

All mice were created in the FVB-C3H/B6 background and remained in a consistent background throughout breeding. The origin and identification of *MMTV-PyMT* has been described previously [20]. *MMTV-GFP* mice were described previously [21] and crossed with the *MMTV-PyMT* mice to produce GFP labeled tumors. Tumors were allowed to grow for 16–18 weeks prior to cell collection to ensure late stage carcinomas and increased metastasis.

Preparation and handling of collection needles

We have combined needle collection of invasive cells with multiphoton-based intravital imaging in mice with mammary tumors produced by the expression of the polyomavirus middle T oncogene. Expression of the polyomavirus middle T antigen in the mammary gland (MMTV-PyMT) results in transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas [22]. MMTV-MiddleT transgenic mice crossed with MMTV-GFP transgenics were allowed to grow tumors for 16 weeks. On the day of the experiment 33-gauge needles were prepared by filling them with 1:10 matrigel and L15-BSA (the isotonic equivalent of 5% FBS), or L15-BSA with a final concentration of 25nM EGF. All needles contained 0.01 mM EDTA (pH 7.4) to sequester heavy metals that might be released from the needle. A mouse was anesthetized using 5% isoflurane and laid on its back. The isoflurane was reduced to 2 %, and a small patch of skin was removed to expose the tumor. Three 25-gauge

needles, with inserted blocking wires, were positioned with the needle holder held in a micromanipulator for stability, into the tumor. The guide wires were removed and the matrigel containing needles were inserted. The animal was kept under monitored anesthesia for 4 hours. Afterwards, the needle contents were expelled onto a cover slip, mixed 1:1 with DAPI and counted immediately.

Macrophage separation from cultured cells

As described in our previous work [13], carcinoma cells comprised approximately 73% of the total cell population, while macrophages comprised 26%, together accounting for over 99% of the cells collected in response to EGF. To test the possibility of removing the macrophages from this total cell population, the following experiment was performed. BAC-1.25 macrophages and MTLn3-GFP adenocarcinoma cells grown in culture were removed with PBS/ 2 mM EDTA, pH 7.5. After counting cells, approximately 1000 carcinoma cells were mixed with 350 macrophages in 90 μl of PBS/0.5% BSA/ 2 mMEDTA in a 500 μl eppendorf tube. Cells were then mixed with 10 μl of MACS CD11b Microbeads (Miltenyi Biotec). These microbeads are colloidal super-paramagnetic beads conjugated with monoclonal anti-mouse CD11b (Mac-1α) antibodies. The cells were placed at 4°C for 15 min and then placed in a magnetic separator (LifeSep HGS-1.5; Dexter Magnetic Technologies) for 15 min at 4°C. The supernatant was removed while still in the magnetic separator and the cells were stained with DAPI and counted immediately. Different cells types were identified by their expression or lack of expression of GFP.

Macrophage separation from microneedle collected cells

Cells were collected from MMTV-PyMT mice. The needle contents were extruded into a 500 μl eppendorf tube with PBS/BSA/EDTA and diluted to 100 μl in the same buffer. 10 μl was removed and stained with DAPI to get an approximate cell count. The other 90 μl was treated with the microbeads as described above. After cell separation, cells were mixed 1:1 with 10% buffered Formalin on a poly-l-lysine coated Mattek dish and stained for anti-pan-keratin and anti-F480.

Real Time PCR was performed on mRNA isolated from the collected cells before and after microbead separation using macrophage (MAC-1) and carcinoma cell (keratin) specific primers.

Conventional total RNA extraction and microarray hybridization

For isolation of RNA from large numbers of cultured cells, standard RNA extraction and microarrays hybridization we followed standard protocols as described elsewhere [6,16].

RNA Extraction and cDNA amplification from small numbers of cells and microneedle collection samples

Cell collection was performed using needles with 25 nM EGF [5], followed by microbead separation. Cells collected from each needle were divided into 1/10 of the collection volume for counting. The remaining 9/10 of cells from the microneedle were placed into a 1.5-ml microcentrifuge tube containing 350 μl of guanidine thiocyanate buffer, homogenized by passing the lysate through a 20-G needle, attached to a sterile plastic syringe, at least 5–10 times. 500 μl of double-distilled water and 10 μl of Qiagen Proteinase K solution was added to the homogenate and mixed thoroughly by pipeting and incubated at 55°C for 10 minutes. Extraction was continued using the RNeasy kit (QIAGEN), and RNA molecules selectively bound to the silica gel base were eluted with 30 μl RNase-free water. The RNA was then concentrated by ethanol precipitation and re-dissolved in 3.5 ul DEPC water.

