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Oddziaływanie PARP1 z chromatyną jako mechanizm regulujący nabywanie tolerancji na bakteryjną endotoksynę przez ludzkie monocyty i makrofagi

PARP1-chromatin interaction as mechanism regulating the acquisition of tolerance to baterial endotoxin by human monocytes and macrophages

Praca doktorska

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Publikacje przeglądowe

1. **Pietrzak J**, Spickett CM, Płoszaj T, Virág L, Robaszkiewicz A. PARP1 promoter links cell cycle progression with adaptation to oxidative environment. Redox Biol. 2018 Sep;18:1-5. doi: 10.1016/j.redox.2018.05.017.
   IF_{2018}= 7.77, MNiSW= 40pkt

Publikacje doświadczalne

1. **Pietrzak J**, Płoszaj T, Pułaski Ł, Robaszkiewicz A. EP300-HDAC1-SWI/SNF functional unit defines transcription of some DNA repair enzymes during differentiation of human macrophages. Biochim Biophys Acta Gene Regul Mech. 2019 Feb;1862(2):198-208. doi: 10.1016/j.bbagrm.2018.10.019.
   IF_{2019}= 3.51, MNiSW= 40pkt
2. Sobczak M, **Pietrzak J**, Płoszaj T, Robaszkiewicz A. BRG1 Activates Proliferation and Transcription of Cell Cycle-Dependent Genes in Breast Cancer Cells. Cancers (Basel). 2020 Feb;12(2):349. doi: 10.3390/cancers12020349.
   IF_{2019}= 6.126, MNiSW= 140pkt (Według punktacji MNiSW z dnia 31 lipca 2019)
3. **Pietrzak J**, Gronkiewicz K, Robaszkiewicz A. PARP Traps Rescue the Pro-Inflammatory Response of Human Macrophages in the In Vitro Model of LPS-Induced Tolerance. Pharmaceuticals. 2021 Feb; *14*(2), 170; doi: 10.3390/ph14020170.
   IF_{2019}= 4.286, MNiSW= 100pkt (Według punktacji MNiSW z dnia 31 lipca 2019)

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Pozostały dorobek naukowy

1. Tempka D, Tokarz P, Chmielewska K, Kluska M, **Pietrzak J**, Rygielska Ż, Virág L, Robaszkiewicz A. Downregulation of PARP1 transcription by CDK4/6 inhibitors sensitizes human lung cancer cells to anticancer drug-induced death by impairing OGG1-dependent base excision repair. Redox Biol. 2018 May;15:316-326. doi: 10.1016/j.redox.2017.12.017.
   IF_{2018}= 7.77, MNiSW= 40pkt

Komunikaty zjazdowe

- **Pietrzak J**, Robaszkiewicz A: PARP-1 as a principal protein in immunotolerance inhibition in cells of myeloid origin, Young Scientists Conference on Molecular and Cell Biology 2019, Warsaw Poland, April 2019;
- **Pietrzak J**, Bryszewska M, Robaszkiewicz A: PARP-1 inhibitors modulate cytokine transcription in THP-1 model of acute monocytic leukemia, Kiev Ukraine (oral presentation), May 2018;
- Płoszaj T, **Pietrzak J**, Robaszkiewicz A: Bioinformatic ChIP-Seq-based approach to identify PARP1dependent genes in cancer cells, The 43rd FEBS CONGRESS PRAGUE 7-12/07/2018;
- Tempka D, Tokarz P, Chmielewska K, **Pietrzak J**, Robaszkiewicz A: By downregulating transcription of PARP1, CDK4/6 inhibitors sensitise human lung cancer cells to oxidative stress-induced DNA damage triggered by WP631 and etoposide, JOINT SFRRE-OCC MEETING 2017 At: Berlin.

Kursy naukowe

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Wstęp

Sepsa, zwana posocznicą, jest stanem zagrażającym życiu, spowodowanym dysfunkcją odpowiedzi immunologicznej gospodarza na infekcje. Wywołana jest m.in. przez monocyty i makrofagi należące do pierwszej linii obrony immunologicznej [1,2]. Według danych Narodowej Organizacji Zdrowia (WHO), tylko w 2017 roku u prawie 50 milionów pacjentów na całym świecie zdiagnozowano posocznicę, z czego 11 milionów przypadków zgłoszono jako śmiertelne, co stanowi 20% wszystkich zgonów [3]. Obecnie, powodzenie leczenia zależne jest od wczesnego rozpoznania oraz doboru właściwych środków przeciwdrobnoustrojowych. Jednak ostatnie dane pokazują, że stosowanie antybiotyków wiąże się z rozwojem antybiotykooporności, zaś antybiotyki o szerokim spektrum działania zwiększają ryzyko zgonu o 20% w przypadku leczenia niektórych szczepów bakterii [4]. Z tego względu poszukuje się alternatywnych metod leczenia pacjentów z sepsą, by między innymi obniżyć negatywne skutki obecnych terapii. Ostatnie lata badań rozwinęły nowe techniki leczenia, opierające się na przeciwciałach neutralizujących TNFα, białkach bakteriobójczych oraz czynnikach aktywujących płytki krwi. Prowadzone są również badania kliniczne wykorzystujące analogi lipidu A jako czynnika przeciw-endotoksynowego [5,6].

Odpowiedź immunologiczną w sepsie definiuje się jako: „nadmierną reakcję prozapalną na cząsteczki związane z patogenami, takimi jak endotoksyny (np. lipopolisacharyd (LPS)), oraz obniżony mechanizm przeciwzapalny". Wielokrotna stymulacja endotoksyną powoduje osłabienie reakcji prozapalnej, wywołując zjawisko opisywane jako „tolerancja immunologiczna" [7]. LPS po zawiązaniu się z receptorem TLR4 (Toll-like receptor 4) występującym na powierzchni makrofagów, aktywuje szlaki wewnątrzkomórkowe odpowiedzialne za wydzielanie cytokin i czynników prozapalnych, takich jak czynnik martwicy nowotworów α (TNFα) czy interleukiny: IL-1β, IL-6, IL-8 [8]. Stymulacja receptora TLR4 aktywuje ścieżkę sygnałową zależną od czynnika transkrypcyjnego NFκB, który na drodze fosforylacji i degradacji białka inhibitorowego κB (IκB) ulega translokacji do jądra komórkowego w postaci heterodimeru (p50-p65), aktywując transkrypcję [9]. Tak przetransportowany czynnik transkrypcyjny, często wymaga dodatkowych ko-faktorów, takich jak PARP1 (polimeraza poli-(ADP-rybozy)-1), która wraz z acetylotransferazą p300, promuje tworzenie kompleksu preinicjacyjnego, aktywując ekspresję genów w odpowiedzi na stymulację lipopolisacharydem [10].

Rola białka PARP1 w procesach biologicznych, została obecnie dość dobrze poznana. Jego aktywacja związana jest z różnego rodzaju zaburzeniami zapalnymi, w tym ze

wstrząsem septyczny, cukrzycą czy zaburzeniami neurodegeneracyjnymi takimi jak choroby Alzheimera i Parkinson [11]. Najnowsze badania naukowe donoszą, że wykorzystanie odpowiednich inhibitorów PARP1 można wykorzystać w leczeniu chorób immunologicznych. Jednym ze znanych inhibitorów, działających na zasadzie utrzymywania PARP1 związanego z nicią DNA jest Olaparib (Lynparza®, AZD-2281), wykorzystywany w leczeniu guzów z niedoborem BRCA1/2. Działa on w miejscu pęknięcia nici DNA zapobiegając naprawie uszkodzonej nici, jednocześnie stymulując pęknięcia dwuniciowe [12].

W poniższej pracy w komórkach, w których wywołano tolerancję na bakteryjną endotoksynę, podjęto próbę nowego wykorzystania „pułapkowania" PARP1 na chromatynie przy użyciu inhibitora Olaparib. Badania zakładały utrzymanie odpowiedzi prozapalnej zależnej od NFκB w komórkach prestymulowanych bakteryjną endotosyną, co może przyczynić się do zahamowania rozwoju sepsy.

Cel pracy

Celem niniejszej pracy było określenie udziału białka PARP1 oraz procesu poli(ADP-rybozylacji) w powstawaniu zjawiska immunotolerancji. Cel ten osiągnięto poprzez:

1. Ocenę poziomu ekspresji PARP1 na poziomie mRNA oraz białka w monocytach i makrofagach;
2. Określenie markerów powierzchniowych CD11b oraz CD14;
3. Określenie statusu proliferacji w monocytach i makrofagach oraz powiązanie go z występowaniem zjawiska tolerancji i poziomem PARP1 w badanych komórkach;
4. Określenie wpływu zmian epigenetycznych katalizowanych przez białka EP300, HDAC oraz BRG1 na poziom ekspresji PARP1 w monocytach i makrofagach, jak również wpływu tych białek na kontrolę cyklu komórkowego w silnie proliferujących liniach nowotworowych MDA-MB-231 i MCF7;
5. Określenie wpływu inhibicji białka PARP1 na zależną od szlaku NFκB odpowiedź prozapalną w stymulowanych bakteryjnym lipopolisacharydem makrofagach oraz linii nowotworowej THP1;
6. Określenie roli białek p50 i p65 w powstawaniu zjawiska tolerancji po stymulacji komórek lipopolisacharydem.

Niniejsze badania pozwolą na rozważenie inhibitorów PARP1 jako potencjalnych czynników zapobiegających rozwojowi sepsy, które utrzymują prozapalny fenotyp makrofagów.

Materiały i Metody

Materiał do badań stanowiły ludzkie monocyty oraz pierwotne linie nowotworowe THP1, MDA-MB-231 i MCF7. Monocyty izolowane były z kożuszka leukocytarnego zdrowych dawców, udostępnionego przez Centrum Krwiodawstwa w Łodzi. Monocyty różnicowane były w makrofagi za pomocą czynnika różnicującego GM-CSF przez 7 dni. W przypadku linii nowotworowej THP1, różnicowanie zostało przeprowadzone za pomocą octanu forbolu (PMA) przez 72h. Monocyty i niezróżnicowana linia THP1 utrzymywana była w hodowli zawiesinowej, natomiast po zróżnicowaniu komórki hodowane były w monowarstwie hodowli adherentnej.

Pierwszy etap badań określający poziom ekspresji białka PARP1, wpływu białek remodelujących histony: EP300, HDAC1 i BRG1, oraz zatrzymanie cyklu komórkowego w obecności inhibitora CDK4/6 (iCDK4/6 - PD0332991), rozpoczęto od izolacji całkowitego RNA oraz białka z monocytów i makrofagów. Następnie, wyizolowane RNA zostało przepisane na matrycę cDNA przy użyciu reakcji odwrotnej transkrypcji i wykorzystane do analizy ekspresji PARP1 za pomocą reakcji łańcuchowej polimerazy w czasie rzeczywistym (Real-Time PCR), która została znormalizowana do genów referencyjnych *ACTB* i *GAPDH*. W przypadku wyizolowanego białka, próby zostały wykorzystane w technice Western Blot, w celu określenia ekspresji PARP1 na poziomie białka. Jako kontrolę wewnętrzną wykorzystano histon H3. Fenotyp makrofagów został potwierdzony przez porównanie liczebności receptorów CD11b i CD14 na powierzchni świeżo wyizolowanych monocytów i makrofagów zróżnicowanych czynnikiem GM-CSF, przy użyciu skoniugowanych z PE-Cy7 przeciwciał anty-CD11b i anty-CD14 z analizą fluorescencji za pomocą cytometru przepływowego.

Postęp cyklu komórkowego został zbadany za pomocą cytometrii przepływowej poprzez pomiar zawartości DNA w utrwalonych monocytach i makrofagach wybarwionych jodkiem propidyny. W celu potwierdzenia podziałów komórkowych w makrofagach zbadano ich odpowiedź na obecność inhibitora CDK 4/6 (PD0332991; 1 μM) po 72 h. Związek ten blokuje wejście komórek w fazę S cyklu komórkowego. Dodatkowo, w celu potwierdzenia zdolności makrofagów do proliferacji, zmierzono fluorescencję wybarwionych jodkiem propidyny prób za pomocą czytnika wielomodowego przy długości fal 528/590 nm. W tym

przypadku fluorescencja odpowiadała zawartości DNA w próbach. Jako linie kontrolne potwierdzające rolę EP3001, HDAC1 oraz kompleksu SWI/SNF w kontroli cyklu komórkowego i utrzymania proliferacji wybrano ulegające szybkim podziałom mitotycznym linie komórkowe MDA-MB-231 oraz MCF7.

Do badania odpowiedzi prozapalanej na bakteryjną endotoksynę, w obecności inhibitora PARP1 - Olaparibu, wykorzystano ludzkie monocyty, makrofagi oraz linię nowotworową THP1, reprezentującą premonocyty. Wybrane linie charakteryzują się, silną odpowiedzią immunologiczną po potraktowaniu bakteryjnym lipopolisacharydem, jak również łatwością hodowli i sposobem różnicowania. W 7 dniu różnicowania makrofagi wstępnie traktowano inhibitorem PARP przez 1 godzinę, a następnie pierwszą dawką LPS w różnych stężeniach (0,01 ng/ml, 0,1 ng/ml, 1 ng/ml, 10 ng/ml) przez 24 godziny. Następnego dnia komórki traktowano drugą dawką LPS (10 ng/ml) przez 2 godziny. W przypadku linii nowotworowej THP1 stężenie pierwszej dawki endotoksyny wynosiło 50 ng/ml, zaś drugiej 10 ng/ml. Pierwsza dawka LPS została we wszystkich typach badanych komórek dobrana w taki sposób, aby wywołać tolerancję, czyli zablokować odpowiedź prozapalną przy kontakcie komórek z kolejną dawką endotoksyny. W przypadku linii THP1 konieczność zastosowania wyższej dawki podyktowany był mniejszą ekspresją receptorów powierzchniowych CD14 oraz TLR4 na powierzchni tych komórek. Wymienione białka tworzą kompleks sygnalizacyjny w odpowiedzi na stymulowanie lipopolisacharydem [13].

By rozróżnić możliwy wpływ zahamowania ADP-rybozylacji i pułapkowania PARP1 na chromatynie na rozwój tolerancji, pod uwagę zostały wzięte kolejne dwa inhibitory PARP, które różnią się potencjałem wiązania PARP1 z DNA - Niraparib (MK-4827) i Veliparib (ABT-888). Inhibitory PARP stosowano w następujących stężeniach: Olaparib (1 μM), MK-4827 i ABT-888 (0,5 μM, 2,5 μM). Jako wyznacznik odpowiedzi immunologicznej na stymulowanie LPS mierzono zmiany ekspresji *TNFα*. Produkt tego genu uważany jest za marker odpowiedzi zapalnej makrofagów, a więc także tolerancji na endotoksynę [14].

Efekt działania Olaparibu na rozwój zjawiska tolerancji został również sprawdzony na monocytach i zróżnicowanych komórkach nowotworowych THP1 w celu powiązania powstawania immunoparaliżu z ekspresją PARP1 oraz statusem proliferacji komórek. Schemat eksperymentów został wykonany zgodnie z procedurą opracowaną dla makrofagów i niezróżnicowanych THP1 (wyniki nie znalazły się w manuskryptach stanowiących podstawę rozprawy o nadanie stopnia doktora).

Obecność białek PARP1, p50 i p65 w promotorze *TNFα*, została sprawdzona wykorzystując immunoprecypitację chromatyny połączoną z oceną ilościowa wykonaną techniką real-time PCR (ChIP-qPCR). Badane miejsce zostało wybrane technikami bioinformatycznymi i stanowiło miejsce wiązana komponentów kompleksu NFκB - białek p50 oraz p65.

W zróżnicowanej linii THP1 testowano również interakcję PARP1 z chromatyną w obrębie promotora *TNFα*. Pod uwagę wzięto stopień acetylacji histonów katalizowany przez acetylotransferazę EP300. W tym celu wykonano analizy ChIP dla zmian acetylacji w pozycji H3K27, oraz obecności białek EP300 i PARP1 w promotorze *TNFα*. Do eksperymentów wykorzystano inhibitor EP300 w stężeniu 1μM, który dodawano do komórek 24h przed pierwszą stymulacją LPS-em. Wykonano również analizę zmian ekspresji *TNFα* techniką Real Time PCR w obecności lub braku iEP300 (wyniki te nie znalazły się w manuskryptach będących podstawą nadania stopnia doktora).

Wszystkie wyniki zostały porównane wykorzystując test t-Studenta gdy testowano różnicę między dwiema średnimi. Za istotne przyjęto różnicę dla wartości $p<0,05$. Jednokierunkową analizę wariancji (ANOVA) wykorzystano w celu porównania średnich pomiędzy kilkoma grupami testowymi. Do wszystkich wyników zostały policzone średnie ± odchylenie standardowe średniej (SEM).

Omówienie wyników

Pierwszy etap pracy obejmował optymalizację różnicowania monocytów w makrofagi za pomocą GM-CSF, która polegała na doborze odpowiedniego czasu różnicowania i stężenia czynnika różnicującego, aby zaobserwować zmiany morfologiczne w makrofagach. Umożliwiło to wyodrębnienie schematu hodowli komórek do dalszych eksperymentów (wyniki te nie znalazły się w manuskryptach stanowiących podstawę nadania stopnia doktora).

Następne etapy pracy obejmowały analizę ekspresji PARP1 w monocytach i makrofagach. Oznaczono markery powierzchniowe CD11b oraz CD14 w obydwu typach komórek w celu potwierdzenia pożądanego działania GM-CSF i właściwego doboru czasu różnicowania i dawek czynnika różnicującego. Przekształcenie monocytów pochodzących z krwi do makrofagów potwierdziło utratę receptora powierzchniowego CD14 przy jednoczesnym wzroście ekspresji receptora CD11b. Zmiana ta związana była ze znacznym

wzrostem mRNA i białka PARP1 w komórkach zdefiniowanych jako makrofagi. Dodatkowo, w związku ze zmianami ekspresji PARP1, zaobserwowano również zmiany w kontroli cyklu komórkowego. W przeciwieństwie do monocytów, które wykazały zatrzymanie w fazie G0, w makrofagach znaczna część populacji występowała w fazach S i G2. Aby potwierdzić proliferację makrofagów komórki potraktowano inhibitorem CDK4/6 w celu zatrzymania cyklu komórkowego. Spowodowało to spadek ekspresji PARP1 na poziome mRNA oraz białka [15].

Kolejnym krokiem w badaniach było sprawdzenie roli białek biorących udział w zmianach epigenetycznych na ekspresję PARP1. Potraktowanie komórek inhibitorami EP300 oraz HDAC1 przyczyniło się do powstania zmian w transkrypcji *PARP1*. Obecność iEP300 powodowało spadek (w makrofagach), zaś iHDAC1 wzrost poziomu mRNA PARP1 (w makrofagach i monocytach). Zastosowanie obu inhibitorów jednocześnie jednoznacznie wskazało dominujący wpływ aktywności EP300 nad HDAC prowadząc do zmniejszonej ekspresji PARP1 w makrofagach. Wskazuje to na istotną rolę acetylotransferazy w kontrolowaniu aktywności transkrypcyjnej w zróżnicowanych, proliferujących fagocytach [15].

Aby potwierdzić hipotezę, że białko BRG1 należące do rodziny białek SWI/SNF związane jest z relaksacją chromatyny w różnicujący makrofagach prowadząc do wzrostu transkrypcji PARP1, komórki potraktowano iSWI/SNF. Badanie pokazało, że użycie iSWI/SNF, podobnie jak w przypadku iEP300, wpływa na obniżenie ekspresji PARP1 w tych komórkach, przy jednoczesnym braku zmian ekspresji w monocytach. Brak zmian w ekspresji przy równoległym traktowaniu makrofagów iSWI/SNF oraz iEP300, sugeruje że zarówno acetylotransferaza jak i kompleks działają w tym samym szlaku regulacyjnym [15].

Zaangażowanie białek remodelujących chromatynę takich jak EP300 oraz BRG1 w kontrolę cyklu komórkowego i utrzymanie proliferacji komórek zostało dodatkowo potwierdzone na silnie proliferujących liniach komórkowych MDA-MB-231 oraz MCF7. Inhibicja tych białek na tym etapie badań również potwierdziła, że aktywność białka EP300 jest niezbędna do utrzymania statusu proliferacji [16].

