Published in final edited form as:

Methods Mol Biol. 2009 ; 539: 49–57. doi:10.1007/978-1-60327-003-8_3.

Microarrays for Protease Detection in Tissues and Cells

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Summary

Expression of a given protease and of the endogenous inhibitors that regulate protease activity can be readily determined at the transcript level by using whole genome microarray chips. In the case of proteases and protease inhibitors, however, determining which cells are expressing them is often critical to understanding the functional roles of the proteases. For example, in cancer many of the proteases are derived from cells that are found in the microenvironment surrounding the tumor, e.g., fibroblasts and inflammatory cells. Proteases from both fibroblasts and inflammatory cells have been implicated in malignant progression. Therefore, it is important to recognize the origin of these molecules if one is to develop effective therapies. In this regard, mouse transgenic models and xenograft models in which human tumor cells are implanted in mice are useful tools. To profile human and mouse proteases, protease inhibitors, and protease interactors, we have developed in partnership with Affymetrix a custom, single platform, dual species chip: the Hu/Mu ProtIn chip. The Hu/Mu ProtIn chip has been validated for its ability to identify human and mouse transcripts in single species specimens and to identify and distinguish between human and mouse transcripts in dual species specimens such as xenografts. In the latter specimens, the Hu/Mu ProtIn chip has enabled us to identify host (mouse) proteases that play a protective role in development of lung tumors. Here we outline a protocol for using the Hu/Mu ProtIn chip to profile proteases, protease inhibitors, and protease interactors in tissues and cells.

Keywords

Protease; Protease inhibitors; Protease interactors; Microarray; Hu/Mu ProtIn chip; Xenograft; Tumor microenvironment

1. Introduction

Proteases are involved in the regulation of physiologic and pathologic processes including inflammation, angiogenesis, tumorigenesis, invasion, and metastasis (reviewed in **refs.** (1–5). On the basis of their common evolutionary origin, proteases have been divided into 47 clans of more than 175 families that share similar tertiary structures or catalytic sites (6). Detailed information on known proteases and their relationships can be found in the MEROPS database online (<http://merops.sanger.ac.uk>, (6). The activities of proteases are regulated by various endogenous protease inhibitors (for review *see* **refs.** (7–9). Like their protease counterparts, the inhibitors are numerous and diverse, and have been classified into 33 clans of 55 families (6). Other proteins, such as activators and receptors, affect the biological functions of proteases by modulating their behavior, trafficking, and localization.

To understand the significance of proteolysis in any physiologic and/or pathologic process, one has to consider proteolysis in the context of the tissue microenvironment and the interplay therein among proteases, protease inhibitors, and protease interactors. Suitable model systems that can be experimentally manipulated are helpful as they provide the ability to compare proteolytic pathways under various conditions. One example for elucidating tumor–host interactions is xenografts of human cancer cells implanted orthotopically in mice. Furthermore, to determine the role played by proteases, protease inhibitors, and protease interactors in any given process, whether pathologic or physiologic, they first need to be identified and their levels of expression in the affected tissues/cells measured.

Oligonucleotide microarrays have greatly facilitated our ability to measure levels of gene expression in diseases such as cancer (for review, *see ref.* (10)). The increasing evidence that development and progression of cancers is affected both positively and negatively by interactions between the tumor and its microenvironment (11–16) suggests that the ability to profile gene expression in both compartments will be critical. Xenograft models of human cancer provide a system in which the genes in the two compartments are from different species: host (mouse) and graft (human). Typical oligonucleotide microarrays contain probes for profiling gene expression from only one species and as a consequence expression of both host and graft genes cannot be determined simultaneously (*see Note*¹). Although one could profile sequentially with two microarrays, one specific for human and one for mouse, this doubles the expense. More importantly, such dual-species analyses are likely to lead to misinterpretation as probes on single-species arrays are not designed to prevent binding of transcripts from other species but rather to profile transcripts of only one species. Therefore, cross-hybridization could be interpreted as a change in expression (*see Note*¹). To address this, in partnership with Affymetrix, we have designed and developed a dual species oligonucleotide microarray, the Hu/Mu ProtIn chip, to profile human and murine protease, protease inhibitor, and protease interactor genes. All of the probes on the custom Hu/Mu ProtIn chip were blasted against both human and mouse genome sequences to minimize cross-species hybridization (17). The ability of the Hu/Mu ProtIn chip to differentiate murine from human and human from murine gene expression and the specificity and sensitivity of the probe sets on this chip was determined based on comparative gene expression analyses in pure mouse or human samples and orthotopic xenograft models of human breast and lung cancers (Fig. 1, (17)). Thus, the Hu/Mu ProtIn chip, although