The total RNA was reverse-transcribed directly using the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol. Annealing was conducted using a modified oligo(dT) at 70°C for 2 minutes in the presence of the SMART II oligonucleotide in a total volume of 5.5 μl. The reaction was followed by the addition of Superscript II (200 units) RNase H^- reverse transcriptase (RT) (Gibco-BRL, Gaithersburg, MD) and incubated at 42°C for 1 hour. The reaction was stopped by adding 40 μl of Tris-EDTA buffer and heatingat 72°C for 7 minutes. Representative double-stranded cDNAs were then generated by exponential PCR amplification. The optimal number of cycles for each sample was determined by analyzing the PCR products of a series of PCR amplifications using different numbers of cycles by electrophoresis. Four microliters from the 50-μl single-stranded cDNA stocks were amplified in 50-μl reactions using the SMART PCR primer by the predetermined exponential number of cycles. Amplified cDNAs from the cells of the primary tumor and needle collection were diluted and used for further analysis by cDNA microarrays and real-time PCR.

Microarray hybridization and analysis

Microarray analysis was performed by using cDNA microarrays made at AECOM. About 9,700 mouse genes (Incyte Genomics) were precisely spotted onto a single glass slide. Detailed descriptions of microarray hardware and procedures are available from http://www.aecom.yu.edu/cancer/new/cores/microarray/default.htm.

After amplification, cDNAs were purified using the QIAquick PCR Purification Kit (Qiagen) and eluted with TE buffer. For each probe, labeling was conducted by incorporation of Cy5 or Cy3-dUTP (Amersham Pharmacia) during random hexamer-primered primer extention

in the presence of Klenow DNA polymerase (Life Technology). Briefly, 2.5 μg dscDNA was mixed with 3 μl (0.5 μg/μl) random primer, adjusted to 23 μl, boiled at 95°C for 5 minutes, and put on ice. The above was then mixed with 3 μl reaction 2 buffer (Biolabs), 3 μl 10X dNTP (1.2 mM dCTP, dGTP and dATP, 0.6 mM dTTP), 3 μl Cy5 dUTP and 1 μl Klenow DNA polymerase and incubated at 37°C for 2 hours and stopped with EDTA. The two resulting cDNA probes were then mixed together, purified and concentrated, denatured at 94°C, and hybridized to an arrayed slide overnight at 50°C. Details of slide washing, image collection and data normalization and analysis were described in previous study [16]. In this study, comparisons of normalized data were graphed using Log_2 (ch1/ch2) ratio scatter plots. The linear regression function in Excel was used to calculate R^2 (the correlation coefficient).

Quantitative Real-Time PCR (QRT-PCR)

Quantitative RT-PCR analysis of the abundance of mRNA in cell and tissue samples was performed by using the iCycler apparatus (Bio-Rad) with sequence specific primer pairs for selected genes. The SYBR Green PCR Core Reagents system (Perkin-Elmer Applied Biosystems) was used for real-time monitoring of amplification. Results were evaluated with the ICYCLER IQ REAL TIME DETECTION SYSTEM software (Bio-Rad) [16].

Quality control of the RNA prepared from cells collected in microneedles

Total RNA from cells in microneedles was extracted. To verify successful RNA isolation and the intactness of the RNA samples, the RNA 6000 Pico kit and Agilent 2100 Bioanalyzer (Agilent Technologies) were used. The system automatically calculates the ratio of ribosomal bands in total RNA samples and shows the percentage of ribosomal impurities in RNA samples.

Total RNA from mammary tumors of *MMTV-PγMT* mice was prepared, and different amounts of RNA (correlated with the numbers of cells, 30 pg/cell) were used to generate a standard curve by real-time PCR. The abundance of β-actin mRNA was measured by the CT (threshold cycle) values of the real time PCR reaction. Cells were collected from *MMTV-PγMT* tumors of the mammary using the microneedle technique. One fourth of the cDNA (equivalent to that from 200 and 400 cells, in three repeats) from two independent cell collection experiments was used in real-time PCR with the same β-actin primers used to construct the standard curve. The CT values for these two samples were fit to the standard curve.

Author's Contributions

WW carried out microneedle collection, amplification validation, microarray analysis and drafted the manuscript. JW and YW carried out the microneedle assay, macrophage separation and Immunoassays. EB participated in the microarray analysis. JS and JC conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Acknowledgements

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