Highly Specific and Sensitive Nanoimmunoassay of PARP1: A Toolkit to Measure Cell Death

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ABSTRACT

We developed and validated a highly specific toolkit to analyze PARP1 using a panel of control and siRNA knockdown cell lysates by multiple immunoassays. We can specifically and quantitatively distinguish PARP1 from 17 other PARP family members using highly specific antibodies and optimized methods.

Poly (ADP-ribose) polymerase-1 (PARP1) is a chromatin associated, ADP-ribosylating enzyme essential for multiple cellular functions, including cardiac remodeling, vasoconstriction, regulation of astrocyte and microglial function, long term memory, aging, transcription regulation, and DNA repair. More recently, it has been implicated in a new form of cell death termed parthanatos. PARP1 can also promote tissue survival by shifting the balance of cell death programs between autophagy and necrosis. Since PARP1 can promote tumorigenicity, it has gained traction as a therapeutic target in cancer. In that regard, clinical studies have shown vulnerability to PARP inhibitors in DNA repair defective cancers. One of the difficulties in analyzing PARP1 activity is the promiscuity of the reagents toward other PARP family members. Furthermore, the ability to examine multiple tissue samples in parallel has been limited.

In this study we developed highly sensitive and specific antibodies against PARP1 fragments. Using siRNAs against 18 PARP family members, we validated antibody specificity by western blotting and nanoimmunoassay (NIA). NIA is a highly sensitive platform capable of screening up to 96 cancer samples at sub-microliter volume. Moreover, this platform permits simultaneous quantitative analysis of non-modified PARP1 as well as post-translational modifications such as PARylation and phosphorylation using a single antibody for the measurement of all species. We anticipate this workflow will be amenable to a wide range of protein targets, ushering in a new frontier in diagnostic analysis.

INTRODUCTION

PARPs are currently in the spotlight due to their potential as cancer drug targets. The therapeutic potential of PARP inhibition is based on the role of PARP proteins in DNA repair. Paradoxically to their role in DNA repair, which promotes cell survival, PARPs can also promote cell death in some contexts. Because inhibiting a death-promoting function counteracts the goal of chemotherapy, and also because PARP inhibition has been suggested to cause secondary cancers, the rationale for therapeutically targeting PARP has been called into question. Hence, there is urgent need to more thoroughly characterize PARP biology, to define the contexts in which PARP inhibition is desirable, and to identify biomarkers capable of predicting response to PARP inhibition so that non-responsive patients can be spared from detrimental toxicity. To facilitate rapid advances in our understanding of PARP biology, we are focused on the development of high-fidelity tools to modulate and measure specific PARP variants. Because off-target activity plagues the use of affinity reagents for investigating biological pathways, we have focused on developing optimized workflows for validation of affinity reagents.

Validated siRNA of all PARP-family members (Table 1) will serve as positive and negative control cell lysates in the production of tools specific to analyze PARP biology. Since target knockdown is reliably achieved within 48 – 72 h after siRNA transfection, siRNA proffers significant time savings over the heralded CRISPR/Cas9 genome editing technology. The resulting collection of validated siRNAs and corresponding cell lysates are key components of our toolkit for studying PARP biology. Highly specific multi-assay validated antibodies also represent a major tool that we are developing for studying PARP biology. We have developed a powerful antibody validation workflow based on the ProteinSimple Peggy platform. Peggy Sue™ is an nanoimmunoassay system designed for high-throughput separation and analysis of proteins in biological samples based on molecular weight (size) or isoelectric point (charge). When combined with validated siRNA, Peggy provides a powerful approach to validating antibody specificity.

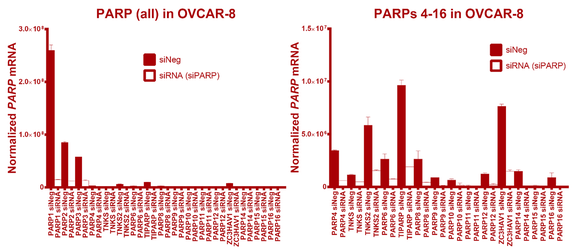
Using this platform and the validated tools, we will examine PARP protein expression and post-translational modifications in cancer cell lines following DNA damage and PARP inhibition. This work will provide the research community with a comprehensive panel of validated reagents and assays for incisive analysis of PARP family proteins and by extension the perturbation of cell death pathways induced by specific anti-cancer drugs.