¹**Alternative methods:** Proteases, protease inhibitors, and protease interactors can be profiled in human samples, mouse samples, and samples containing both human and mouse genes by the sequential use of human genome chips and mouse genome chips available commercially. These single-species chips, however, are designed to profile transcripts of only one species and may misinterpret cross-hybridization as a change in expression. Costs will of course be double for analysis with two chips. Proteases and protease inhibitors, but not protease interactors, can be profiled in human samples, mouse samples, and samples containing both human and mouse genes with the hybrid version of the Clip-ChipTM, a spotted oligonucleotide array, which is available through collaboration with Overall and colleagues (26, 27). One advantage of this chip is that it contains probe sets for more proteases and inhibitors than on the Hu/Mu ProtIn chip. At this time, however, the hybrid Clip-ChipTM “is still under validation to determine cross-species hybridization between the oligonucleotides” (27). Thus, whether the Clip-ChipTM will prove suitable for analysis of xenografts is not yet known. In a dual-species array when proteases and protease inhibitors of the two species are known to be highly homologous (7, 28, 29), the use of shorter probes, e.g., the 25-mer probes used for the Hu/Mu ProtIn chip, is desirable as longer probes increase the probability for cross-hybridization. Sensitivity is higher with longer probes such as the 70-mer probes used in the Clip-ChipTM. When 25- and 60-mer probes were compared, the 25-mer probes were found to be 20-fold more specific and the 60-mer probes threefold more sensitive (30), indicating that the Clip-ChipTM will be less likely to distinguish expression of human and mouse genes. Depending on the design of individual experiments, either the Hu/Mu ProtIn or Clip-ChipTM array may be most appropriate or one may want to use the two arrays in tandem.

designed for interrogation of xenograft samples, also can determine expression of proteases, protease inhibitors, and protease interactors in single-species human and mouse samples (*see Note 1*). Furthermore, the Hu/Mu ProtIn chip has successfully identified proteases that function in proteolytic processes that protect against development of murine tumors of the intestine (18) and the lung (19). The former study was a single species analysis of mouse tissues, and the latter was a dual-species analysis of human tumor cells orthotopically implanted in mouse lung. In both, the Hu/Mu ProtIn chip proved an important tool for the identification of new tumor suppressive roles for proteases in cancer (20), i.e., roles that in the case of matrix metalloproteinases may explain the failure of the clinical trials using broad spectrum inhibitors of these enzymes (4).

Here we outline protocols for using the Hu/Mu ProtIn chip to profile proteases, protease inhibitors, and protease interactors in tissues and cells.

2. Materials

2.1. Sample Preparation and RNA Isolation

1. Tissue, fresh or snap-frozen.
2. Cultured cells grown as monolayers or in a 3D matrix.
3. RNALater reagent (Ambion, Austin, TX).
4. QIAshredder tissue homogenizer (Qiagen, Valencia, CA) or equivalent.
5. Dulbecco's phosphate buffered saline (PBS) without calcium or magnesium (Invitrogen, Carlsbad, CA).
6. Dispase (BD Biosciences, San Jose, CA).
7. Collagenase (Invitrogen, Carlsbad, CA).
8. TRIZOL™ (Invitrogen, Carlsbad, CA).
9. RNeasy Mini-Kits (Qiagen, Valencia, CA).
10. RNase free DNase set (Qiagen, Vaencia, CA).
11. Hu/Mu ProtIn chip: Protease520066F (Affymetrix, Santa Clara, CA).
12. XpressRef universal reference total RNA from mouse or human tissues (SuperArray Bioscience, Frederick, MD).