Podczas stymulacji monocytów czynnikiem GM-CSF dochodzi do wzrostu transkrypcji PARP1 w następstwie aktywacji proliferacji komórek. Również kontrola ekspresji PARP1 przez acetylotransferazę EP300 oraz kompleks SWI/SNF związana jest z pobudzaniem monocytów do różnicowania. Zmiany w poziomie białka PARP1 biorącego

udział w m.in. naprawie DNA związane są z funkcją makrofagów jaką pełnią, m.in. eliminacją patogenów w organizmie na drodze fagocytozy, która naraża je na liczne czynniki takie jak reaktywne formy tlenu, azotu i chloru uszkadzające wewnątrzkomórkowe struktury.

Ponieważ przeprowadzone doświadczenia wskazały brak ekspresji PARP1 w monocytach, do dalszych badań nad tolerancją na endotoksynę wybrano makrofagi. Wprowadzono także drugą monocytarną linię komórek nowotworowych THP1 charakteryzującą się zarówno wysokim poziomem PARP1 jak i tempem proliferacji.

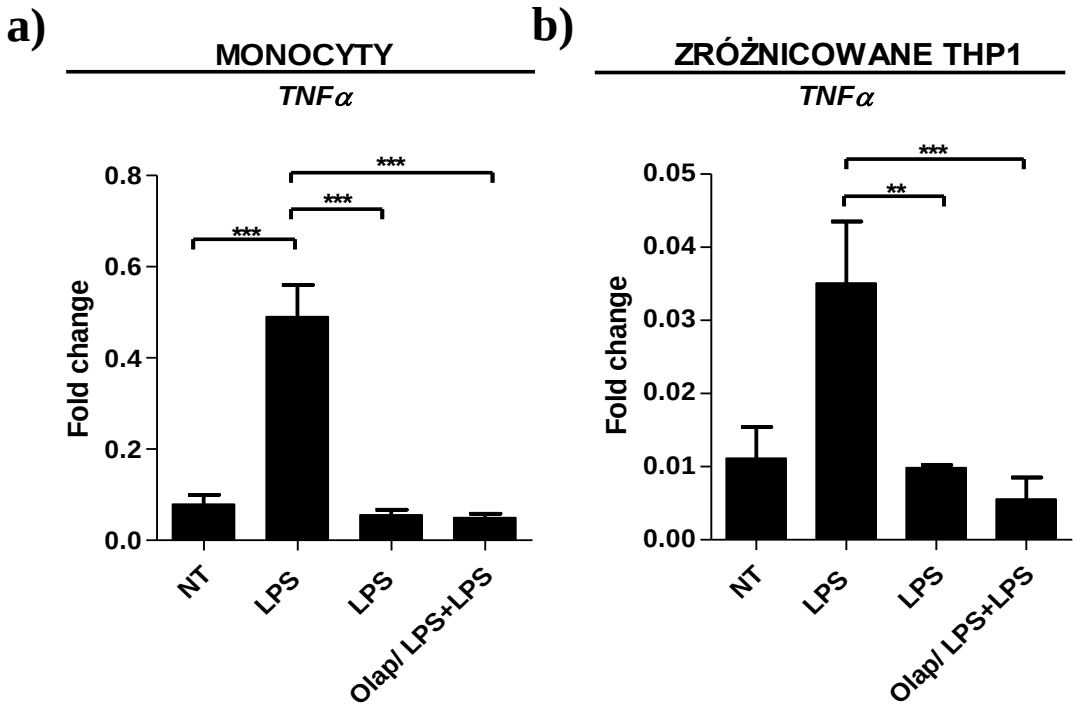
Pierwszy etap doświadczeń polegał na optymalizacji dawek lipopolisacharydu, które wywołają paraliż odpowiedzi prozapalnej fagocytów. Do tego celu komórki traktowano szeregiem wybranych dawek LPS, które pozostawiono na okres 24 h. Po tym czasie komórki potraktowano drugą wysoką dawką przez 2 h. Wyznacznikiem tolerancji była ekspresja TNFα (mRNA), która umożliwiła sprawdzenie poziomu odpowiedzi prozapalnej. Następnie wybrano dwa stężenia, które zasadniczo blokowały ekspresję TNFα, jednoznacznie wskazując rozwój tolerancji makrofagów po stymulacji endotoksyną. Dla tych stężeń został sprawdzony potencjał Olaparibu do hamowania rozwoju tolerancji. Związek ten hamuje aktywność PARP1 i tym samym ADP-rybozylację białek, ale także wiąże enzym z DNA. Komórki były traktowane Olaparibem przez 1 h przed indukcją tolerancji. Jako kontrole wykorzystano komórki nietraktowane oraz potraktowane jedną wysoką dawką LPS, aby sprawdzić czy makrofagi prawidłowo rozwijają odpowiedź immunologiczną po aktywacji endotoksyną. W badaniach tych do dalszych eksperymentów został wybrany jeden model traktowania LPS ze względu na niską zmienność danych pomiędzy niezależnymi eksperymentami [17].

W celu rozróżnienia wpływu rybozylacji ADP oraz pułapkowania PARP1 w makrofagach podczas rozwoju tolerancji, przetestowano dodatkowe dwa inhibitory PARP o różnym potencjale wiązania enzymu z DNA. Do badań wybrano Niraparib (MK-4827), który podobnie jak Olaparib ma znacząco wyższy potencjał wiązania PARP1 z chromatyną niż drugi wybrany inhibitor - Veliparib (ABT-174 888), który działa wyłącznie jako inhibitor PARylacji. Dla każdego z inhibitorów przetestowany został szereg stężeń, z których to zostały wybrane dwa do dalszej analizy. Eksperyment wykazał, że Niraparib podobnie jak Olaparib, zapobiega powstawaniu immunotolerancji, szczególnie znacząco przy wyższej dawce. Potraktowanie komórek takimi samymi dawkami Veliparibu nie wpłynęło na transkrypcję TNFα w komórkach z wywołaną tolerancją, co dostarczyło przesłanek o

wpływie interakcji białka PARP1 z chromatyną, nie zaś procesu ADP-rybozylacji, na paraliż makrofagów [17].

Jako drugi model do badań nad tolerancją immunologiczną została wybrana linia THP1. Stosowana jest ona jako model *in vitro* reprezentujący monocyty i makrofagi w chorobach zapalnych człowieka. Komórki zostały przetestowane pod kątem indukowania tolerancji w odpowiedzi na stymulację LPS oraz potencjalnego wpływu inhibicji PARP1 w blokowaniu powstawiania tolerancji. Wyniki wykazały, że komórki THP1, podobnie jak ludzkie makrofagi, rozwijają oporność na bakteryjną endotoksynę, co wiązało się ze znacznym spadkiem ekspresji TNFα. Potraktowanie komórek Olaparibem chroniło TNFα przed represją wywołaną przez LPS i utrzymywało wysoki poziom ekspresji tej cytotoksyny [17].

Jako kontrolę oceniającą swoistość działania inhibitorów PARP1 wykorzystano monocyty i zróżnicowane komórki nowotworowe THP1. W obu liniach charakteryzujących się brakiem proliferacji, a przez to również niskim poziomem PARP1, nie zaobserwowano efektu działania Olaparibu (Rys.1 a,b). Poprzednie badania zespołu [18] wykazały, że PARP1 tworzy kompleks z acetylotransferazą EP300 i kontroluje ekspresję genów w monocytach i makrofagach. W związku z tym, białko EP300 oraz acetylacja histonów została poddana dalszym rozważaniom jako potencjalne przyczyny braku odpowiedzi immunologicznej w stymulowanych lipopolisacharydem, zróżnicowanych komórkach THP1 (wyniki nie znalazły się w manuskryptach stanowiących podstawę nadania stopnia doktora).

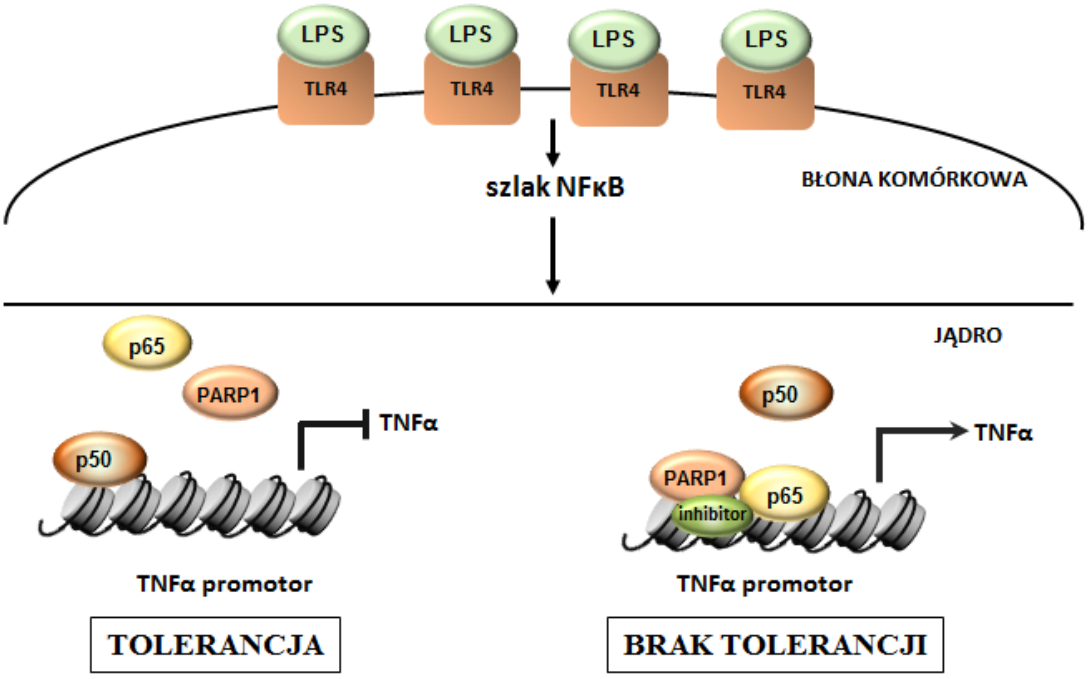


Rys.1. Wykresy przedstawiają brak efektu pułapkowania PARP1 na ekspresję TNFa w stymulowanych bakteryjną endotoksyną a) monocytach oraz b) zróżnicowanych komórkach THP1 charakteryzujących się niskim poziomem PARP1.

Wiedząc że PARP1 jest udokumentowanym współregulatorem transkrypcji, występowanie białka zostało sprawdzone na promotorze w komórkach nietraktowanych, tratowanych endotoksyną oraz w obecności Olaparibu w niezróżnicowanych THP1. Miejsce wiązania zostało wybrane na podstawie analizy bioinformatycznej i odpowiadało miejscu wiązania czynnika transkrypcyjnego NFκB. Wyniki ChIP-qPCR potwierdziły obecność PARP1 w promotorze *TNFα* oraz jego usunięcie po stymulacji LPS. Pułapkowanie za pomocą Olaparibu utrzymywało PARP1 w badanym fragmencie promotora *TNFα*. Potwierdziło to założenie, że odpowiednie utrzymanie obecności PARP1 w miejscu kontroli transkrypcji badanego genu zapobiega powstawaniu immunotolerancji w odpowiedzi na dawkę LPS [17].

Analiza ChIP-qPCR w promotorze *TNFα* wykazała natychmiastowe wiązanie podjednostki p65 przy jednoczesnym oddysocjowaniu p50 po stymulacji LPS-em. W komórkach, u których wywołano tolerancję, mechanizm był odwrotny - kolejna dawka LPS-u blokowała możliwość wiązania p65 oraz rekrutowanie p50 do badanej sekwencji promotora. Utrzymanie PAPR1 związanego z chromatyną za pośrednictwem Olaparibu przed pierwszą dawką LPSu utrzymywało stały, wysoki poziom wiązania p65 i blokowało oddziaływanie p50 z promotorem genu. Wyniki te sugerują, że usunięcie PARP1 z miejsca promotorowego

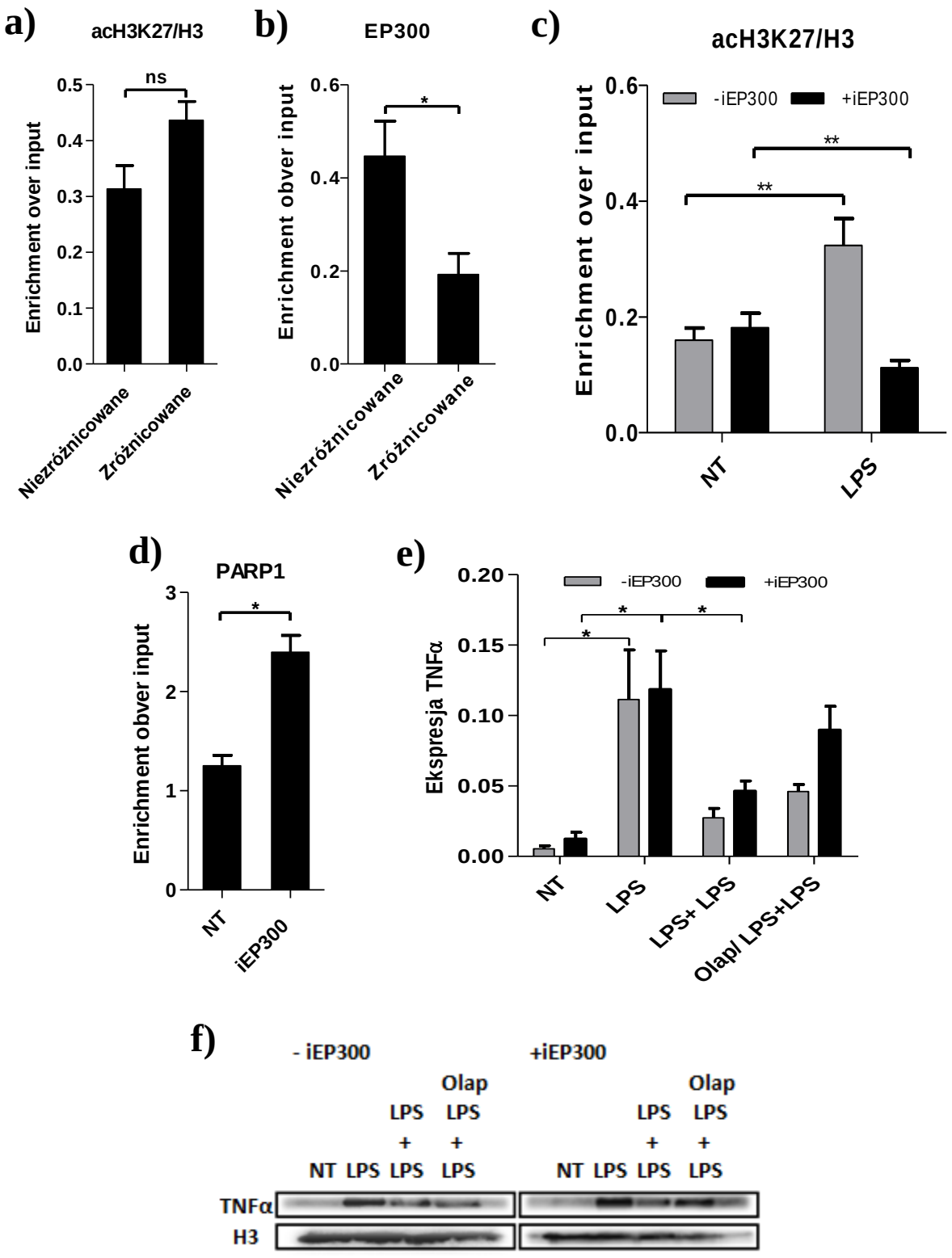
badanego genu przyczynia się do przebudowy chromatyny, zapobiegając zawiązaniu p65 i sprzyjając wiązaniu podjednostki p50, co przyczynia się do powstania tolerancji (Rys.2) [17].



Rys.2 Schemat obrazujący oddziaływanie białka PARP1 oraz komponentów czynnika transkrypcyjnego NFκB, białek p50 i p65 w powstawaniu tolerancji immunologicznej w komórkach stymulowanych lipopolisacharydem.

Na koniec, poszukiwano przyczyny braku ekspresji TNFα w zróżnicowanych THP1 w odpowiedzi na stymulowanie lipopolisacharydem. Pierwszym krokiem było określenia stopnia acetylacji lizyny histonu 3 w pozycji 27 (acH3K27) w trakcie różnicowania. Immunoprecypitacja chromatyny powiązana z oceną ilościową techniką real-time PCR wykazała wzrost acetylacji (Rys. 3a) i usunięcie białka EP300 z promotora TNFα w trakcie różnicowania komórek THP1 (Rys. 3b). Wskazuje to na działanie innych niż EP300 acetylotransferaz w remodeling-u chromatyny towarzyszącemu specyfikacji komórek. EP300 okazała się być kluczowa dla dalszej acetylacji promotora *TNFα* w komórkach zróżnicowanych, ale stymulowanych endotoksyną (Rys. 3c). Ten rodzaj zmiany epigenetycznej, związany jest z luźniejszą strukturą chromatyny i łatwiejszym dostępem czynników transkrypcyjnych, które aktywują ekspresję genów. Sprawdzono również czy dochodzi do funkcjonalnej interakcji pomiędzy białkiem PARP1 a EP300 w obrębie promotora *TNFα*. Eksperyment wykazał, że obecność inhibitora EP300 w zróżnicowanych THP1 zwiększa poziom białka PARP1 w obrębie badanej sekwencji (Rys. 3d). Wiedząc, że

usunięcie PARP1 z sekwencji promotorowej *TNFα* odpowiada za powstawanie tolerancji na bakteryjną endotoksynę, zatrzymanie enzymu na chromatynie za pomocą iEP300 sugerowało możliwą inhibicję zjawiska tolerancji. Ponieważ sama inhibicja EP300 nie była wystarczająca dla zahamowania paraliżu komórek wywołanego LPS-em, odpowiedź zróżnicowanych komórek THP1 testowano w obecności dwóch inhibitorów: iEP300 i Olaparibu. Jednoczesne ograniczenie aktywności acetylotransferazy i wiązanie PARP1 z chromatyną okazało się skutecznym sposobem na zatrzymanie negatywnego wpływu pierwszej dawki endotoksyny bakteryjnej na kolejne stymulowanie komórek zróżnicowanych lipopolisacharydem, co zostało potwierdzone na poziomie mRNA i białka (Rys. 3e,f). Wyniki te pokazują że PAPR1 jest białkiem zależnym od EP300 i aktywność acetylotransferazy w obrębie promotora *TNFα* jest ważnym czynnikiem wpływającym na dalszy przebieg występowania tolerancji w zróżnicowanych THP1. Jednakże, wiedząc że stymulacja lipopolisacharydem przyczynia się do usunięcia PARP1 z promotora, zahamowanie aktywności EP300 nie jest wystarczające aby zapobiec wystąpieniu tolerancji. Jednoczesne pułapkowanie PAPR1 za pomocą Olaparibu przy zahamowanej aktywności EP300 w promotorze *TNFα* pozwala na podtrzymanie ekspresji tego genu w komórkach, które mają przynajmniej dwukrotny kontakt z bakteryjną endotoksyną (wyniki nie znalazły się w manuskryptach będących podstawą nadania stopnia doktora).



Rys. 3. Wykresy przedstawiają zmiany w a) poziomie acetylacji H3K27 oraz b) obecności białka EP300 w trakcie różnicowanie THP1; c) rolę EP300 w acetylacji promotora *TNFα* podczas stymulacji endotoksyną oraz d) zmiany obecności PARP1 w promotrze *TNFα* w zróżnicowanych THP1; wpływ jednoczesnej inhibicji acetylotransferazy EP300 i białka PARP1 na powstawanie tolerancji na poziomie e) mRNA i f) białka w zróżnicowanych komórkach THP1.