PARP Family	MW (kDa)	Isoelectric Point (pI)
PARP1	113.1	8.99
PARP2	66.2	9.02
PARP3	60.1	6.28
PARP4	192.6	5.43
TNKS (PARP5a)	142.0	6.58
TNKS2 (PARP5b)	126.9	6.76
PARP6	71.1	8.60
TIPARP (PARP7)	76.2	6.19
PARP8	95.9	8.58
PARP9	96.3	8.12
PARP10	110.0	4.90
PARP11	38.7	7.62
PARP12	79.1	8.84
ZC3HAV1 (PARP13)	101.4	8.72
PARP14	202.8	6.81
PARP15	72.5	6.76
PARP16	36.4	9.17
PARG	111.1	6.03

Table 1: PARP family members with MW and Isoelectric point

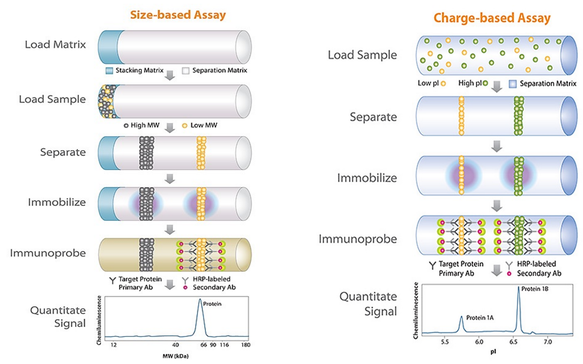
STRATEGY

Antibody Validation 1



1D Western Blot:
Validation against other PARP family members

Antibody Validation 2



NIA
Size based assay :
SDS and DTT denatured, sample separated by size

Charge-based assay:
Not denatured, native sample separated by charge

Antibody Validation 3

2D –Western Blot Analysis

Final Assay

human tumor lysates from a variety of tissues, organs and xenografts analysis

PARP Measurement in Clinical Samples

REFERENCES

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ANTI-PARP1 ANTIBODIES VALIDATION

One of the pitfalls of existing PARP1 antibodies is their cross reactivity to other PARP-family members. Here we show the specificity of newly generated anti-PARP1 antibodies using a panel of siRNA knockdown cell lysates specific for each PARP family member.

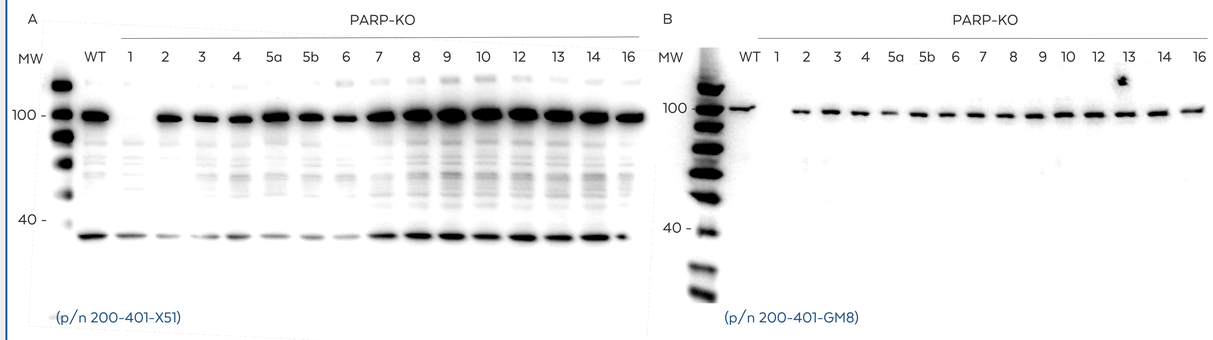

Figure 1: Western Blot analyses of OVCAR-8 lysates and PARP-KO Lysates using PARP1 antibodies.