3. Methods

In general, microarray chips are processed and analyzed by an institutional genomics core facility. This is in part due to the fact that high-end, specialized instrumentation is needed that is beyond the research budget of most individual investigators. Core facilities may prefer to handle the entire protocol (from RNA isolation through chip processing) to ensure adequate quality control.

3.1. Sample Preparation and RNA Isolation

Total RNA can be isolated in one's laboratory or the samples may be given to a genomics core for isolation of RNA. If RNA is to be prepared in the laboratory, one must meet the minimum requirements of the genomics core in regard to RNA integrity and purity. Tissue samples or cultured cells may be fresh or frozen (snap frozen and stored at -80°C) as long as ample precaution has been exercised to protect RNA from degradation. The following are the protocols we have utilized successfully in our laboratory to provide the highest quality RNA to our genomics facility.

3.1.1. Isolation of RNA from Tissue Samples

1. Place all tissue samples, fresh or frozen, immediately in RNALater and store for at least 24 h at 4°C . To ensure rapid and sufficient diffusion of RNALater throughout the tissue, make sure that no tissue dimension exceeds 0.5 cm.
2. Centrifuge briefly at 300 g (2 min) to remove the preservative and freeze the sample at -80°C . Transfer tissue (50–100 mg) to a QIAshredder tissue homogenizer or equivalent and briefly centrifuge ($1,000 \times g$, 2 min) at room temperature to remove residual RNALater.
3. Remove the tissue from the QIAshredder and transfer to a nuclease-free microcentrifuge tube containing 1 mL of TRIZOL™ on ice.
4. Isolate total RNA from TRIZOL™ as recommended by the manufacturer.
5. Following isolation, further purify RNA with an RNeasy Mini-Kit.

3.1.2. Isolation of RNA from Monolayer Cell Cultures

1. Discard the media.
2. Wash the cells with Dulbecco's PBS for three times.
3. Aspirate the residual wash.
4. Remove the cells directly into 1 mL TRIZOL™ per 35-cm^2 dish, using a plastic cell scraper to aid in lysing the cells.
5. Collect cell lysates and incubate at room temperature for 10 min.
6. Store the lysates at -80°C until RNA isolation.
7. Follow **steps 4 and 5 in Subheading 3.1.1** to isolate RNA.

3.1.3. Isolation of RNA from 3D Cell Cultures—If cells are grown in a 3D extracellular matrix such as Matrigel (BD Bioscience, San Jose, CA), collagen I (BD Bioscience, San Jose, CA) or Cultrex (Trevigen, Gaithersburg, MD), the following procedure should be followed to remove the matrix.

1. Incubate 3D cultures with 1 U Dispase or 0.1% w/v collagenase (if the matrix is collagen) in Dulbecco's PBS at 37°C for approximately 20 min to release the cells.

2. Centrifuge briefly ($300 \times g$, 2 min) and wash the cells three times with Dulbecco's PBS.
3. Resuspend cell pellet in 1 mL TRIZOL™.
4. Follow **steps 5–7** in **Subheading 3.1.2** above to isolate RNA.

3.2. RNA Integrity and Yield

At this point the purity, integrity, and quantity of RNA needs to be determined by the genomics core facility. Please note that in most cases this is a requirement set by the facility because of the high cost of microarray processing and analysis. In general, any sample with an rRNA ratio of <1 (as determined by the core) is not suitable for processing.

3.3. RNA Processing, cRNA Production, and cRNA Labeling

Since the Hu/Mu ProtIn chip is based on an Affymetrix platform, the processing of RNA for hybridization, including cRNA synthesis and labeling, has to precisely follow standard protocols described in the Affymetrix GeneChip Expression Analysis Technical Manual. These are performed with platform-specific instrumentation available only through genomic cores.

3.4. Data Analysis

Analysis of the expression data obtained from the Hu/Mu ProtIn chip or any other microarray chip requires extensive bioinformatics expertise and sophisticated computer software. Again, as with chip processing, unless there is bioinformatics expertise in one's laboratory, data should be transmitted to a bioinformatics core facility for analysis. We have used Microarray Suite version 5 (MAS5, Affymetrix) (21), dChip (22), and trimmed-mean algorithm (TM) methods (23, 24), to characterize and validate the Hu/Mu ProtIn chip. Details of this extensive analysis are reported in **ref.** (17).