Przeprowadzone badania wskazują nowe możliwości zastosowania inhibitora PARP1 - Olaparibu w blokowaniu rozwoju tolerancji na bakteryjną endotoksynę. Zwiększona ekspresja PARP1 w trakcie różnicowania monocytów może przyczyniać się do przygotowania makrofagów do pełnionej przez nie funkcji, zaś obecność LPS-u w środowisku stymuluje do zmian w kontroli szlaku zależnego od NF-κB. Inhibicja PARP1 poprzez utrzymanie wiązania z chromatyną w sekwencjach regulatorowych czynników odpowiedzi immunologicznej przyczynia się do zablokowania rozwoju immunotolerancji i jednocześnie utrzymania stałej ekspresji czynnika prozapalnego TNFα.

Wnioski

Niniejsza rozprawa doktorska pozwalana na sformułowanie następujących wniosków:

- Proces różnicowania monocytów do makrofagów przyczynia się do zwiększenia ekspresji PARP1 na poziomie mRNA i białka;
- Zwiększona ekspresja PARP1 w ludzkich makrofagach związana jest z ich statusem proliferacji;
- Kontrola ekspresji PARP1 w makrofagach jest zależna od acetylotransferazy EP300, deacetylazy HDAC1 oraz kompleksu SWI/SNF;
- Obecność PARP1 zawiązanego w sekwencji promotorowej *TNFα* pozwala na zapobiega postania zjawiska tolerancji immunologicznej;
- Powstawanie tolerancji związane jest z wiązaniem podjednostki p50 oraz blokowaniem wiązania podjednostki p65 czynnika transkrypcyjnego NFκB w sekwencji promotorowej *TNFα;*
- Acetylacja promotora *TNFα* spowodowana działaniem LPS-u odpowiada za niski poziom PARP1 na chromatynie i sprzyja rozwojowi tolerancji, podczas gdy jednoczesna inhibicja EP300 i pułapkowanie PARP1 na chromatynie zatrzymuje rozwój tolerancji w modelowej linii THP1 zróżnicowanej w makrofagi.

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Introduction

Sepsis is a life-threatening condition caused by a dysfunction of the host's immune response to infections caused by monocytes and macrophages, belonging to the first line of immune defense [1,2]. According to data from the National Health Organization (WHO), only in 2017, almost 50 million patients worldwide were diagnosed with sepsis, of which 11 million cases were reported as fatal, accounting for 20% of all deaths [3]. Currently, treatment success depends on early diagnosis and the selection of appropriate antimicrobials. However, recent data show that the use of antibiotics is associated with the development of antibiotic resistance, and broad-spectrum antibiotics increase the risk of death by 20% when treating selected bacterial strains [4]. Therefore, alternative treatments for patients with sepsis are being sought to reduce the negative effects of current therapies. Recent years of research have developed new treatment techniques based on antibodies neutralizing TNFα, bactericidal proteins and factors activating platelets, as well as clinical trials using analogues of lipid A as an anti-endotoxin factor [5,6].

The immune response in sepsis is defined as: "an excessive pro-inflammatory response to pathogen related molecules such as endotoxins eg. lipopolysaccharide (LPS), and a reduced anti-inflammatory mechanism". Repeated stimulation with endotoxin reduces the pro-inflammatory reaction, causing the phenomenon described as "immune tolerance" [7]. After binding to the TLR4 receptor (Toll-like receptor 4) on the surface of macrophages, LPS activates intracellular pathways responsible for the secretion of cytokines and pro-inflammatory factors, such as tumor necrosis factor α (TNFα) or interleukins: IL-1β, IL-6, IL-8 [8]. Persistent stimulation of the TLR4 receptor activates a signaling pathway dependent on the NFκB transcription factor, which, by phosphorylation and degradation of the κB inhibitor protein (IκB), is translocated into the nucleus as a heterodimer (p50-p65), where it activates transcription [9]. The transcription factor transported in this way often requires additional co-factors, such as PARP1 (poly (ADP-ribose) -1 polymerase), which, together with p300 acetyltransferase, promotes the formation of a pre-initiation complex, activating gene expression in response to stimulation with lipopolysaccharide [10].

The role of PARP1 protein in biological processes has now been recognized at a high level. Its activation is associated with various types of inflammatory disorders, including septic shock, diabetes and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases [11]. The latest scientific research reports that the use of appropriate PARP1 inhibitors can be used in the treatment of immune diseases. One of the known inhibitors that act by maintaining

PARP1 at the DNA strand is Olaparib, which is used to treat BRCA1/2-deficient tumors. It works at the site of DNA strand breakage, preventing the damaged strand from repairing, stimulating double strand breaks [12].

The following work attempts to novelize the use of PARP1 "trapping" on chromatin using an Olaparib inhibitor in cells that have been tolerated to bacterial endotoxin. The research aim was to maintain the NFκB-mediated pro-inflammatory response, which may help to inhibit the development of sepsis.

Aim of the study

The aim of this study was to determine the role of PARP1 protein and the poly (ADP-ribosylation) process in the development of the phenomenon of immunotolerance. This goal was achieved through:

1. Assessment of PARP1 expression level at the mRNA and protein level in monocytes and macrophages;

2. Determination of the CD11b and CD14 surface markers;

3. Determining the proliferation status in monocytes and macrophages and relation with the tolerance occurrence;

4. Determination of the influence of epigenetic changes mediated by EP300, HDAC and BRG1 proteins on the level of PARP1 expression in monocytes and macrophages, as well as the influence of these proteins on the control of the cell cycle in highly proliferating tumor lines MDA-MB-231 and MCF7;

5. Determination of the effect of inhibition of PARP1 protein on the NFκB pathway-dependent pro-inflammatory response in macrophages stimulated with bacterial lipopolysaccharide and in the THP1 tumor line;

6. Determination of the role of p50 and p65 proteins in the development of tolerance after stimulation of cells with lipopolysaccharide.

The present studies will consider PARP1 inhibitors as potential sepsis inhibitors that maintain the pro-inflammatory phenotype of macrophages.

Materials and methods

The experiments were performed with human monocytes and the primary tumor lines THP1, MDA-MB-231 and MCF7. Monocytes were isolated from buffy coat from healthy donors, provided by the Blood Donation Center in Lodz, and then differentiated using the GM-CSF differentiating factor for 7 days. In the case of the THP1 tumor line, differentiation was performed with phorbol acetate (PMA) for 72h. The monocytes and undifferentiated THP1 line were maintained in a suspension culture, while after differentiation, the cells were grown in a monolayer of adherent culture.

The first stage of the research, determining the level of PARP1 protein expression, the effect of histone remodeling proteins: EP300, HDAC1 and BRG1, and cell cycle arrest in the presence of the CDK4 / 6 inhibitor (iCDK4 / 6 - PD0332991), began with the isolation of total RNA and protein from monocytes and macrophages. Subsequently, the isolated RNA was transcribed into a cDNA template using reverse transcription reactions and used to analyze PARP1 expression by Real-Time PCR chain reaction, which had been normalized to the *ACTB* and *GAPDH* reference genes. In the case of the isolated protein, the samples were used for Western Blot to determine PARP1 expression at the protein level normalized to histone H3. Additionally, using a flow cytometer, the macrophage phenotype was confirmed by comparing the abundance of CD11b and CD14 receptors on the surface of freshly isolated monocytes and differentiated macrophages with GM-CSF, using PE-Cy7 conjugated with anti-CD11b and anti-CD14 antibodies.

The progress of the cell cycle was examined by flow cytometry by measuring the DNA content of fixed monocytes and macrophages, stained with propidium iodide. In order to confirm cell division in macrophages, their response to the presence of the CDK 4/6 inhibitor (PD0332991; 1 μM) was examined after 72 h, blocking their entry into the S phase of the cell cycle. Additionally, to confirm the ability of macrophages to proliferate, the fluorescence of propidium iodide stained samples was measured with a multimode reader at a wavelength of 528/590nm, which corresponded to the DNA content of the samples. The highly proliferating MDA-MB-231 and MCF7 cell lines were selected as control lines confirming the role of EP3001, HDAC1 and the SWI / SNF complex in the control of the cell cycle and maintenance of proliferation.

Human monocytes, macrophages and the THP1 tumor line representing pre-monocytes were used to study the pro-inflammatory response to bacterial endotoxin in the

presence of the PARP1 inhibitor- Olaparib. The selected lines are characterized by a strong immune response after treatment with bacterial lipopolysaccharide, as well as ease of cultivation and a method of differentiation. At 7^{th} day of differentiation, macrophages were pretreated with a PARP inhibitor for 1 hour followed by a first dose of LPS at various concentrations (0.01 ng / ml, 0.1 ng / ml, 1 ng / ml, 10 ng / ml) for 24 hours. The next day, cells were treated with a second dose of LPS (10 ng / ml) for 2 hours. In the case of the THP1 tumor line, the concentration of the first dose of endotoxin was 50 ng / ml, and the second dose was 10ng / ml. The first dose of LPS was selected in all types of cells tested to block the immune response after the second dose and induce tolerance. In the case of the THP1 line, usage of higher dose was dictated by the lower expression of the CD14 and TLR4 surface receptors, which form a signaling complex in response to stimulation with lipopolysaccharide [13].

To distinguish the possible effect of ADP-ribosylation and PARP inhibition on the development of tolerance, another two PARP inhibitors that differ in the potential of PARP1 binding to DNA - Niraparib (MK-4827) and Veliparib (ABT-888) were considered. PARP inhibitors were used at the following concentrations: Olaparib (1 μM), MK-4827 and ABT-888 (0.5 μM, 2.5 μM). Changes in TNFα expression were measured as a determinant of the immune response to LPS stimulation. The product of this gene is considered to be a marker of the inflammatory response of macrophages, and thus also of endotoxin tolerance [14].

The effect of Olaparib on the development of tolerance has also been tested in monocytes and differentiated THP1 tumor cells in order to link the induction of immunoparalysis with PARP1 expression and cell proliferation status. The design of the experiments was carried out according to the procedure developed for macrophages and undifferentiated THP1 (the results were not included in the manuscripts that are the basis for awarding the doctoral degree).

The presence of PARP1, p50 and p65 proteins in the *TNFα* promoter was checked using chromatin immunoprecipitation with quantification by real-time PCR (ChIP-qPCR). The test site was selected by bioinformatics techniques and was the binding site of the components of the NFκB complex - p50 and p65 proteins.

In the differential THP1 lineage, interaction of PARP1 with chromatin within the *TNFα* promoter was also tested, in dependence of the degree of histone acetylation catalyzed by EP300 acetyltransferase. For this purpose, ChIP analyzes were performed for changes in

acetylation at position H3K27, and for the presence of EP300 and PARP1 proteins in the *TNFα* promoter. In the experiments, the EP300 inhibitor in a concentration of 1 μM was used, which was added to the cells 24 hours before the first LPS stimulation. The analysis of changes in TNFα expression using the Real Time PCR technique in the presence or absence of iEP300 in the experimental setting for tolerance was also performed, as in the case of undifferentiated THP1 (the results were not included in the manuscripts that are the basis for awarding the doctoral degree).

All results were compared using a Student's t-test between two means for p values <0.05, and a one-way analysis of variance (ANOVA) was used to compare the means of several groups. The mean ± standard deviation of the mean (SEM) was calculated for all results.

Results and discussion

The first stage of the work involved the optimization of monocyte to macrophage differentiation with GM-CSF, which consisted in selecting the appropriate differentiation time and differentiating factor concentration to observe morphological changes in macrophages. This made it possible to extract the cell culture pattern for further experiments (results not were included in the manuscripts covered by the basis for awarding the title of doctor).

The next steps of the work included the analysis of PARP1 expression in monocytes and macrophages. For this purpose, the surface markers CD11b and CD14 were marked. Differentiation of blood-derived monocytes into macrophages confirmed the loss of the CD14 surface receptor, while the expression of the CD11b receptor was increased. This change was associated with a significant increase in PARP1 mRNA and protein in cells defined as macrophages. In addition, changes in the control of the cell cycle were also observed due to changes in PARP1 expression. In contrast to monocytes that showing G0 arrest, macrophages had a fraction of the population in the S and G2 phases. To confirm the macrophage proliferation status, cells were treated with a CDK4/6 inhibitor to arrest in the cell cycle. Such inhibition was associated with a decrease in PARP1 expression at the mRNA and protein levels [15].

The next step in the research was to check the role of proteins involved in epigenetic changes in PARP1 expression. Treatment of cells with EP300 and HDAC1 inhibitors contributed the changes in PARP1 transcription. The presence of iEP300 modulated the

decrease (in macrophages), and iHDAC1 the increase (in macrophages and monocytes) in the mRNA level of the *PARP1* gene. The simultaneous use of both inhibitors clearly indicated the dominant effect of EP300 activity over HDAC leading to decreased PARP1 expression in macrophages, indicating the importance of acetyltransferase in controlling transcriptional activity in differentiated proliferating phagocytes [15].

To support the hypothesis that the BRG1 protein belonging to the SWI / SNF family, is involved in chromatin relaxation in differentiating macrophages leading to increased PARP1 transcription, cells were treated with iSWI/SNF. The study showed that the use of iSWI/SNF, as in the case of iEP300, reduces the expression of PARP1 in these cells, while not changing the expression in monocytes. The lack of changes in expression with the parallel treatment of iSWI/SNF and iEP300 macrophages suggests that both the acetyltransferase and the complex act in the same regulatory pathway [15].

The involvement of chromatin remodeling proteins such as EP300 and BRG1 in the control of the cell cycle and maintenance of cell proliferation was further confirmed in the highly proliferating cell lines MDA-MB-231 and MCF7. Inhibition of these proteins at this stage of the study also confirmed that the activity of EP300 protein is necessary to maintain the proliferation status [16].

When monocytes are stimulated with GM-CSF, PARP1 transcription increases as a result of activation of cell proliferation. The control of PARP1 expression by EP300 acetyltransferase and the SWI/SNF complex is also related to the stimulation of monocytes to differentiate. Changes in the level of PARP1 protein involved in e.g. DNA repair is related to the function of macrophages, including elimination of pathogens in the body by phagocytosis, which exposes them to numerous factors such as reactive forms of oxygen, nitrogen and chlorine, damaging intracellular structures.

Since the conducted experiments showed how PAPR1 expression is controlled during the differentiation of monocytes into macrophages, macrophages were selected for further studies on endotoxin tolerance. A second monocytic THP1 tumor cell line was also introduced, characterized by both a high PARP1 level and a proliferation rate.

The first stage of the experiments consisted in the optimization of lipopolysaccharide doses which would induce paralysis of the pro-inflammatory response of phagocytes. For this purpose, cells were treated with a series of selected doses of LPS which were left for 24h, after time with a second high dose for 2h. The determinant of tolerance was TNFα (mRNA)

expression to test the level of the pro-inflammatory response. Two concentrations were then selected that essentially blocked TNFα expression, clearly indicating the development of macrophage tolerance upon endotoxin stimulation. For these concentrations, the potential of Olaparib to inhibit PARP1 activity by blocking protein ADP-ribosylation, but also binding the enzyme to DNA, was tested. Cells were treated with Olaparib for 1h before induction of tolerance. Untreated cells and cells treated with one high dose of LPS were used as controls to check that the macrophages properly developed an immune response upon endotoxin activation. In these studies, one LPS treatment model was chosen for further experiments, due to the low variability of the data between independent experiments [17].

To distinguish the effects between the ADP-ribosylation and the PARP1 trapping in macrophages during tolerance development, two additional PARP inhibitors with different enzyme-DNA binding potential were tested. Niraparib (MK-4827), which, like Olaparib, has a significantly higher PARP1-chromatin binding potential than the second selected inhibitor - Veliparib (ABT-174 888), which acts solely as a PARylation inhibitor. A number of concentrations were tested for each inhibitor, two of which were selected for further analysis. The experiment showed that Niraparib, like Olaparib, prevents the development of immunotolerance, especially significantly at the higher dose. At the same concentrations, Veliparib treatment of cells did not affect TNFα transcription in tolerant cells, providing evidence that PARP1 protein-chromatin interaction, rather than ADP-ribosylation, influences macrophage paralysis.[17]

The THP1 line, used as an *in vitro* model representing monocytes and macrophages in human inflammatory diseases, was selected as the second model for studies on immune tolerance. Cells were tested for tolerance induction in response to LPS stimulation and the potential effect of PARP1 inhibition in blocking tolerance development. The results showed that THP1 cells, like human macrophages, developed resistance to bacterial endotoxin, which was associated with a significant decrease in TNFα expression. Treatment of cells with Olaparib protected TNFα from LPS-induced repression and maintained high expression levels [17].

Monocytes and differentiated THP1 tumor cells were used as controls to evaluate the specificity of the action of PARP1 inhibitors. Those cell lines are characterized as non-proliferating cells and no effect of Olaparib was observed in both lines (Fig. 1 a, b). In the case of monocytes, this was associated with low PARP1 expression. Knowing from previous research by the team [18] that PARP1 forms a complex with EP300 acetyltransferase to

control gene expression in monocytes and macrophages, EP300 protein and histone acetylation were further considered as potential causes of immune failure in lipopolysaccharide stimulated differentiated THP1 cells (results not were included in the manuscripts covered by the basis for awarding the title of doctor).

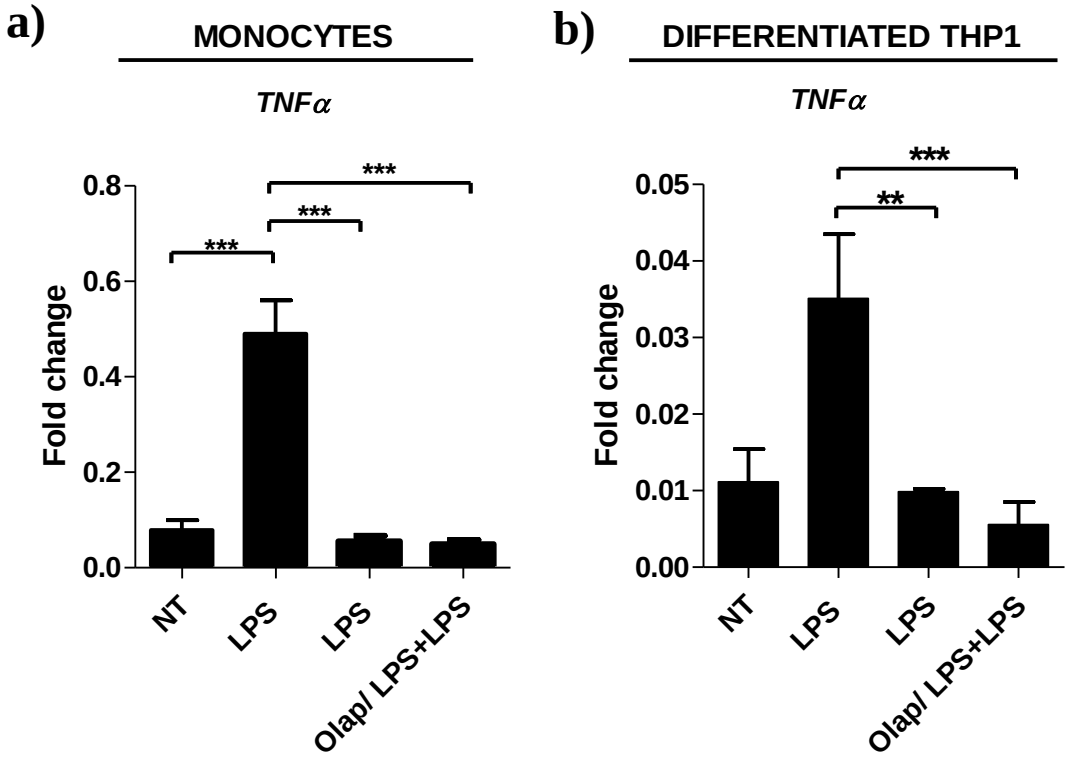

Figure 1. The graphs show no effect of PARP1 trapping after bacterial endotoxin stimulation in a) monocytes and b) differentiated THP1 cells characterized by low PARP1 levels.

Then, knowing that PARP1 is a documented co-regulator of transcription, the presence of the protein was checked on the promoter in untreated, endotoxin-treated cells and in the presence of Olaparib, in undifferentiated THP1. The binding site was selected on the basis of bioinformatics analysis and corresponded to the binding site of the NFκB transcription factor. The ChIP-qPCR results confirmed the presence of PARP1 in the *TNFα* promoter and its removal after LPS stimulation. Trapping with Olaparib maintained PARP1 in the *TNFα* promoter fragment tested. This confirmed the assumption that adequate maintenance of PARP1 presence in the transcription control site of the studied gene prevents the development of immunotolerance in response to the LPS dose [17].