(A) PARP1 antibody specific for the autocatalytic domain of PARP1, (B) PARP1 antibody specific for the zinc finger 1 domain of PARP1. Lysates loaded on the gel are as follows: WT: wild type; 1: PARP1-KO; 2: PARP2-KO; 3: PARP3-KO; 4: PARP4-KO; 5a: PARP5a-KO; 5b: PARP5b-KO; 6: PARP6-KO; 7: PARP7-KO; 8: PARP8-KO; 9: PARP9-KO; 10: PARP10-KO; 12: PARP12-KO; 13: PARP13-KO; 14: PARP14-KO; 16: PARP16-KO. Only the PARP1-KO lysate shows the absence of PARP1 demonstrating antibody specificities.

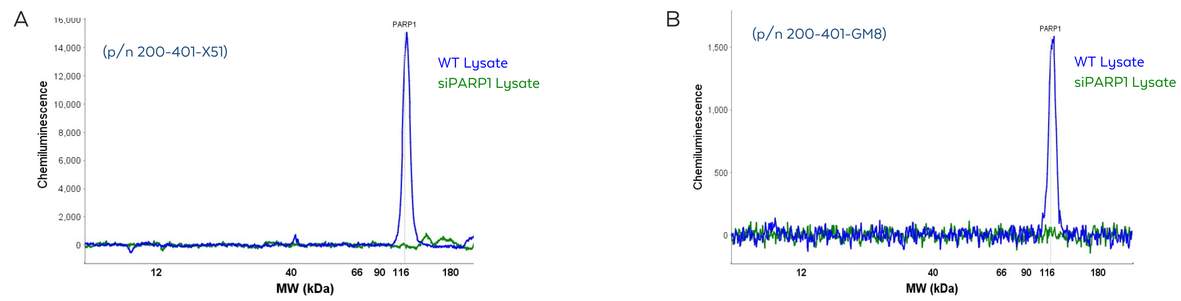

Figure 2: Gel electrophoresis, denaturing NIA.

WT (blue) and siPARP1 (green) OVCAR-8 lysates were analyzed using the PARP1 antibodies. (A) Detection using PARP1 antibody specific for the autocatalytic domain of PARP1, (B) PARP1 antibody specific for the zinc finger 1 domain of PARP1. In WT cells >94% of the signal is shown at a MW consistent with PARP1 (MW ~113 kDa). Specific peak reduction with siPARP1 confirms on-target antibody signal. Cells were lysed in RIPA buffer to result in a sample concentration of 500 µg/mL protein. Experimental parameters are as follows: Separation- 250 V for 45 min. ; UV immobilization- 250 s ; 1° antibody incubation- 180 min. ; 1° antibody concentration- 20 µg/mL (A) and 40 µg/mL (B) ; Exposure time: 2 s (A) and 6 s (B).

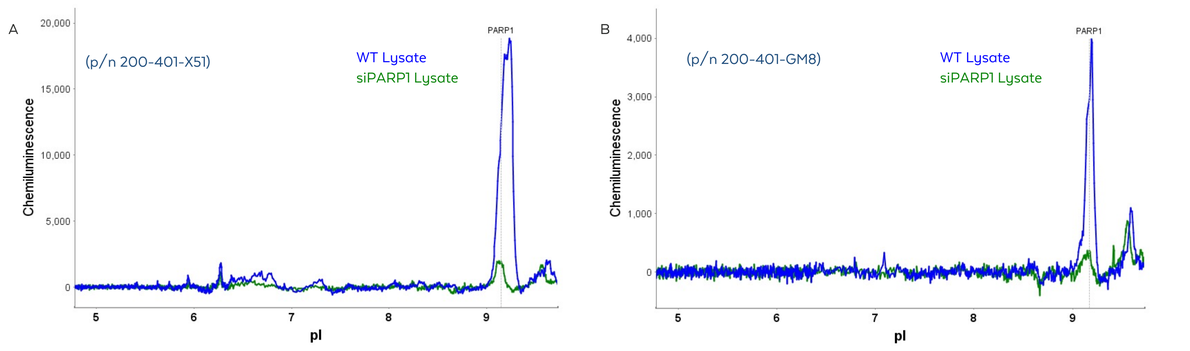

Figure 3: Isoelectric focusing, non-denaturing NIA.