3.4.1. Calculations

1. In MAS5, set expression analysis parameters to default values (e.g., scale factor = 500, normalization = 1.0 and no masking).
2. Generate a p value for the likelihood of the presence of a transcript. The detection algorithm in MAS5 software utilizes a one-sided Wilcoxon's signed rank test of the discrimination value (R) relative to a user-defined threshold Tau (default = 0.015).
3. Calculate the Discrimination value by adjusting the intensity difference, PM (Perfect Match) – MM (Mismatch), to the overall hybridization intensity (PM + MM). A Discrimination value greater than Tau indicates the presence of the transcript and less than Tau for the absence of the transcript.
4. In dChip, calculate the frequency of a detection call. Make sure that 100% of the samples are present at the p value generated in **Step 2** (e.g., 0.05).

3.5. Validation of Probe Design

To compare arrays of different samples, the raw data from each array need to be normalized using the invariant set normalization algorithm within the dChip software. Gene expression values are calculated using the Model Based Expression Index (MBEI). For some experiments, normalization and MBEI can be performed with PM and MM probe information (dChip PM + MM). In some experiments, gene expression values may be calculated using a TM followed by quantile normalization to a standard array as previously reported (18). TM can be performed by removing either 20 (TM20) or 25 (TM25) percent of the PM-MM probe pair values within each probe set prior to calculating the gene expression value (17).

3.6. Verification of Expression

Although the Hu/Mu protIn chip has been extensively characterized and validated (17), any microarray expression data should be verified through other techniques. At the transcript level, the data can be validated with RT-PCR utilizing TaqMan primers (25). Ultimately, however, validation must be accomplished at the protein level through the use of tissue microarrays or tissue sections through immunohistochemical analysis.

Acknowledgments

This work was supported by a Department of Defense Breast Cancer Center of Excellence (DAMD17-02-1-0693).

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Fig. 1.

Validation of the Hu/Mu ProtIn chip: **(a)** Three independent replicates of XpressRef (SuperArray Bioscience) universal reference total RNA from mouse or human tissues were profiled with the Hu/Mu ProtIn microarray. The presence of a transcript was determined by the detection algorithm in MAS5 software (Affymetrix), which assigns P as a measure of the detection call confidence. The number of probe sets, giving present call P values smaller than 0.01, for all three replicates are indicated. In the presence of only mouse transcripts, there were extremely few present detection calls by human probe sets (*gray bars*), and vice versa (*black bars*), despite the high sequence similarity among protease and protease inhibitor genes and their potential for cross-hybridization. **(b)** Total RNA derived from spontaneous mammary carcinomas from MMTV-PyMT⁺ (FVB/n) ($n = 8$) and MMTV-PyMT⁺ /uPARAP^{-/-} (FVB/n) ($n = 2$) transgenic mice or human breast ductal carcinoma biopsies ($n = 18$) from women with stage II or III disease was profiled. The number of probe sets, giving present call P values smaller than 0.01, for 80% are indicated. Transcripts derived from mammary carcinoma were rarely detected by nonspecies identical probes (human, *gray bars*; mouse, *black bars*). In C, total RNA derived from normal mouse mammary fat pads ($n = 4$), MDA-MB-231 breast carcinoma cells ($n = 3$), and orthotopically implanted xenografts of MDA-MB-231 cells ($n = 3$) was profiled. Number of probe sets, giving present call P values smaller than 0.01, for all replicates in each group is indicated. Nonspecies-identical probes (*gray bars*, human and *black bars*, mouse) rarely detect transcripts in mouse mammary fat pads, and MDA-MB-231 cells. Importantly, transcripts from MDA-MB-231 orthotopic xenografts were detected by both mouse (*black bars*) and

human probes (*gray bars*), suggesting that both mouse (host) and human (tumor) transcripts present in the xenograft can be detected simultaneously by the Hu/Mu ProtIn microarray. Frequency represents the number of probe sets that satisfy the selection criterion. Adapted from **ref.** (17)