ChIP analysis in the *TNFα* promoter showed immediate binding of p65 subunit while p50 eviction upon LPS stimulation. In tolerant cells, the mechanism was opposite - the next dose of LPS blocked the possibility of p65 binding and the recruitment of p50 to the promoter

sequence under study. The maintenance of Olaparib-mediated chromatin bound PAPR1 maintained a constant high level of p65 binding and p50 site blocking. These results suggest that removal of PARP1 from the promoter site of the studied gene contributes to chromatin remodeling, preventing p65 binding and promotes p50 binding, which contributes to tolerance (Fig. 2) [17].

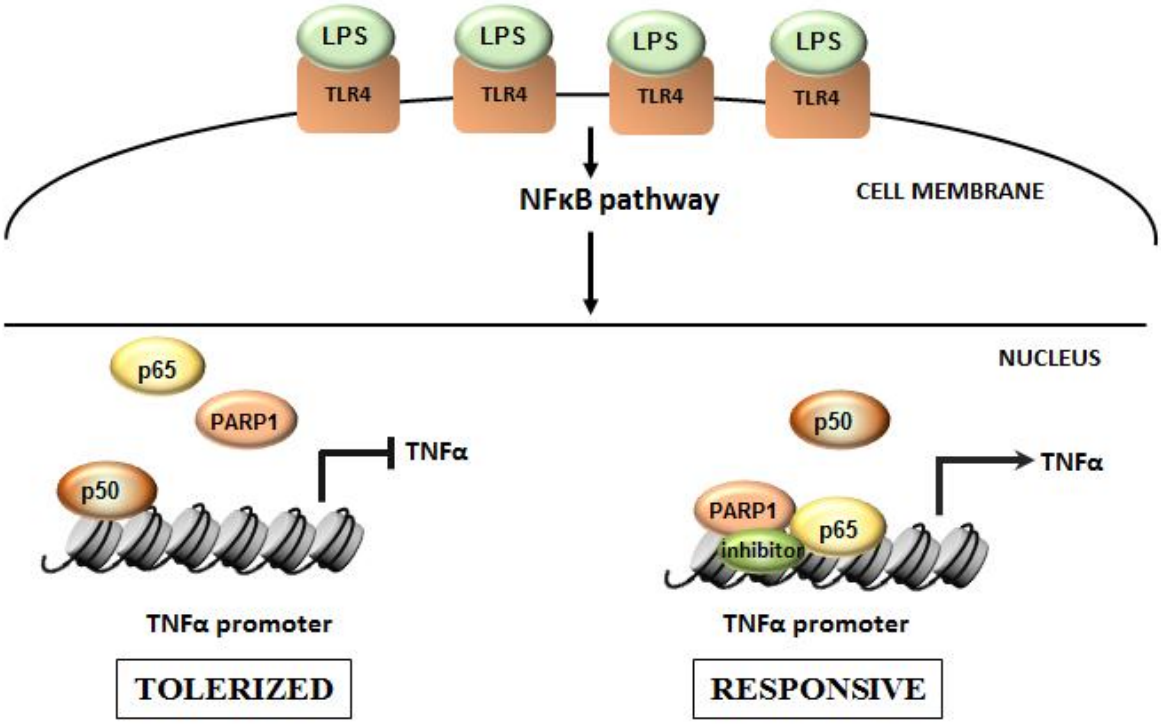

Figure 2. The figure shows the interaction of PARP1 protein and components of the NFκB transcription factor-p50 and p65 proteins in the development of immune tolerance in cells stimulated with lipopolysaccharide.

Finally, the reasons for the lack of TNFα expression in differentiated THP1 in response to lipopolysaccharide stimulation were investigated. The first step was to determine the degree of histone 3 lysine acetylation at position 27 (acH3K27) during differentiation. Chromatin immunoprecipitation associated with quantification by real-time PCR showed an increase in acetylation (Fig. 3a) and removal of EP300 protein from the *TNFα* promoter during THP1 cell differentiation (Fig. 3b). This indicates the effect of non-EP300 acetyltransferases in the chromatin remodeling associated with cell specification. EP300 turned out to be crucial for further acetylation of the *TNFα* promoter in cells differentiated but stimulated with endotoxin (Fig. 3c). This type of epigenetic change is related to a relaxed chromatin structure and easier access of transcription factors to activate gene expression. It was also checked the functional interaction between PARP1 and EP300 proteins. The experiment showed that the presence of an EP300 inhibitor in differentiated THP1 increases the level of PARP1 protein within the

studied sequence (Fig. 3d). Knowing that removal of PARP1 from the *TNFα* promoter sequence is responsible for tolerance to bacterial endotoxin, retention of the enzyme on chromatin with iEP300 suggested a possible inhibition of the tolerance phenomenon. Since inhibition of EP300 alone was not sufficient to inhibit LPS-induced cell paralysis, the differentiated THP1 cell response was tested in the presence of two inhibitors: iEP300 and Olaparib. Simultaneous limitation of acetyltransferase activity and binding of PARP1 to chromatin turned out to be an effective method to stop the negative influence of the first dose of bacterial endotoxin on subsequent stimulation of cells differentiated with lipopolysaccharide on mRNA and protein level (Fig. 3e,f). These results show that PAPR1 is an EP300-dependent protein and the maintenance of acetyltransferase within the *TNFα* promoter is an important factor influencing the subsequent development of tolerance in differentiated THP1. However, knowing that lipopolysaccharide stimulation contributes to the removal of PARP1 from the promoter, inhibition of EP300 activity is not sufficient to prevent tolerance from occurring. Simultaneous trapping of PAPR1 with Olaparib in the inhibited activity of EP300 in the *TNFα* promoter allows the gene expression to be restored (results not were included in the manuscripts covered by the basis for awarding the title of doctor).

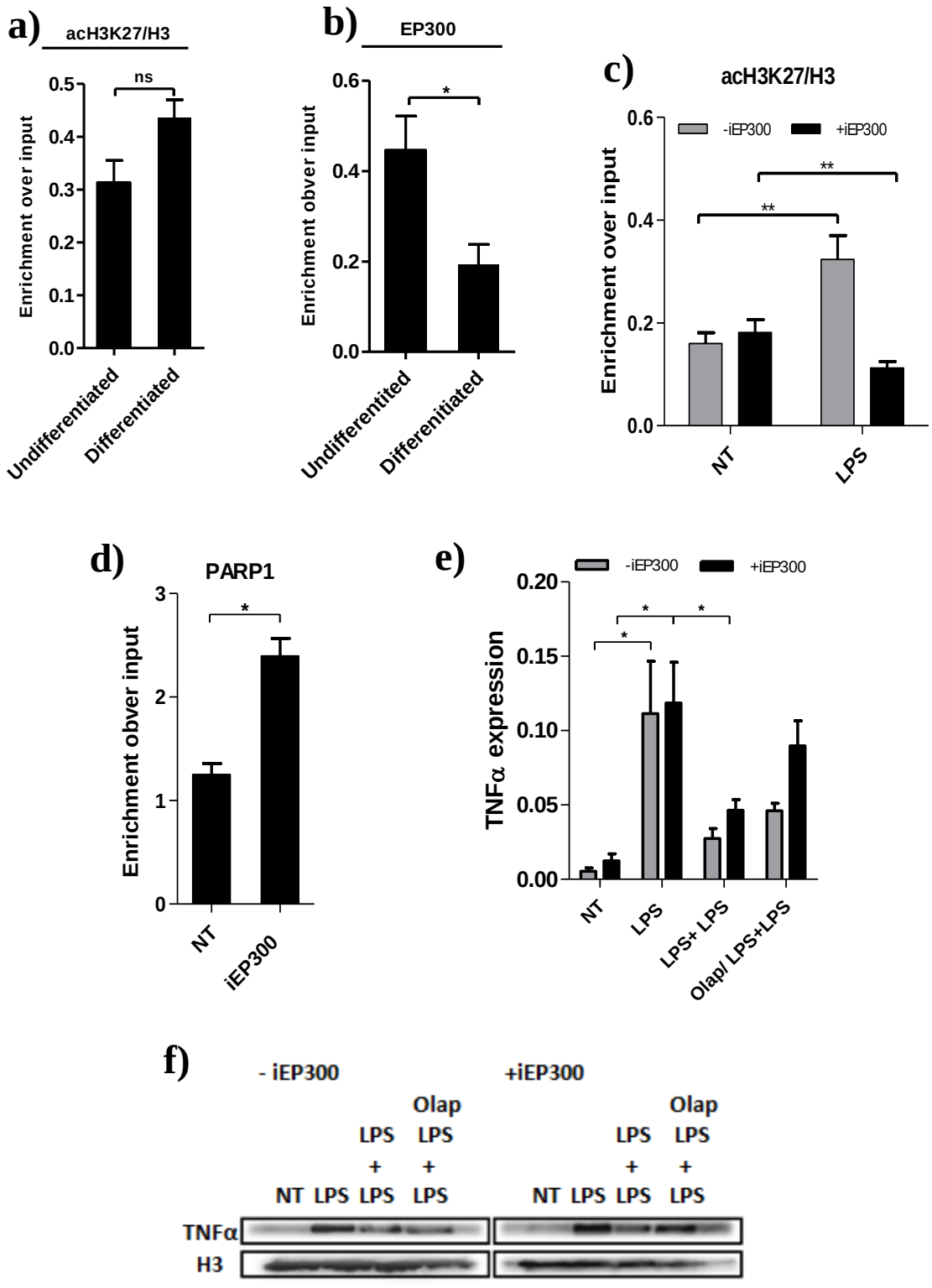

Figure 3. Graphs show changes in a) level of H3K27 acetylation and b) presence of EP300 protein during THP1 differentiation; c) the role of EP300 in acetylation of the *TNFα* promoter during endotoxin stimulation and d) alteration of the presence of PARP1 in the *TNFα* promoter in differentiated THP1; the effect of the simultaneous inhibition of EP300 acetyltransferase and PARP1 protein on the development of tolerance in differentiated THP1 cells on e) mRNA and f) protein levels.

The conducted studies indicate new possibilities using of the Olaparib- PARP1 inhibitor in blocking the development of tolerance to bacterial endotoxin. Increased PARP1 expression during monocyte differentiation contributes to the preparation of macrophages for their function, and the presence of LPS in the environment stimulates changes in the control of the NFκB-dependent pathway. The inhibition of PARP1 by maintaining the binding to chromatin in the regulatory sequences of the immune response factors contributes to blocking the development of immunotolerance and at the same time maintaining the constant expression of the pro-inflammatory factor TNFα.

Conclusions

This doctoral dissertation allows for the following conclusions:

- The process of differentiation of monocytes into macrophages contributes to the increase of PARP1 expression at the mRNA and protein level;
- Increased PARP1 expression in human macrophages is related to their proliferation status;
- Control of PARP1 expression in macrophages is dependent on EP300 acetyltransferase, HDAC1 deacetylase and SWI / SNF complex;
- The presence of PARP1 bounded to the *TNFα* promoter sequence prevents the occurrence of the immune tolerance phenomenon;
- The development of tolerance is related to the binding of the p50 subunit and the blocking of the binding of the p65 subunit of the NFκB transcription factor in the *TNFα* promoter sequence;
- Acetylation of the *TNFα* promoter by LPS action is responsible for the low level of PARP1 on chromatin and promotes the development of tolerance, while the simultaneous inhibition of EP300 and entrapment of PARP1 on chromatin stops the development of tolerance in the macrophage-differentiated model THP1 lineage.

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Review article

PARP1 promoter links cell cycle progression with adaptation to oxidative environment

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ABSTRACT

Although electrophiles are considered as detrimental to cells, accumulating recent evidence indicates that proliferating non-cancerous and particularly cancerous cells utilize these agents for pro-survival and cell cycle promoting signaling. Hence, the redox shift to mild oxidant release must be balanced by multiple defense mechanisms. Our latest findings demonstrate that cell cycle progression, which dictates oxidant level in stress-free conditions, determines *PARP1* transcription. Growth modulating factors regulate CDK4/6-RBs-E2Fs axis. In cells arrested in G1 and G0, RB1-E2F1 and RBL2-E2F4 dimers recruit chromatin remodelers such as HDAC1, SWI/SNF and PRC2 to condense chromatin and turn off transcription. Release of retinoblastoma-based repressive complexes from E2F-dependent gene promoters in response to cell transition to S phase enables transcription of *PARP1*. This enzyme contributes to repair of oxidative DNA damage by supporting several strand break repair pathways and nucleotide or base excision repair pathways, as well as acting as a co-activator of transcription factors such as NRF2 and HIF1a, which control expression of antioxidant enzymes involved in removal of electrophiles and secondary metabolites. Furthermore, PARP1 is indispensible for transcription of the pro-survival kinases MAP2K6, ERK1/2 and AKT1, and for maintaining MAPK activity by suppressing transcription of the MAPK inhibitor, MPK1. In summary, cell cycle controlled *PARP1* transcription helps cells to adapt to a pro-oxidant redox shift.

1. Pro-oxidant physiology of proliferating cells

Human cells proliferate in a variety of contexts. Controlled cell divisions play a particular function for development of the embryo, while in the adult organism mainly stem and some immune cells retain the ability to proliferate. Cancer cells, as a special type of transformed cells, are capable of unlimited and uncontrolled growth. Regardless of the type of dividing cell, proliferation imposes a requirement for energy and reducing power. Although mitochondrial oxidative phosphorylation is the most efficient source of ATP, it can cause extensive release of $O_2^{\bullet-}$, which is dismutated to H_2O_2 either in mitochondria (by SOD2) or in the cytoplasm (by SOD1). Therefore, above some critical threshold value of this oxidant in cell compartments, aerobic glycolysis becomes more favorable than oxidative phosphorylation in order to limit the hazardous waste products resulting from the mitochondrial metabolic pathway [1]. During fatty acid oxidation, $O_2^{\bullet-}$ and H_2O_2 can also be produced by xanthine oxidase in peroxisomes, which duplicate and are segregated between progeny cells. Although metabolically unrelated, NADPH oxidases act as a primary source of oxidants in macrophages and some cancer cells [2].

Depending on the cell type, proliferation-inducing agents such as growth factors (platelet-derived, fibroblast, epidermal, insulin-like and transforming growth factor β), cytokines (type I interferons, granulocyte-macrophage colony-stimulating factor), mutant K-ras or small GTPase Rac-1 elevate intracellular $O_2^{\bullet-}$ through NADPH oxidase and/or mitochondria [3,4]. Due to pressure induced by an elevated and sustained redox shift to a mild oxidative environment, cells have developed efficient mechanisms of adaptation and functional transformation of „bad" to „good" molecules, which promote cell proliferation and survival at different signaling levels [5].

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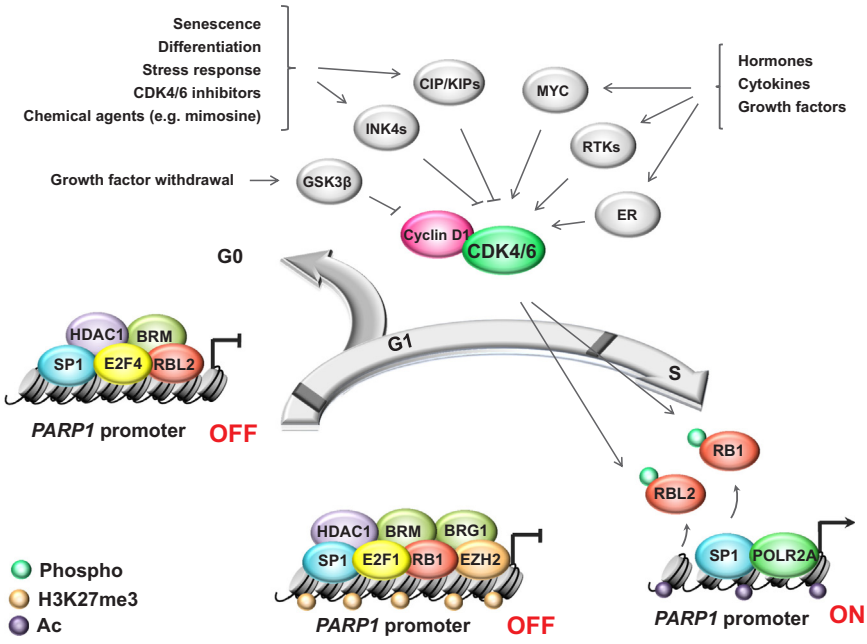

Fig. 1. Cell cycle progression dictates *PARP1* transcription via growth factors/inhibitors-G1/G0-CDK4/6-RBs axis. Cell cycle machinery is controlled by external signals in order to adapt cells to environmental requirements and conditions. Stimulation of receptor tyrosine kinases (*RTKs*), MYC protooncogene or estrogen receptor (ER) in response to peptide and non-peptide growth-promoting agents activates cyclin-dependent kinase 4 and 6 (CDK4/6), which associate with cyclin D1 and phosphorylate retinoblastoma proteins (RB1, RBL2). This modification keeps retinoblastoma proteins released from promoters of PARP1 and cell cycle promoting genes, thereby allowing active gene transcription and enabling cell transition from G1 to S phase. Upon cell growth arrest in G1 or cell cycle exit to G0, CDK4/6 inhibition results in hypophosphorylation of retinoblastoma proteins, their binding to E2F-driven gene promoters and recruitment of chromatin remodelers, which are capable of inactivating gene expression by removing transcription-promoting indicators and/or inserting transcription-inhibiting histone modification(s). It leads to an increase in nucleosome density and chromatin condensation. Notably, composition of the repressive complex varies between cells arrested in G1 and in G0. Limiting PARP1 expression in G0 is achieved solely by histone deacetylase 1 (HDAC1) for histone deacetylation, while in G1 HDAC1 additionally requires PRC2 (polycomb repressor complex 2) activity and trimethylation of H3K27 by enhancer of zeste homolog 2 (EZH2) to repress PARP1 transcription. Cell cycle arrest in G2 does not affect the mRNA and protein levels of PARP1.

2. Cell cycle progression regulates PARP1 transcription

Poly-ADP-ribose polymerase 1 (PARP1) is a multitasking enzyme that regulates many intracellular processes, including DNA repair, metabolism, signaling and transcription, by direct interaction with other proteins and DNA, involving their ADP-ribosylation and auto-ADP-ribosylation of PARP1. The data acquired and published in the EMBL-EBI Expression Atlas indicate high PARP1 abundance in proliferating cancerous and non-cancerous cells (e.g. macrophages) [6]. In search for the link between *PARP1* transcription and cell cycle progression, we recently revealed that cell arrest in G1 or exit to G0 lead to *PARP1* repression by retinoblastoma-based multiprotein complexes, which are also known to repress transcription of E2F-dependent genes encoding proteins responsible for cell transition to S phase [7]. The mode of growth inhibition determines the composition of the repressive complex at the *PARP1* promoter, giving priority to E2F1-RB1 dimers under G1 arrest in cancer, as well as in CD34 + hematopoietic progenitor/stem cells treated with cyclin-dependent kinases 4 and 6 (CDK4/6) pharmacological inhibitors or depleted of nucleotides by mimosine. E2F4-RBL2-based complexes were found to be prevalent in differentiated cells (Fig. 1). Since PARP1 is involved in cell protection against oxidants, one may think that *PARP1* repression in response to proliferation arrest may sensitize cells to agents that challenge redox homeostasis. Some ongoing and recruiting clinical trials have been testing FDA approved CDK4/6 inhibitors Palbociclib (IBRANCE®, PD0332991) and Ribociclib (LEE011, Kisqali) in combination with drugs such as doxorubicin, carboplatin and paclitaxel, which trigger acute redox imbalance [8].

Furthermore, PARP1 enhances cell proliferation. Hormone-activated cyclin-dependent kinase 2 (CDK2) phosphorylates and activates PARP1, thereby facilitating H1 displacement and transcription of the majority of hormone-responsive genes in breast cancer [9]. In urinary bladder carcinoma cells, PARP1 regulates cyclin E expression, cell cycle re-entry and G1/S progression [10]. Thus, high levels of PARP1 in cancer cells promote cell cycle progression, which is associated with an increased level of oxidants, thereby maintaining PARP1 transcription and creating a self-promoting cycle.