WT (blue) and siPARP1 (green) OVCAR-8 lysates were analyzed using the PARP1 antibodies. (A) Detection using PARP1 antibody specific for the autocatalytic domain of PARP1, (B) PARP1 antibody specific for the zinc finger 1 domain of PARP1. In WT cells >90% of the signal is shown at a pI consistent with PARP1 (pI ~8.99). Specific peak reduction with siPARP1 confirms on-target antibody signal. Cells were lysed in no-salt buffer to result in a sample concentration of 250 µg/mL protein. Experimental parameters are as follows: Separation matrix- ProteinSimple G2 pH 3-10 + 0.5% TEMED + 10 mM NaCl ; Separation: 40,000 µW for 35 min.; UV immobilization- 120 s; 1° antibody incubation- 180 min.; 1° antibody concentration- 15 µg/mL (A) and 20 µg/mL (B); Exposure: 4 s (A) and 6 s (B).

ANTIBODY VALIDATION BY 2D ELECTROPHORESIS

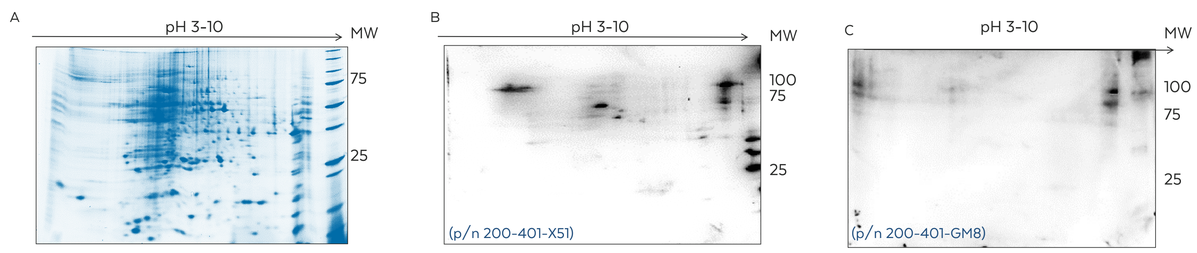

Figure 4: 2D Western blot analysis of anti-PARP1 antibodies against OVCAR-8 lysates.

Lysates were separated on 2D SDS-PAGE and blotted on PVDF to analyze immunocoverage of the best antibody. Antibodies were purified by Protein A and used to assay for coverage levels. (A) Oriole stained protein gel, (B) Detection using PARP1 antibody specific for the autocatalytic domain of PARP1, (C) PARP1 antibody specific for the zinc finger 1 domain of PARP1.

CONCLUSION AND FUTURE DEVELOPMENT

- Highly sensitive and specific antibodies against PARP1 have been developed and characterized and are available at Rockland Immunochemicals [PARP1 (zinc finger domain) - 200-401-GM8; PARP1 (autocatalytic domain) - 200-401-X51].
- Antibody specificity was validated by multiple assays. Validations were facilitated using siRNAs against 18 PARP family members in conjunction with western blotting and nanoimmunoassay (NIA).
- A robust NIA for PARP1 has been developed and is capable of screening up to 96 cancer samples at sub-microliter volume in both denatured and non-denatured formats, suggesting that the validated antibodies will be compatible with a broad range of applications.
- NIA and validated antibodies will be used to characterize PARP status in cancer cell lines and patient samples, including quantitative analysis of non-modified PARP1 and post-translational modifications such as PARylation and phosphorylation.
- This workflow will be tested with a wide range of protein targets to usher in a new frontier in diagnostic analysis.