3. PARP1 co-activates expression of proteins that enzymatically decompose oxidants and remove secondary metabolites

The primary role in antioxidant defense and in cell adaptation to excessive oxidant or electrophile production is fulfilled by enzymatic antioxidant defense, which comprises direct scavengers of electrophiles, but also enzymes that detoxify the secondary metabolites. Many such enzymes are under transcriptional control of nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2), a basic leucine zipper (bZIP) protein, which dissociates from its repressor Keap1 and translocates to nucleus in response to a physiological shift in redox homeostasis towards oxidant production. NRF2 requires PARP1 for full transcriptional activity, because PARP1 facilitates interaction of NRF2 and NRF2-partner (small MAF protein; MAFG) with the antioxidant response element (ARE) (Fig. 2) [11]. An inhibitory effect of PARP1 knockdowns was found in breast cancer cells and proliferating mouse fibroblasts. Although in normal cells NRF2 suppresses tumor promotion and progression, this pathway is constitutively activated in various cancers by mutation and transcriptional repression of Keap-1, accumulation of Keap-1-NRF2 disruptors, transcriptional and post-translational NRF2 induction. In view of NRF2 targets, this transcription factor provides chemoresistance and, like PARP1, has become a target for anticancer interventions [12].

Another oxidant-counteracting mechanism that involves PARP1 is represented by its interaction with the transcription factor hypoxia-inducible factor 1-alpha (HIF1α), which undergoes activation during hypoxia and hypoxia-triggered redox imbalance [13,14]. PARP1 co-activates HIF1α-dependent transcription of genes, which promotes cell survival. In murine embryonic fibroblasts, PARP1 caused accumulation of HIF1α via upregulation of NO and oxidant production in cells treated with deferoxamine [15]. In addition to PARP1, HIF1α binds EP300/CBP acetylase(s) for full transcriptional activity. A similar observation was made for nuclear factor kappa B (NF-κB), activation of which required synergistic interaction with PARP1 and EP300/CBP. However, for HIF1α mutual dependence between these two types of co-activators has not been documented yet.

Promoters of some antioxidant enzymes such as catalase, SOD1 or SOD2 carry the binding motif for NF-κB, but the role of PARP1 in transcription activation of these genes has not been confirmed. Instead,

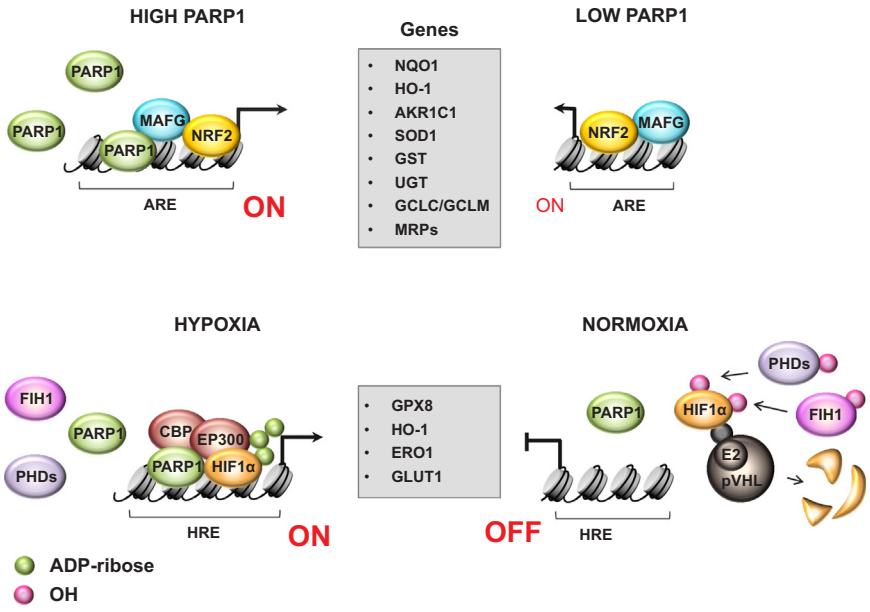

Fig. 2. PARP1 contributes to antioxidant cell defense by enhancing transcription of enzymatic scavengers of electrophiles and secondary metabolites. Under normal oxygen conditions, PARP1 determines intracellular redox homeostasis by intensifying nuclear factor erythroid 2 (NFE2)-related factor 2 (NRF2)-dependent transcription of enzymatic redox-balancing enzymes (NAD(P)H quinone oxidoreductase 1, NQO1; heme oxygenase-1, HO-1; aldo-keto reductase family 1, member C1, AKR1C1; superoxide dismutase 1, SOD1), as well as phase II detoxifying enzymes (glutathione S-transferase, GST; UDP-glucuronosyltransferase, UGT; catalytic and modifier subunits of glutamate cysteine ligase, GCLC and GCLM respectively) and drug transporters (multidrug resistance-associated proteins, MRPs). In the absence of PARP1, transcription of the above-mentioned genes is restricted as NRF2 moderately associates with small MAF proteins (in this case with MAFG) and the antioxidant response element (ARE), which is localized within the promoter of NRF2 target genes. When abundant, PARP1 enhances the interaction among NRF2, MAFG and ARE, thereby acting as a co-activator of NRF2-dependent gene transcription. PARP1 also functions actively in cell adaptive responses to match O_2 supply under hypoxia by supporting hypoxia-inducible factor 1-alpha (HIF1α) at different signaling levels. This protein, together with PARP1, acts as a key modulator of the transcription response in cells that experience a low O_2 level. Under normal oxygen condition, factor inhibiting HIF1α (FIH1; asparaginyl hydroxylase) and propyl hydroxylase domain-containing enzymes (PHDs) hydroxylate HIF1α, thereby preventing transcription factor interaction with EP300/CBP coactivators and marking HIF1α for proteasomal degradation by E3 ubiquitin ligase, the von Hippel-Lindau (pVHL) complex. Low O_2 concentration inhibits hydroxylases and stabilizes HIF1α. PARP1 forms complex and co-activates HIF1α in a PARP1 enzymatic activity-dependent manner, therefore enabling expression of genes controlled by hypoxia response element (HRE)-positive promoters. This group comprises antioxidant defense enzymes such as heme oxygenase-1 (HO-1), glutathione peroxidase 8 (GPX8), ER oxidoreductin 1 (ERO1) and glucose transporter 1 (GLUT1), the activity of which helps to maintain glutathione homeostasis.

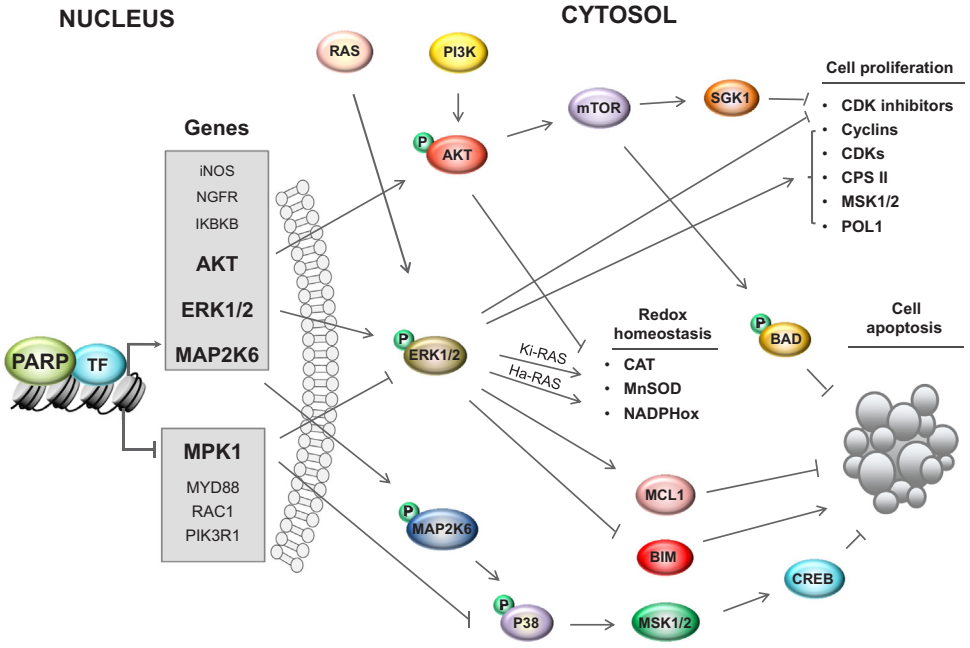

Fig. 3. PARP1 contributes to regulation of redox-related signaling pathways. PARP1 regulates both positively and negatively transcription of numerous enzymes involved in the transmitting signals to and from oxidant-releasing intracellular systems or extracellular sources. Expression of MAP2K6 and ERK1 are, like PARP1, controlled by cell cycle progression and RB-based repressive complexes. In proliferating cells, PARP1 is indispensable for their transcription, because it mediates EP300 recruitment to their promoters. Although AKT and ERK2 are not directly repressed by RBs, PARP1 maintains their expression. All these kinases were shown to protect proliferating cells facing mild or physiological increases in the electrophile abundance from death. In the AKT pathway, PI3 acts as a redox sensor and after activation phosphorylates AKT, which in turn activates mTOR kinase. This enzyme stimulates cell proliferation via the SGK1-FOXO3 pathway, which represses transcription of CDK inhibitors. Moreover, mTOR phosphorylates and inactivates BAD, thus blocking the release of cytochrome c from mitochondria. AKT contributes to H_2O_2 accumulation by stimulating oxidative metabolism and FOXO-dependent repression of catalase. Growth factors and oxidants switch on ERK signaling via RAS, which, depending on the isoform expressed in a particular cell type, shifts up or down the intracellular level of oxidants. Ha-RAS isoform promotes $O_2^{\bullet-}$ accumulation by activating NADPH oxidase, while the Ki-RAS pathway upregulates transcription of SOD2. There are numerous ERK targets, which implicate this enzyme in cell proliferation: carbamoyl phosphate synthetase (CPS II, source of pyrimidine nucleotides), MSK1/2 (chromatin remodeling and induction of cell cycle-related gene transcription), RNA polymerase I (transcription of the ribosomal RNA genes), CDK inhibitors or MYC (transcription of cyclin D1). ERK protects cells from death by repressing pro-apoptotic BIM and stabilizing anti-apoptotic MCL-1 protein. PARP1-MAP2K6 functional cross-talk at the genomic level is not limited to upregulation of kinase transcription. MAP2K6 links PARP1 with transcription of anti-apoptotic genes indirectly by starting the phosphorylation cascade MAP2K6-p38-MSK1/2-CREB. The last component of this axis controls transcription of HO-1 and PGC-1α; the latter promotes mitochondrial biogenesis. Furthermore, PARP1 regulates activity of MAPKs independently of their promoters by regulating transcription of JNK, ER1/2 and the p38 inhibitor MKP1. Downregulation of MKP1 expression by PARP1 blocks dephosphorylation of tyrosine and threonine residues of MAPKs, which undergo activation upon acute cell exposure to H_2O_2.

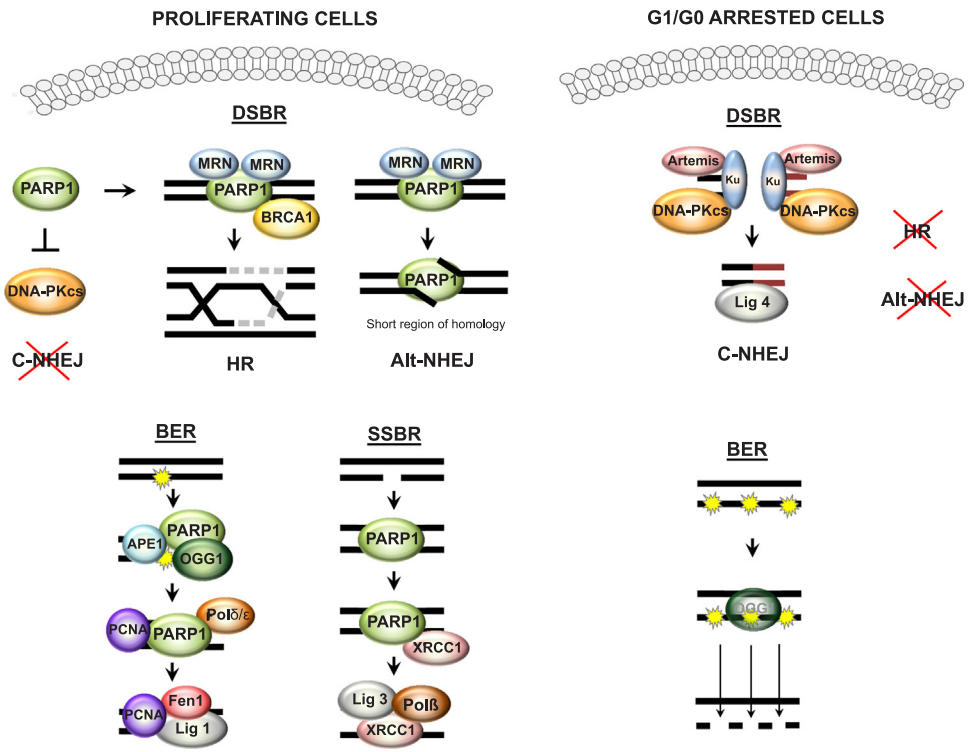

Fig. 4. PARP1 regulates repair of oxidative DNA damages. The cell decision on involvement of particular repair system depends on the type of DNA damage and is strongly related to cell cycle progression. For double strand breaks, the cell is equipped with three repair systems: homologous recombination (HR), classical non-homologous end joining (C-NHEJ) or alternative non-homologous end joining (Alt-NHEJ). Proliferating cells, with high PARP1 levels, make use mainly of two mechanisms: HR and/or Alt-NHEJ, which assure accurate and error-free repair of double strand breaks since they use a replicated DNA template to reconstruct the missing fragment with high fidelity. These two pathways rely on the recognition of detrimental lesions by PARP1, which recruits other proteins to the affected sites: first MRN complex (consisting of MRE11, Rad50, Nbs1), an initiator of repair, then MRE11 determines the composition of proteins for each repair mechanism (HR or NHEJ). The low C-NHEJ involvement in repair is achieved by high expression of PARP1, an inhibitor of DNA-dependent protein kinases (DNA-PKcs), which is crucial for C-NHEJ progression. In G1/G0 arrested cells (deficient in HR and Alt-NHEJ), low expression of PARP1 allows recruitment of the Ku70/80 heterodimer to double strand breaks and sites of DNA-PKcs activation. Repair machinery such as nuclease Artemis, DNA ligase IV and XRCC4 further process the damaged DNA and directly ligate DNA ends, leading to the irreversible loss of genetic material. Base excision repair (BER) is a substantial pathway to repair oxidized bases. The damaged base is recognized and removed by OGG1 glycosylase, which requires PARP1 for proper and efficient functioning. AP endonuclease (APE1) is binds to apurynic sites and produces single-strand breaks (SSBs), which again involve PARP1 in the BER machinery at a later repair step. DNA nick-induced poly-ADP-ribosylation facilitates PARP1 interaction with DNAPδ/ε and PCNA, which further govern repair machinery. Single strand breaks resulting from direct oxidant action also need PARP1 activity to be repaired. Poly-ADP-ribose polymers recruit XRCC1, then the remaining SSBR machinery comprising DNA polymerase (DNAP) and DNA ligase III, which also can be PARylated by PARP1. PARP1 deficiency in growth-arrested cells substantially impairs both pathways. Of note, inhibition of OGG1 in combination with G1-blockade leads to further accumulation of single strand breaks.

PARP1 level negatively correlates with mitochondrial SOD in cancer cells (EMBL-EBI Expression Atlas), where SOD2 overexpression causes a growth inhibitory effect by shifting the $O_2^{\bullet-}/H_2O_2$ balance towards H_2O_2 accumulation [16]. If PARP1 is involved in SOD2 repression, this enzyme could be capable of defining the intracellular repertoire of growth promoting or inhibiting oxidants. Furthermore, low SOD2 level is known to stabilize HIF1α [17].

4. PARP1 regulates redox-sensitive signaling pathways

The roles that small species of oxidizing nature play in cellular signaling are becoming increasingly appreciated. Redox-sensitive pathways allow cells to adapt to mild oxidant/antioxidant imbalance and promote survival by linking redox shifts to post-translational modifications of proteins and to their interactome [5,18]. According to our and previous findings, PARP1 regulates transcription of numerous genes encoding redox sensors and mediators transmitting signals upstream or downstream of electrophile sources (Fig. 3). Redox-sensitive MAP kinase signaling serves as a good example of the PARP1-MAPK-ROS-cell survival axis since PARP1 couples transcription of MAP2K6, ERK1/2 and AKT1 with the cell cycle progression, thereby assuring active transcription of kinases that have a pro-survival function under mild redox imbalance [19–26]. Furthermore, oxidative stress-induced PARP1 activation represses transcription of MPK1, a known MAPK inhibitor [27]. Although in this particular case JNK and p38 were shown to act as pro-death kinases, the beneficial or detrimental activity of kinases and MAPK pathways is determined by the type of intracellular or extracellular stimuli that challenges redox homeostasis, for example whether the action is acute or prolonged, mild or severe, but is also dependent on the cell type. All these aspects also apply to PARP1. In addition to conditions listed above, PARP1-dependent cell life or death fate is determined by the pathway to which PARP1 contributes (pro-survival or suicidal), the direction (inhibition or stimulation) and mode of mutual interdependence with its interacting partner (direct protein-protein binding or covalent modification, i.e. ADP-ribosylation). Oxidants are one of major agents triggering mono- or poly-ADP-ribosylation; the severity of oxidative stress determines the length of ADP polymer synthesized, thus also impinging on the beneficial or detrimental effect of PARP1 activation, since NAD^+ is utilized as a substrate for ADP-ribosylation. High doses of H_2O_2 cause metabolic catastrophe, parthanatos, and activation of detrimental signaling pathways, while moderate PARylation protects cells from mild oxidative stress by attracting DNA repair complexes, clearing and removal of oxidized or damaged proteins, and re-establishing homeostasis [28,29].

5. Cell cycle determines DNA repair mechanisms

PARP1 actively contributes to numerous repair pathways of oxidative DNA lesions, which comprise covalent modifications of nucleobases as well as single and double strand breaks (SSB and DSB, respectively). The deformability of DNA within SSB is recognized by two flexibly linked N-terminal zinc fingers, and initiates self-assembly of remaining PARP1 domains leading to activation of the C-terminal catalytic domain [30]. In case of DSB induced by oxidative stress, JNK 6 phosphorylates SIRT6. This enzyme is rapidly mobilized to break sites, where it potentiates recruitment and activation of PARP1, which in turn stimulates DSB repair [31]. In proliferating cells all repair mechanisms are active, and PARP1 is highly expressed to support SSB repair (SSBR), base excision repair (BER), homologous recombination (HR), and alternative non-homologous end joining (Alt-NHEJ), but inhibits classical

non-homologous end joining (C-NHEJ) (Fig. 4). Thus PARP1 has become a target for anticancer interventions. G1/G0 arrest shifts error-free HR and Alt-NHEJ to error-prone C-NHEJ, but low PARP1 impairs also BER and SSBR [32–34]. According to our new data, PARP1 repression by CDK4/6 inhibitors reduces PARP1-dependent 8-oxoguanine glycosylase (OGG1) activity, causing accumulation of single strand breaks and thereby increasing cell vulnerability to anticancer drugs and H_2O_2-induced oxidative stress [35]. The direct binding of OGG1 to PARP1 is stimulated by increased oxidant level, and for full activity OGG1 requires acetylation by EP300, which physically interacts with PARP1 and is recruited to some genomic loci by PARP1 [36,37].

PARP1 has been postulated to cooperate with transcription factor(s) that activate expression of genes encoding proteins contributing to DNA repair [38]. Although such a premise must be experimentally confirmed, PARP1 is a bona fide co-regulator of NF-κB, p53, AP-1, E2F1, and BRCA1, which control promoter activation, epigenetic landscape and miRNA transcription of DNA repairing machinery. The confirmation for likely contribution of PARP1 in regulation of DNA repair gene transcription comes from observations in human growth arrested monocytes differentiating to proliferating macrophages [39]. This process was associated with increased PARP1 expression, but also with transcriptional activation of XRCC1, ligase IIIα, OGG1 and catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}). Severe DNA repair defects that impacted base excision repair and double-strand break repair in monocytes sensitized these cells to death by t-butyl hydroperoxide and irradiation with γ-rays, while macrophages revealed almost complete resistance to these redox-challenging agents.

To conclude, PARP1 provides cell with protection against oxidants at different levels: by activating expression of proteins setting up antioxidant defense and redox sensitive signaling pathways, and by fine tuning of DNA repair machinery. Therefore PARP1 expression, which is defined by the proliferative status of cells, can determine cell resistance to oxidants, even though an adaptive response is only apparent within a narrow dose window.

Acknowledgments

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EP300-HDAC1-SWI/SNF functional unit defines transcription of some DNA repair enzymes during differentiation of human macrophages

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ABSTRACT

Differentiation of human macrophages predisposes these cells to numerous tasks, i.e. killing invading pathogens, and this entails the need for enhanced intracellular defences against stress, including conditions that may increase DNA damage. Our study shows that expression of DNA repair enzymes, such as *PARP1*, *BRCA1* and *XRCC1*, are activated during macrophage development by the SWI/SNF chromatin remodelling complex, which serves as a histone acetylation sensor. It recognises and displaces epigenetically marked nucleosomes, thereby enabling transcription. Acetylation is controlled both in monocytes and macrophages by the co-operation of EP300 and HDAC1 activities. Differentiation modulates the activities of individual components of EP300-HDAC1-SWI/SNF functional unit and entails recruitment of PBAF to gene promoters. In monocytes, histone-deacetylated promoters of repressed *PARP1*, *BRCA1* and *XRCC1* respond only to HDAC inhibition, with an opening of the chromatin structure by BRM, whereas in macrophages both EP300 and HDAC1 contribute to the fine-tuning of nucleosomal acetylation, with HDAC1 remaining active and the balance of EP300 and HDAC1 activities controlling nucleosome eviction by BRG1-containing SWI/SNF. Since EP300-HDAC1-SWI/SNF operates at the level of gene promoters characterized simultaneously by the presence of E2F binding site(s) and CpG island(s), this allows cells to adjust *PARP1*, *BRCA1* and *XRCC1* transcription to the differentiation mode and to restart cell cycle progression. Thus, mutual interdependence between acetylase and deacetylase activities defines the acetylation-dependent code for regulation of histone density and gene transcription by SWI/SNF, notably on gene promoters of DNA repair enzymes.

1. Introduction

In eukaryotes, gene transcription by RNA polymerase II (RNA Pol II) requires loosening of chromatin structure at gene regulatory regions, which include changes in epigenetic histone modifications, and consequential nucleosome eviction or sliding, before initiation of transcription can occur. This may happen passively as a consequence of preinitiation complex formation or actively by the action of chromatin remodelling complexes, such as mammalian SWI/SNF. This large heterogeneous multi-subunit complex in mammalian cells utilizes 2 alternative ATP-dependent enzymes: Brahma-related gene 1 (BRG1) and Brahma (BRM), which modulate histone density. Both enzymes are parts of the BAF complex, whereas PBAF exclusively uses BRG1 as the catalytic subunit [1]. In terms of their impact on transcriptional regulation, the relationship between complexes containing BRM and BRG1 comprises all the obvious options: antagonism, synergism or mutual exclusivity, but the factor(s) determining the outcome of their cross-talk remain(s) unknown [2]. The most frequently described paradigm regarding biological relevance of histone displacement considers SWI/SNF-mediated activity as a determinant of chromatin accessibility to transcription factors and RNA polymerase during formation of pre-initiation complex at certain gene promoters, but also during elongation, when SWI/SNF moves through coding regions along with RNA Pol II [3]. No unified mode of SWI/SNF recruitment has hitherto been reported, and there is little data on histone modifications or variants that may trigger displacement activity or on the preferred type of gene promoters (constitutive or inducible). It is known, however, that post-translational modifications of histones may contribute to nucleosome binding and processing by SWI/SNF – relevant results have been reported using both in vitro and in vivo models. At the endothelin promoter, BRM/BRG1 interacted with the H3K4 methylation complex (Ash2 alone or with Wdr5) via the myocardin-related transcription

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1874-9399/

factor A (MRTF-A) to activate transcription in response to angiotensin II [4]. In an immobilized nucleosome array, SAGA-acetylated histones interacted with the SWI/SNF bromodomain, thereby predisposing them to nucleosome displacement [5]. Recent findings have emphasized the role of histone acetylation by SAGA subunit Ada2 (Gcn5) as well as Sas3 over acetylation-independent histone eviction in the derepression of FLO1 promoter in *Saccharomyces cerevisiae*; however, histone acetylation and displacement seemed to occur in parallel to facilitate transcription, but may remain independent of each other [6]. In our model, macrophage differentiation-associated histone acetylation marks nucleosomes for eviction by SWI/SNF and the opening of chromatin structure, not acetylation alone, dictates transcription of *PARP1*, *BRCA1* and *XRCC1* in human monocytes and macrophages. This range of histone acetylation is defined by the resultant activity of EP300 and HDAC1, which co-occur at gene promoters with BRM and BRG1.

Antagonistically acting histone acetylases (HATs) and deacetylases (HDACs) are simultaneously targeted to promoters of transcriptionally active and MLL-primed genes that were occupied by phosphorylated RNA Pol II [7]. Promoter occupancy by the initiating form of RNA Pol II (phosphorylated at Ser 5 of the C-terminal domain) is characteristic for CpG islands, which may also evolve both into active and inactive chromatin. This state constitutes a checkpoint in transcriptional initiation, allowing effective triggering upon transcription factor-dependent recruitment of P-TEFb, which then phosphorylates RNA Pol II and releases inhibited transcription [8]. Identification of HATs together with HDACs at RNA Pol II-enriched promoters, as well as the lack of HDAC presence at promoters of silent genes, has paved the way for a new concept in the regulation of nucleosome acetylation, in addition to the well-described exchange (recruitment and release) of HATs and HDACs under certain conditions (such as inhibition or re-initiation of cell proliferation). Our previous studies, as well as others, have documented HDAC1 as a co-repressor within retinoblastoma-based complexes that recognize and bind promoters controlled by E2F transcription factors (and therefore also by the cell transition from G1 to S phase), thus regulating HDAC1 presence in the specific chromatin section [9,10]. Regardless of composition of the basic E2F-RB complex, which corresponds to the inhibitory trigger for proliferation of hematopoietic stem and progenitor cells (E2F1-RB1 upon G1 cell synchronization, E2F4-RBL2 upon monocyte differentiation), HDAC1 is always enriched at the *PARP1* promoter in response to cell cycle arrest. *PARP1*, like some other genes encoding proteins involved in base excision and double-strand break DNA repair (*XRCC1*, *LIG3*, *LIGI*, *OGG1*), also becomes de-repressed during monocyte differentiation into macrophages, thereby i.e. giving macrophages enhanced resistance to oxidative stress [11]. The increased abundance of DNA repair enzymes protect macrophages from the accumulation of DNA lesions and cell death after treatment with chemical agents, irradiation or oxLDL exposure, whereas in monocytes defects in BER and activation of NHEJ lead to apoptosis.

In this study, we show that macrophage differentiation from monocytes modulates histone acetylation and nucleosome density at the E2F-driven and CpG island-containing promoters of *PARP1*, *BRCA1* and *XRCC1*. This occurs by fine-tuning of EP300 and HDAC1 activities and the subsequent displacement of acetylated nucleosomes by SWI/SNF (BAF complex in monocytes, but PBAF in macrophages). Differentiation-induced cell cycle re-entry was not followed by HDAC1 release from the chromatin, but PBAF was recruited to gene promoters and switched function between BRM and BRG1. Thus, the EP300-HDAC1-SWI/SNF multiprotein functional unit contributes to defining macrophage physiology from its early differentiation stages.

2. Materials and methods

2.1. Materials

RosetteSep™ Monocyte Enrichment Cocktail was purchased from STEMCELL Technologies (Grenoble, France), cell culture media were from Biowest (CytoGen, Zgierz, Poland), granulocyte-macrophage colony-stimulating factor (GM-CSF) from PeproTech (London, UK), iEP300 (C646), iHDAC (sodium butyrate), iSWI/SNF (PFI-3), anti-rabbit IgG (whole molecule) (A0545) and anti-mouse IgG (whole molecule) (A4416) peroxidase-labeled antibodies produced in goat, anti-E2F4 antibody (05–312), BLUeye prestained protein ladder (#94964), oligonucleotides for real-time PCR were from Sigma Aldrich (Poznan, Poland). Anti-CD11b (25–0118-41) and anti-CD14 (25-0149-41) PE-Cy7®-labeled antibodies, anti-acetyl-histone H3 (Lys9 + Lys14) (PA5–16194), anti-HDAC1 (PA1–860), anti-EP300 (PA1848) antibodies, Lipofectamine RNAiMAX, OptiMem, Dynabeads™ Protein G, High-Capacity cDNA Reverse Transcription Kit, Click-iT™ Nascent RNA Capture Kit, SuperSignal™ West Pico Chemiluminescent Substrate, TRI Reagent™, Silencer™ Select Pre-Designed siRNA ID:s531223 (BRM), ID:s13141 (BRG1), ID:s73 (HDAC1) and ID:s534247 (EP300) were from Thermofisher Scientific (Thermofisher Scientific, Warsaw, Poland). KapaSybr Fast qPCR Master Mix and KAPA HiFi™ HotStartReadyMix (2×) were purchased from KapaBiosystems (Polgen, Łódź, Poland). EvaGreen® Dye, 20× in water was purchased from Biotium (Hayward, USA). Advanced TC™ culture plates were from Greiner Bio-One (Biokom, Janki/Warsaw, Poland). WB antibodies: anti-PARP1 (sc-8007), anti-BRG1 (sc-17,796), anti-Ki-67 (sc-23,900), anti-PCNA (sc-25,280), anti-cyclin E (sc-377,100) and anti-cyclin B (sc-166,210) and anti-PBRM1 (anti-PB1; sc-390,095) were from Santa Cruz Biotechnology (AMX, Lodz, Poland). ChIP grade antibodies: anti-PARP1 (#9532), anti-BRM (#11966), anti-BRG1 (#49360), anti-E2F1 (#3742), anti-acH3K27ac (#4353), anti-histone H3 (#4620), normal rabbit IgG (#2729) were purchased from Cell Signaling Technology (LabJOT, Warsaw, Poland).

2.2. Monocyte isolation and differentiation of macrophages

Human monocytes were isolated from buffy coats from healthy donors using RosetteSep Monocyte Enrichment Cocktail as described previously [12]. After attachment, monocytes were differentiated in RPMI with 10% FBS, penicillin/streptomycin (50 U/ml and 50 μg/ml, respectively) and GM-CSF (10 ng/ml) for 7 days. Macrophage phenotype was confirmed by comparing CD11b and CD14 abundance on the surface of freshly isolated monocytes and GM-CSF differentiated macrophages (Suppl. Fig. 1A). Briefly, cells were washed three times with PBS, suspended in PBS supplemented with 1% BSA, incubated with anti-CD11b and anti-CD14 PE-Cy7® conjugated antibodies at room temperature for 30 min, washed and cell fluorescence was measured using a flow cytometer (LSRII BD Flow Cytometer).

2.3. Evaluation of macrophage proliferation

Cell cycle progression was monitored by flow cytometry in cultures of monocytes and macrophages after cell fixation (1% formaldehyde in PBS for 15 min), permeabilization (0.1% Tween in PBS for 15 min), RNA digestion (1 mg/ml RNAse A for 1 h) and staining with propidium iodide (5 μg/ml) as described previously [13]. To confirm cell divisions, non-treated macrophages were cultured in parallel with cells supplemented with CDK4/6 inhibitor (PD0332991; 1 μM) for 72 h thus the latter were prevented from entering the S phase. Then cells were processed exactly as for the cell cycle analysis and sample fluorescence that corresponds to DNA content and cell number was read at 528/590 nm with SYNERGY/HTX multi-mode reader (BioTek®, Biokom, Janki/Warsaw, Poland).

2.4. Quantification of gene expression

For mRNA quantification, total RNA was extracted with TRI Reagent™, reverse transcribed with High-Capacity cDNA Reverse Transcription Kit and selected cDNA fragments were amplified by real-

time PCR (KapaSybr Fast qPCR Master Mix; CFX96 C1000 Touch, BioRad Warsaw, Poland). Primer pairs used for cDNA amplification are listed in Supplementary Table Primer List.

The median expression of ACTB, GAPDH and B2M (housekeeping genes, HSKG) was taken for normalization of gene expression and it is presented as a Log2 of calculated fold change.

Nascent *PARP1* mRNA was measured using Click-iT® Nascent RNA Capture Kit as described previously and was normalized to 1 μg of total RNA isolated from cells [9].

For protein detection by Western blotting, cell lysates were separated by SDS-PAGE, transfered to nitrocellulose membranes and stained overnight with primary antibodies at 4 °C. After staining with HRP-conjugated secondary antibodies, the signal was developed using SuperSignal™ West Pico Chemiluminescent Substrate and acquired with ChemiDoc-IT2 (UVP, Meranco, Poznan, Poland).

2.5. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out according to a previously described protocol [9]. A fragment spanning the E2F-binding site in the *PARP1* promoter was amplified using KAPA HiFi™ HotStartReadyMix supplemented with EvaGreen® Dye and 7% DMSO, while other promoters and a control region (exon 8 of *PARP1*) were amplified using KapaSybr Fast qPCR Master Mix. Primer pairs used in ChIP are listed in Supplementary Table Primer List.

2.6. Transient gene silencing

For *BRM* and *BRG1* silencing, freshly isolated monocytes (that were allowed to adhere to the plate bottom in RPMI) and differentiated macrophages were transfected with RNAiMAX-siRNA complexes prepared in OptiMem according to the following ratio: 200000 cells, 20 nmol siRNA and 3 μl of transfection reagent. Silencing efficiency was confirmed and *PARP1* transcription was studied by Western blotting and/or real-time PCR 48 h after cell transfection.

2.7. DNAse I digestion

DNAse I hypersensitive sites were studied according to Song et al. [14] with minor modifications. In brief, monocytes (± iHDAC) and macrophages (± EP300 and ± iSWI/SNF) were washed twice in ice cold PBS, resuspended in 10 mM Tris-HCl (pH = 7.4), 10 mM NaCl, 3 mM $MgCl_2$ and 0.1% NP40 and left on ice for 15 min. After spinning down, nuclei were washed once and suspended in reaction buffer (10 mM Tris-HCl pH = 7.5, 1 mm $CaCl_2$, 10 mM $MnCl_2$). DNAse I (2 U per 200,000 cells) was inhibited after 5 min for *BRCA1* and *XRCC1* promoters and after 15 min for the *PARP1* promoter. The enzyme was inactivated by heating at 65 °C for 10 min and mixed with equal volume of phenol:chlorophorm:isoamyl alcohol. DNA was isolated as described for chromain immunoprecipitation and amplified/quantified by real-time PCR using primers for *PARP1*, *BRCA1*, *XRCC1* promoters and exon 8 of *PARP1,* which served as a negative control. The amount of undigested gene promoters (and *PARP1* exon 8) was normalized to input (DNAse untreated samples).

2.8. Cell treatment with inhibitors

iHDAC (0.5 mM; sodium butyrate), iEP300 (10 μM; C646) and iSWI/SNF (25 μM; PFI-3) were added to cells 24 h prior to analysis. In siRNA-transfected monocytes, iHDAC was added 24 h prior to collecting cells.

2.9. ChIP-Seq analysis

ChIP-Seq analysis was performed in Galaxy version 18.01.rc1 [15] using publicly available data from the GEO database (details in Supplementary Table GEO).

FASTQ format files were unified to Sanger FASTQ encoding with FASTQ Groomer [16]. Reads were aligned to Human Genome (v 19) using Map with Bowtie for Illumina [17] (seed length - 28; if > 1 reportable alignments existed, all alignments were suppressed for a read) and unmapped reads were filtered out. If there was more than one run for the antibody, the aligned reads were merged at this step. ChIP-seq peaks were called in MACS [18] with a *p* value cutoff for peak detection set at 10^{-3}. Co-distribution of EP300 and HDAC1 in the genome was studied by multiBamSummary/plotCorrelation, while HDAC1 occurrence (score file: HDAC1 reads mapped and filtered in BAM format) centered on EP300 peaks (regions to plot: EP300 peaks in BED format) was visualized by computeMatrix/plotHeatmap (DeepTools [19]). Gene promoters enriched in EP300 and HDAC1 were identified by returning intersects of EP300/HDAC1 peaks and genomic regions ± 2000 bp centered on TSSs (overlapping intervals of both datasets). Genomic intervals for E2F1, E2F4 and CpG Islands were taken from UCSC Main tables wgEncodeRegTfbsClusteredV3 (this shows regions of transcription factor binding derived from a large collection of ChIP-seq experiments performed by the ENCODE project on 91 cell lines, together with DNA binding motifs identified within these regions by the ENCODE Factorbook repository) and cpgIslandExt (which describes the CpG Islands (includes observed/expected ratio) i.a. start and end position), respectively. Venn diagrams were created in GeneVenn (http://genevenn.sourceforge.net/) from gene lists. Enriched gene ontology terms (GO) were derived using GOrilla (http://cbl-gorilla.cs.technion.ac.il/) using two unranked lists of genes (target - genes in CD4+ and K562 that had HDAC1-EP300-E2F1/E2F4-CpG in common versus background lists – genes with promoters enriched in E2F1/E2F4 according to wgEncodeRegTfbsClusteredV3).

For identification of gene promoters enriched in H3K27ac in macrophages, peaks for monocytes and macrophages were called in MACS2 and MACS2 bdgdiff (differential peak detection based on paired four bedgraph files (Galaxy Version 2.1.1.20160309.1)) [18,20] was run using IgG as a control for both cell types.

2.10. Statistical analysis

Data are shown as mean ± standard deviation of the mean (SEM). Student's *t*-test was used to determine statistically significant differences between two means (marked with * when $p < 0.05$), while one-way analysis of variance (ANOVA) was carried out in GraphPad Prism 5 to compare means in several groups.

3. Results

3.1. Cell cycle entry during macrophage differentiation is responsible for an increase in PARP1 transcription

Our previous study linked tightly *PARP1* transcription with cell proliferation and showed that repression of *PARP1* in human monocytes is due to the assembly of E2F4-RBL2-HDAC1 repressive complex at the *PARP1* promoter in response to cell cycle exit during the commitment of hematopoietic stem and progenitor cells (HSPC) [9]. Since the later monocyte to macrophage differentiation, when induced by high concentration of GM-CSF (80 ng/ml), was found to substantially restore expression of this gene [11], we addressed 2 major questions: (a) whether stimulation of monocytes with a lower (and more physiologically-relevant) GM-CSF dose (10 ng/ml for 7 days) is followed by higher *PARP1* transcription; and (b) whether macrophage development is associated with cell cycle re-entry that may unlock *PARP1* expression. The differentiation of blood-derived monocytes into macrophages was confirmed by observing the loss of CD14+ expression and gain of CD11b expression (Supplem. Fig. 1A) [21]. This was associated with a significantly higher PARP1 mRNA and protein abundance (Fig. 1A and B), which was due to enhanced gene transcription (Fig. 1C). In contrast

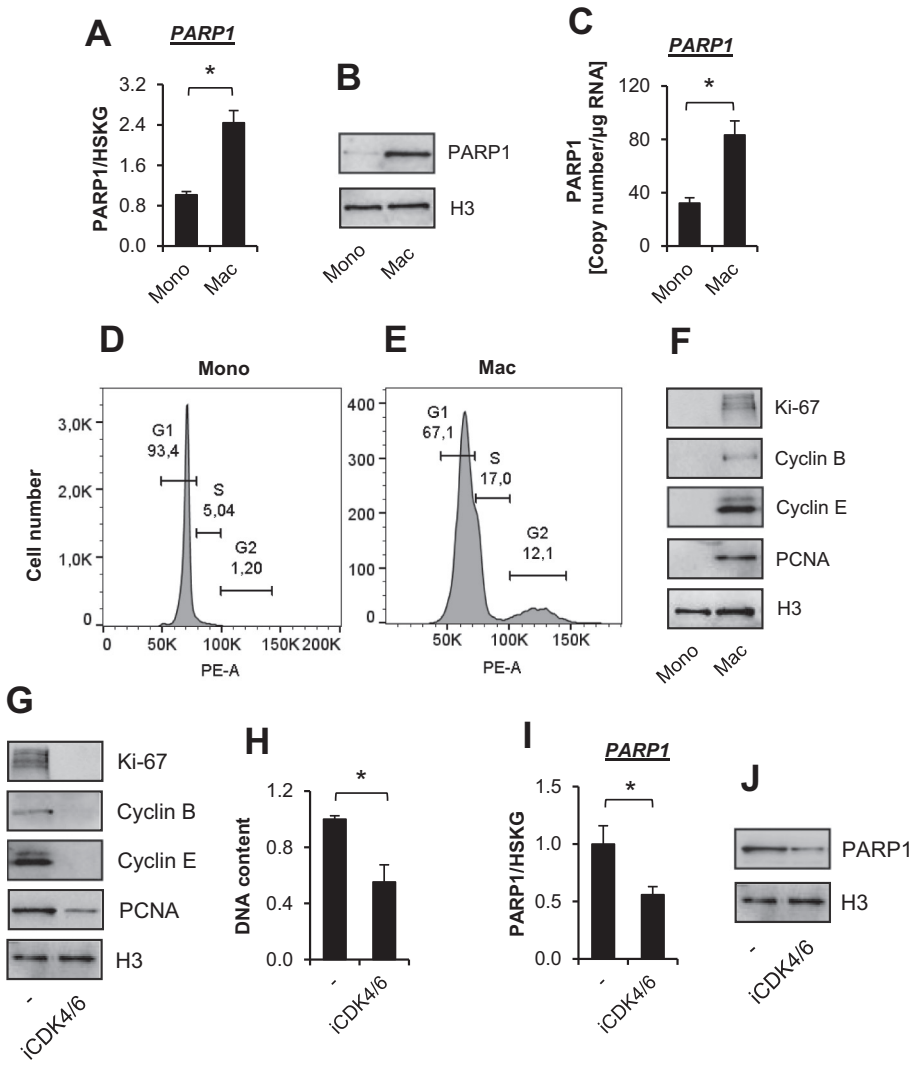


Fig. 1. GM-CSF-induced cell cycle entry restores *PARP1* transcription in human macrophages. Expression of *PARP1* in human monocytes (Mono) and GM-CSF-differentiated macrophages (Mac) was determined at the mRNA (A; real-time PCR; normalized to median of ACTB, GAPDH and B2M) and protein (B; Western blot; normalized to total histone H3) level. PARP1 mRNA synthesis was measured by quantification of ethylene uridine incorporation into de novo transcribed RNA within 6 h (C). Macrophage divisions were confirmed by analyzing the cell cycle progression (D) and by monitoring the expression of proliferation markers by Western blotting (F). Inhibition of cell proliferation in GM-CSF-differentiated macrophages with iCDK4/6 (1 µM PD0332991, 72 h), which led to decrease in expression of proliferation markers (G, Western blot) and cell number in culture corresponding to DNA level (H), dampened PARP1 transcription (I; real-time PCR) and lowered its protein level (J; Western blot). Bars in the figures represent mean ± standard error of the mean (SEM) and statistically significant differences between means are marked with * when $p < 0.05$ (Student's *t*-test was used to compare differences between two means). For gene expression analysis, three independent experiments were run (each of them in two technical replicates); for iCDK4/6 effect on cell proliferation, DNA content was measured twice (two independent experiments in two technical replicates); Western blots and analysis of the cell cycle were repeated three times and representative images are shown.

to G0-arrested monocytes, a fraction of cultured macrophage population was found in S and G2 phases (Fig. 1D and E). Furthermore, cell proliferation markers were also detected in macrophage lysates (Fig. 1F). Treatment of differentiated cells with CDK4/6 inhibitor (iCDK4/6 - PD0332991), which leads to cell synchronization in the G1 phase (loss of proliferation markers is shown in Fig. 1G), considerably reduced both cell number in culture (Fig. 1H) and PARP1 expression (Fig. 1I and J), which parallels the effect seen in proliferating cancer cells [13].

These results indicate that monocyte stimulation with GM-CSF re-activates the CDK4/6-E2F signaling pathway (macrophage differentiation led to E2F4 displacement from the *PARP1* promoter, while E2F1 remained absent – Supplem. Fig. 1B and C; exon 8 of *PARP1* served as a negative control), allowing the re-activation of *PARP1* transcription. Thus, cell proliferation is first reduced and subsequently restored along the hematopoietic HSPC-monocytes-macrophages axis, and this determines *PARP1* level at consecutive differentiation stages.

3.2. SWI/SNF activity mediates the effect of histone acetylation on PARP1 transcription

Since the PU.1 transcription factor acts as a key lineage-determining factor in macrophages [22], *PARP1* promoter was examined for evidence of PU.1 binding in monocytes and macrophages using analysis of peaks in ChIP-Seq data (Supplem. Fig. 2A). PU.1 recruitment to the *PARP1* promoter in monocytes was not enhanced by macrophage differentiation (Supplem. Fig. 2B) and silencing of this factor did not affect *PARP1* transcription (Supplem. Fig. 2C–F), whereas it clearly maintained low *TNFα* transcription after LPS stimulation (Supplem. Fig. 2G).

Since histone deacetylation by E2F4-RBL2-associated HDAC1 is responsible for *PARP1* repression in monocytes, we compared the extent of H3K27ac and H3K9/14 ac modifications within the *PARP1* promoter between monocytes and macrophages. Both analysis of ChIP-Seq data (Fig. 2A and B) and ChIP-qPCR (Fig. 2C) revealed that acetylation of histone lysine residues increased during differentiation, which was followed by a substantial decline in nucleosome density (Fig. 2D). Although abundance of EP300 at the *PARP1* promoter increased slightly (Fig. 2E), HDAC1 remained associated with promoter chromatin whereas E2F4 was displaced from the chromatin (Fig. 2F, Supplem. Fig. 1C), suggesting that EP300 and HDAC1 may regulate transcription and be in a mutual/functional relationship (exon 8 of *PARP1* was used as a negative control for histone acetylation, EP300 and HDAC1 occurrence – Supplem. Fig. 2H). Indeed, both enzymes actively contributed to establishing the new level of *PARP1* transcription in macrophages, since their inhibitors and their silencing modulated the mRNA level of *PARP1* by lowering it (iEP300 and siEP300) or slightly raising it (iHDAC and siHDAC1) (Fig. 2G, Supplem. Fig. 2I and J).

Since inhibition of EP300 activity had a dominant effect over iHDAC (double inhibition still led to decreased *PARP1* expression in macrophages and retained the original low expression levels in monocytes), EP300 seemed to be a key positive regulator of transcriptional activity

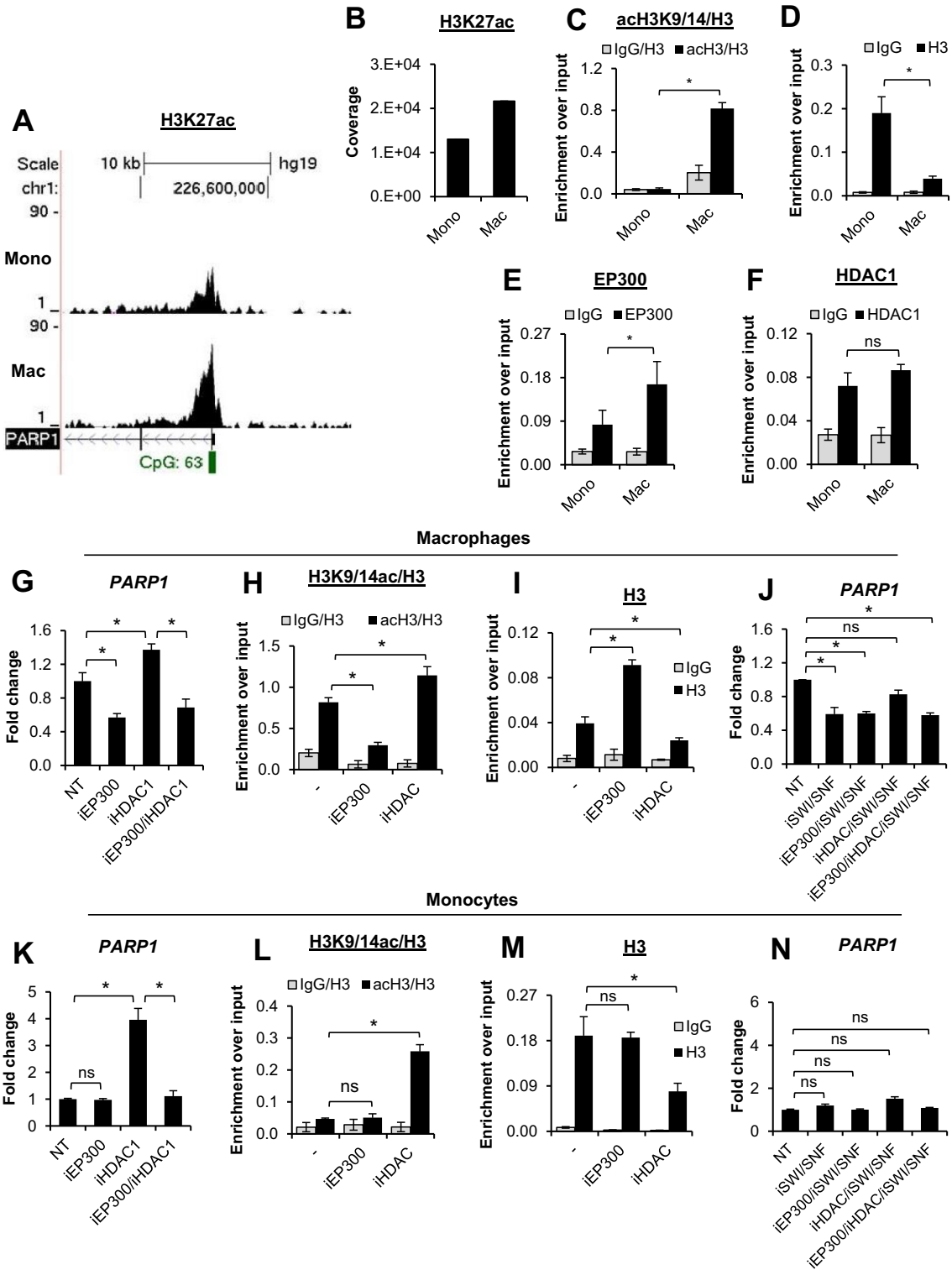


Fig. 2. Histone acetylation influences *PARP1* transcription in a SWI/SNF-dependent manner. Acetylation of H3K27 at the *PARP1* promoter was compared between human monocytes (Mono) and macrophages (Mac) by peak calling (MACS) (A) and quantified using BedCov (data to score: mapped reads in BAM format; genomic interval to plot: acH3K27 peak at *PARP1* promoter in macrophages in BED format; chr1:226592829-226,596,773) (B). Acetylation of histone H3K9/14 (C), density of histone H3 (D), recruitment of EP300 (E) and HDAC1 (F) at the *PARP1* promoter (amplified region spans the binding site of E2Fs) were determined by ChIP-real-time PCR. Effect of EP300 and HDAC inhibitors on *PARP1* transcription in macrophages was measured by real-time PCR (G), while their effect on acetylation of H3K9/14 (H) and density of H3 (I) was measured by ChIP-real-time PCR. To study the influence of SWI/SNF on *PARP1* transcription, SWI/SNF inhibitor was added to cells alone and in combination with iEP300 and iHDAC (J) and the effect was measured by real-time PCR. The same experiments were conducted on freshly isolated monocytes (K–N). Bars in the figures represent mean ± standard error of the mean (SEM) and statistically significant differences between means are marked with * when $p < 0.05$ (Student's *t*-test was used to compare differences between two means), while ns marks lack of difference. For gene expression analysis, four independent experiments were run (each of them in two technical replicates), while ChIP-real-time PCR was repeated twice (two independent experiments in two technical replicates).

of the *PARP1* promoter in macrophages, in which it overcomes ongoing HDAC1 activity, but also in monocytes when the repressor activity of HDAC is removed (Fig. 2E and K). This action of both enzymes at the level of chromatin modification was confirmed by verifying histone acetylation after administration of acetylase and deacetylase inhibitors in macrophages: iEP300 reduced whereas iHDAC increased histone acetylation on the promoter, indicating that HDAC1 remains active after removal of acetyl groups from H3 lysines in macrophages, despite the relatively open chromatin structure and transcriptional activity (Fig. 2H). On the other hand, HDAC1 was again responsible for the high histone acetylation status of the *PARP1* promoter in monocytes. Its activity clearly prevails over EP300 in these cells, since acetylase inhibition was insufficient to alter histone acetylation or density on the closed and transcriptionally inactive promoter (Fig. 2L and M). In general, the pattern of histone acetylation correlated well with *PARP1* transcription levels and chromatin accessibility, regardless of the cell type (Fig. 2G-I and 2K-M).

Bearing in mind that loss of histone occupancy/nucleosome density is a chromatin remodelling event that may have a passive mechanism (e.g. nucleosome sliding, relaxation triggered by acetylation itself, loading of transcription machinery) or be actively catalyzed (e.g. by ATP-dependent SWI/SNF activity), we decided to check this mechanism in our cell model. Since the SWI/SNF complex can displace acetylated nucleosomes in an in vitro model [5], we hypothesized that this complex participates in chromatin relaxation associated with histone acetylation in differentiating macrophages, thus leading to enhanced *PARP1* transcription. Interestingly, inhibition of SWI/SNF by iSWI/SNF (PFI3) turned out to phenocopy the effect of iEP300, decreasing *PARP1* expression in macrophages whilst leaving it unchanged in monocytes (Fig. 2J and N). The lack of synergism in *PARP1* repression in macrophages after simultaneous treatment with iEP300 and iSWI/SNF also suggests that the acetylase and the chromatin remodelling complex operate in the same regulatory pathway. Moreover, iSWI/SNF completely prevented the increase in *PARP1* transcription seen after iHDAC administration in both cell types, underscoring the dependence of acetylation effect on SWI/SNF. Together, the data indicate that SWI/SNF enables increased *PARP1* transcription upon promoter acetylation and, in contrast to some previous findings in other cell types, SWI/SNF activity is an essential prerequisite to initiate enhanced gene expression rather than just nucleosome acetylation.

3.3. Displacement of acetylated histones at the PARP1 promoter is mediated by BRM in monocytes and by BRG1 in macrophages, enabling transcription

Analysis of *PARP1* promoter in monocytes and macrophages confirmed that GM-CSF-induced differentiation did not change the relative content of the 2 catalytic ATPase subunits of SWI/SNF, BRM and BRG1, both of which are present at the promoter (Fig. 3A and B, negative control in Supplem. Fig. 3A). However, their individual silencing revealed that BRG1 acted as a transcription co-activator in macrophages (Fig. 3C and D, Supplem. Fig. 3B and C), whereas neither the depletion of BRG1 nor BRM downregulated the already low *PARP1* transcription level in non-differentiated cells (Fig. 3E and F). This was expected, taking into account the lack of effect of iSWI/SNF treatment on the closed and transcriptionally inactive *PARP1* promoter in monocytes. Interestingly, when the acetylation equilibrium was shifted by the presence of iHDAC in monocytes and SWI/SNF activity controlled the increased *PARP1* transcription, silencing of ATPase genes led to the opposite result compared with macrophages: silencing of BRG1 had no significant effect, whereas BRM proved to be the main mediator of transcriptional stimulation (Fig. 3F). Importantly, neither iEP300 nor iHDAC significantly affected the association of BRM or BRG1 with *PARP1* promoter chromatin (Supplem. Fig. 3D–G). The fact that PBRM1 (the component of PBAF complexes that makes use of BRG1 catalytic activity) is enriched at the *PARP1* promoters in macrophages (Supplem. Fig. 3H) suggests that PBAF complex co-occupies gene promoter with BAF in macrophages and, therefore may be responsible for observed switch between BRM and BRG1 activity.

SWI/SNF inhibition was unable to reduce acetylation of histones in macrophages; a significant decrease in acetylation was seen after combined iSWI/SNF and iEP300 treatment (Fig. 3G), but it was indistinguishable from the effect of iEP300 alone. On the other hand, in the same cell type, iSWI/SNF mimicked the effect of iEP300 in increasing nucleosome occupancy (chromatin compaction), with joint inhibition not providing any further heterochromatizing effect on the promoter (Fig. 3H). Conversely in monocytes, the gain of histone acetylation in response to iHDAC treatment led to nucleosome displacement, and SWI/SNF inhibition could prevent the histone loss triggered by restored acetylation (Fig. 3I and J). The effect of iEP300 and iSWI/SNF on chromatin compaction in macrophages, as well as chromatin relaxation by iHDAC in monocytes, was confirmed by DNA I digestion (Supplem. Fig. 3I, exon 8 of PARP1 served as a negative control – undigested region).

Together these results indicate that SWI/SNF removes EP300-acetylated histones, and that EP300-HDAC-SWI/SNF functional crosstalk defines chromatin structure and transcriptional activity of *PARP1* promoter along the monocyte-macrophage differentiation axis.

3.4. EP300-HDAC1-SWI/SNF functional unit operates at other E2F site/CpG island-containing promoters and determines transcription of their genes

Since both EP300 and HDAC1 bind to the CpG island-containing and E2F-driven *PARP1* promoter in monocytes and macrophages (although EP300 was enriched in macrophages, its lower abundance in monocytes remained sufficient to restore histone acetylation and *PARP1* transcription upon deacetylase inhibition), and that *PARP1* is tightly controlled by cell cycle progression, we have compared the genome-wide distribution of EP300 and HDAC1 in chromatin from non-proliferating CD4+ cells and a proliferating myelogenous leukemia cell line, K562, based on available ChIP-Seq data. Irrespective of proliferation status, these 2 enzymes predominantly occur together on chromatin (Fig. 4A, Supplem. Fig. 4A and B). Further profiling of enzyme association with gene promoters (± 2 kbp from TSSs according to UCSC Genes) revealed that > 80% of EP300 peaks in CD4+ cells and > 70% in K562 cells coincided with E2F1 binding sites (Fig. 4B). Considering deacetylase, most of total HDAC1 peaks co-occurred with EP300 at E2F1 motifs in non-proliferating cells, but independent of EP300 in proliferating cells, suggesting that cell cycle progression is of more relevant as a predictor of HDAC1 than EP300 presence at gene promoters. Furthermore, > 60% representation of HDAC1 at EP300 peaks in CD4+ makes EP300 the major co-operator of this deacetylase. Since most E2F1 binding sites coincide with E2F4 sites due to their virtually identical binding specificity, a highly similar pattern of HDAC1 and EP300 binding was also observed for the latter transcription factor (Supplem. Fig. 4C and D). The great majority of E2F1- and E2F4-regulated gene promoters occupied both by EP300 and HDAC1 in non-proliferating and proliferating cell types (Fig. 4C, Supplem. Fig. 4E and F) were characterized by the presence of a CpG island (> 97%) (Fig. 4D).

The search for biological processes that involve products of genes characterized by E2F/CpG promoters and the presence of EP300-HDAC1 revealed circuits related i.a. to regulation of cell cycle transitions, DNA repair or chromatin remodelling (Fig. 4E; the full list of GO enriched terms is given in Supplem. Table GO). To find genes that are regulated by EP300-HDAC1-SWI/SNF complexes, we identified promoters that are considerably more acetylated at H3K27 when compared to those in monocytes (according to MACS2 bdgdiff) and then selected some of these genes, which were overexpressed in macrophages (Log2FC ≥ 1 in macrophages versus monocytes; expression level of particular genes were taken from the BLUEPRINT epigenome project: strand-specific RNA-Seq of rRNA-depleted total RNA from monocytes and macrophages from healthy individuals). Selected genes taken for further consideration based on their known contribution to repair of

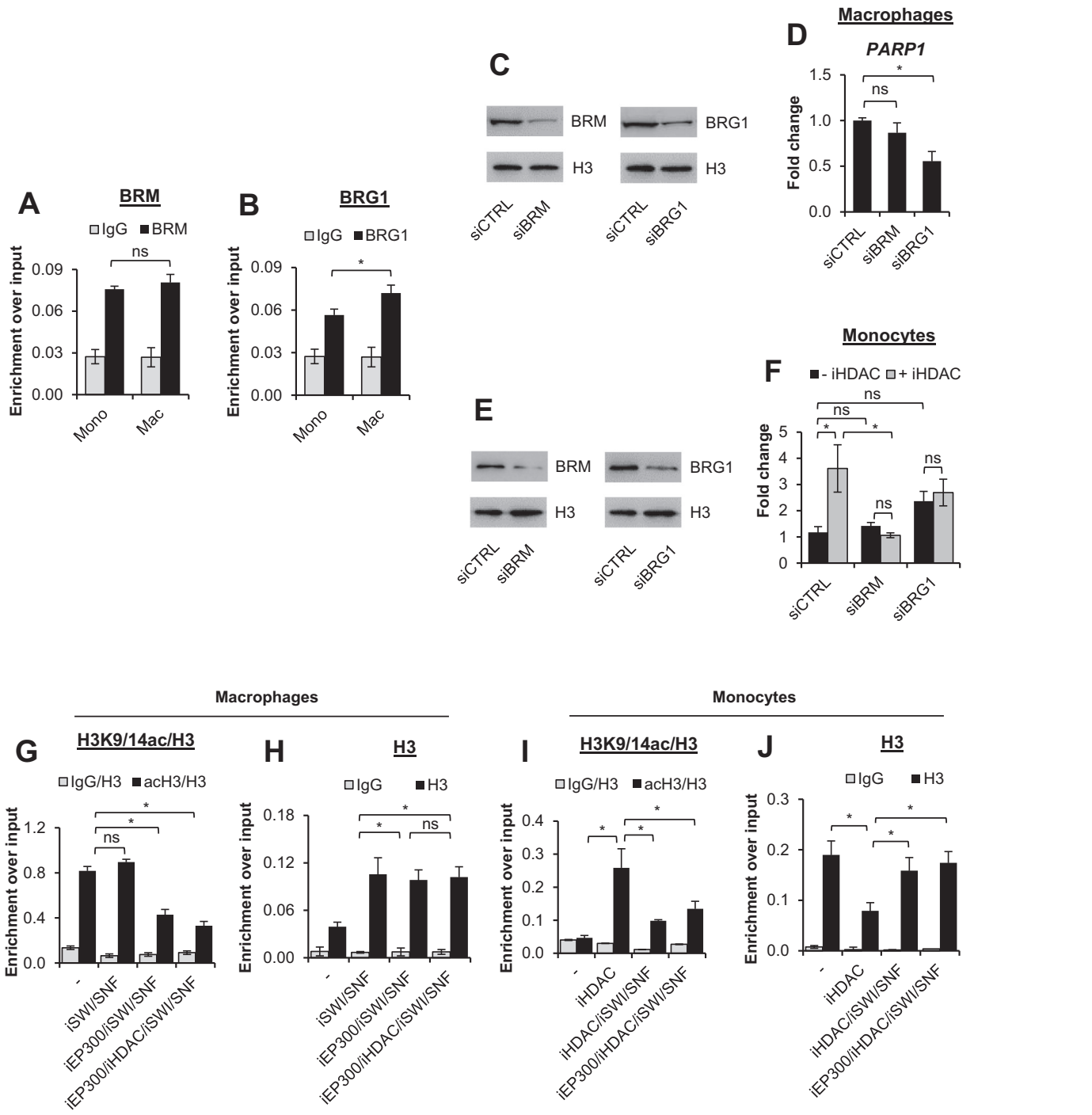
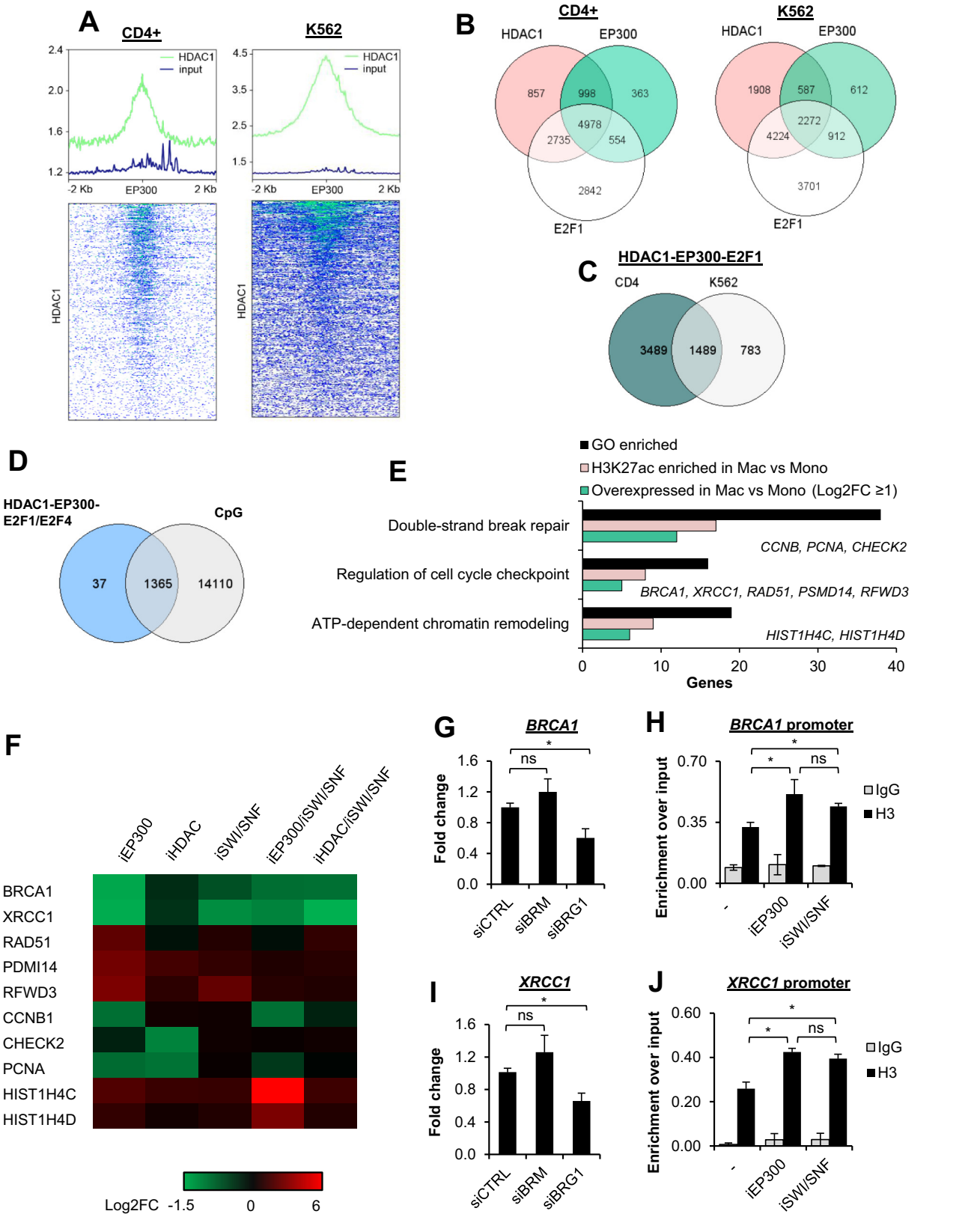


Fig. 3. Histone acetylation is important for SWI/SNF control of nucleosome density at the *PARP1* promoter. BRM (A) and BRG1 (B) presence at the *PARP1* promoter was studied by ChIP-real-time PCR. To study the contribution of both SWI/SNF ATP-ases to regulating *PARP1* transcription in monocytes and macrophages, *BRM* and *BRG1* were silenced and silencing efficiency was confirmed by Western blotting 48 h after cell transfection (C and E), while PARP1 mRNA was quantified by real-time PCR (D and F). To verify the involvement of individual SWI/SNF enzymes ATP-ases in acetylation-dependent recovery of *PARP1* transcription, siRNA-transfected monocytes were treated with iHDAC 24 h prior to analysis (F). Effect of SWI/SNF inhibition on histone acetylation and histone density was measured in macrophages (G, H) and monocytes (I, J) by ChIP-real-time PCR 24 h after administration of iSWI/SNF alone or in combination with iHDAC and iEP300. Bars in the figures represent mean ± standard error of the mean (SEM) and statistically significant differences between means are marked with * when $p < 0.05$ (Student's *t*-test was used to compare differences between two means). For gene expression analysis, two independent experiments were run (each of them in two technical replicates), ChIP-qPCR was repeated twice (two independent experiments in two technical replicates), while Western blots were repeated twice and representative images are shown.

double strand brakes, cell cycle progression and ATP-dependent chromatin remodelling are listed in italics in Fig. 4E. We studied their transcription in response to inhibition of EP300, HDAC1 and SWI/SNF separately or in combination in differentiated macrophages – results are shown in Fig. 4F (extended data in Supplem. Table ANOVA1). Within this cohort, only *BRCA1* and *XRCC1* were transcriptionally controlled by the EP300-HDAC1-SWI/SNF axis described for *PARP1*, whereas for other genes their patterns of response to inhibitors did not lend



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themselves to a similar explanation. Interestingly, genes such as *PCNA* and *CHECK2* that encode proteins involved in cell cycle progression were repressed by iHDAC, whereas *HIST1H4C* and *HIST1H4D* were released from transcriptional repression by simultaneous macrophage treatment with iEP300 and iSWI/SNF. Immunoprecipitation of EP300, HDAC1, BRM and BRG1 demonstrated the lack of these proteins at the promoter of *PCNA* (Supplem. Fig. 5A and B), thereby suggesting that the occurrence of EP300-HDAC1-SWI/SNF at particular gene promoters

Fig. 4. EP300-HDAC1-SWI/SNF functional unit operates at a subset of E2F-dependent promoters to regulate gene transcription. Co-occurrence of EP300 and HDAC1 along the genome was analyzed by studying HDAC1 coverage on EP300 peaks in non-proliferating CD4+ and proliferating K562 cells (A). HDAC1 mapped reads were scored on EP300-enriched regions identified by MACS. The search for overlaps between gene promoters (± 2 kbp from TSSs) and histone acetylase or deacetylase peaks (in BED format) allowed to identify promoters enriched in EP300 and/or HDAC1 in both cell types (B). The same tool was used to find genomic regions characterized by EP300 binding or HDAC1 as well as E2F1 binding motifs (genomic intervals for E2F1 binding sites were derived from table wgEncodeRegTfbsClusteredV3). The corresponding gene lists were then used to create a Venn diagram and indicate E2F1-driven gene promoters occupied simultaneously by EP300 and HDAC1 in CD4+ and K562 (C). The gene promoter list with EP300-HDAC1-E2F1/E2F4 occupancy was compared to the list of CpG island-containing gene promoters (D). The EP300-HDAC1-E2F1/E2F4-binding and CpG island-containing genes were then analyzed for gene ontology; examples of GO enriched terms are shown in (E). Genes listed on the graph were selected as representatives to be further studied for the functional interplay between EP300-HDAC1 and SWI/SNF in human macrophages. mRNA level after 24 h after macrophage treatment with particular inhibitors alone and in combination was measured by real-time PCR and depicted as Log2 fold change heat map (F). *BRCA1* and *XRCC1*, which revealed similar pattern of changes to *PARP1* were further studied with regard to the role of individual SWI/SNF ATPases in regulating their expression by measuring mRNA level of *BRCA1* (G) and *XRCC1* (I) using real-time PCR 48 h after transfection of macrophages with siRNA. Change in H3 density in response to iEP300 and iSWI/SNF (macrophages treated for 24 h) was determined by ChIP-real-time PCR at *BRCA1* (H) and *XRCC1* (J) promoters (the amplified fragments spanned E2F binding motifs). For gene expression analysis, three independent experiments were run (each of them in two technical replicates) and variance among groups was analyzed with one-way ANOVA (genes were marked in bold when $p < 0.05$). Bars represent mean ± standard error of the mean (SEM). Statistically significant differences between means are marked with * when $p < 0.05$ (Student's *t*-test was used to compare differences between two means) for two independent experiments in two technical replicates.

is rather cell type and/or transcription cofactor specific, and/or that EP300 and HDAC1 may act upstream of chromatin in signaling cascades, thereby affecting gene transcription.

Since BRCA1 and XRCC1 are – like PARP1 – involved in DNA repair, we verified the potential similarity of mechanisms regulating their transcription during monocyte to macrophage differentiation. Indeed, their promoters were also occupied by a chromatin remodelling complex comprising EP300, HDAC1, BRM and BRG1, and the composition of subunits did not significantly change during macrophage development (Supplem. Figs. 6A–D and 7A–D). Just like *PARP1*, BRG1 supported *BRCA1* and *XRCC1* transcription from their extensively acetylated promoters in differentiated cells (Fig. 4G and I, Supplem. Figs. 6E–F and 7E–F), and EP300 and SWI/SNF were equally essential for nucleosome depletion (Fig. 4H and J). In monocytes, SWI/SNF also mediated iHDAC-induced chromatin relaxation within these promoters (Supplem. Figs. 6G–H and 7G–H). Promoters of *BRCA1* and *XRCC1* responded to macrophage differentiation with recruitment of PBRM1 and with release of E2F4 (Supplem. Figs. 6I–J and 7I–J). E2F1 that was present at *BRCA1* and *XRCC1* promoters in monocytes (in contrast to *PARP1* promoter) was displaced similarly to E2F4 (Supplem. Figs. 6K and 7K).

Our results indicate that at least 3 functionally linked genes - *PARP1*, *BRCA1* and *XRCC1* responsible for DNA repair and thereby for providing macrophages with increased resistance e.g. to oxidative stress - are transcriptionally controlled by an EP300-HDAC1-SWI/SNF multiprotein complex bound to their E2F-regulated/CpG island-containing promoters. Increased histone acetylation level, determined by the shifted equilibrium between activities of EP300 and HDAC1, primes SWI/SNF for nucleosome eviction, thus activating gene transcription.

4. Discussion

The physiological function of macrophages comprises i.a. their ability to phagocyte and kill invading pathogens. This role, as well as the demanding environmental conditions in tissues in which they reside, makes macrophages potentially vulnerable to several sources of stress and damage, e.g. reactive oxidants released during oxidative burst, pro-inflammatory cytokines or oxidised low-density lipoproteins. Therefore, this cell type has a relatively high requirement for resilient molecular defence mechanisms that should protect intracellular organelles and molecules. One such potential adaptive mechanism is provided by relatively high abundance of proteins contributing to DNA damage response, which, according to our and other findings, is acquired during monocyte commitment to macrophage differentiation, and is controlled at the level of gene transcription [11,23]. Promoters of *PARP1*, *BRCA1* and *XRCC1* genes, all of which encode proteins participating in DNA repair machinery and contributing to maintenance of genomic stability, share the same major features: they contain binding motifs for transcription factors from the E2F family; they include CpG islands; and (as we show here) they undergo transcriptional activation during macrophage differentiation by first gaining EP300-catalyzed histone acetylation and subsequently losing a portion of bound nucleosomes in a SWI/SNF-dependent manner, leading to enhanced gene transcription. Although the PU.1 transcription factor critical for acquiring the macrophage phenotype and function is found at numerous gene promoters in monocytes and macrophages, it does not contribute to activating *PARP1*, *BRCA1* and *XRCC1* expression. This suggests that the EP300-HDAC1-SWI/SNF multiprotein complex may potentially regulate gene transcription and cellular resistance to DNA damage in other cell lineages. Indeed, just as for *PARP1*, *BRCA1* and *XRCC1* have been reported to depend on cell cycle progression, E2F factors that recruit co-repressors (such as RB, RBL2, HDAC1 and PRC2) upon growth inhibition, and CpG methylation status in cancer cells and neurons [24–26]. Although EP300 and RNA Pol II in MCF7 cells were found to associate with the *BRCA1* promoter regardless of estradiol availability and the rate of cell proliferation, HDAC1 (but not EP300) was shown to shuttle to and from this chromatin region depending on the metabolic status, thereby determining nucleosome acetylation and transcription from this promoter. This is in agreement with our data in Fig. 4B and Supplem. Fig. 4C–D, where EP300 peaks are mostly fixed at E2F-driven promoters in both cell types, regardless of proliferation status and HDAC1 occurrence.

Our analysis also indicates that most genes regulated by EP300 are transcribed from E2F-driven promoters. What then is the major foil to EP300 activity at gene promoters? It seems unlikely that the sole recruitment of repressive HDAC1 complexes could single-handedly overcome histone acetylation by EP300, since a relatively large number of gene promoters are simultaneously bound by both enzymes, and yet are transcriptionally active, as in our case are *PARP1*, *BRCA1* and *XRCC1* in macrophages (Fig. 2E and F, Supplem. Figs. 6A–B and 7A–B)). Bearing in mind that HDAC1 inhibition was sufficient to restore histone acetylation and DNA repair gene transcription in monocytes, a plausible hypothesis is that HDAC1 activity modulates directly EP300 activity, a protein capable of autocatalysis and self-acetylation [27]. Deacetylation of this enzyme and its inactivation is normally carried out by SIRT1 and SIRT2; however, the number of acetylated residues in this protein, probably as well as of other post-translational modifications, leaves the gate open for additional action of HDAC1 and HDAC1-associated repressors [28]. Conversely, HDAC1 acetylation by EP300 limits HDAC1 activity, and thus the subtle balance between EP300 and HDAC1 activity likely determines their respective activity and nucleosome acetylation level at equilibrium [29].

Another aspect that requires attention is the permanent and transient distribution of HDAC1 in the genome. While a pool of HDAC1 is recruited to and released from chromatin as a subunit of repressive complexes containing E2Fs and RBs, another subset of the enzyme pool

binds with EP300 to E2F-driven promoters. Although > 97% of gene promoters found to be EP300-HDAC1 enriched both in non-proliferating CD4+ and proliferating K562 cells, and also contained CpG islands, this promoter feature does not in itself determine HDAC1 co-localization with EP300, since both enzymes separately were often found at other E2F1/CpG island-type promoters (Supplem. Fig. 5). Instead, cell type (or rather cell differentiation status within a lineage) may be the deciding factor, as HSPC commitment to monocyte lineage is followed by massive HDAC1 recruitment to the *PARP1* promoter [9]. Following that finding, as we show, HDAC1 is retained on promoter chromatin even during further macrophage development that activated *PARP1* transcription despite the presence of HDAC1. This suggests that other transcription factors and/or transcriptional co-regulators yet to be unidentified mediate HDAC1 deposition on the promoter during initial cell commitment, and that these factors define HDAC1 and EP300 activity balance as well as their mutual interdependence.

Recruitment of EP300-HDAC1-SWI/SNF multiprotein complexes to E2F/CpG island-regulated promoters allows fast and synchronous gene regulation in response to differentiation, cell cycle progression and external stimuli, since all necessary components are in place to allow active elongation upon RNA Pol II release from pausing or repression (e.g. by pause factors, such as DSIF and NELF) [30]. Some selectivity of E2F-RB repressive complexes (e.g. E2F1-RB1 preferably downregulates gene expression upon G1 arrest, whereas E2F4-RBL2 acts in conditions of differentiation or quiescence), along with modulation of the composition of chromatin-associated remodelling enzymes or multiprotein complexes (HDAC1, EP300 and SWI/SNF, but also SUV39H1 or PRC2) provide a further possibility to adapt the transcriptional response to a particular cellular condition or state [9,31,32]. As CpG islands are constitutively enriched in histone modifications associated with ongoing transcription, such as acetylation, this post-translational modification pattern is not entirely lost at the *PARP1* promoter even in its repressed state in monocytes (Fig. 2A and B), which enables fast and efficient activation of gene transcription during macrophage differentiation [33]. With regard to the ubiquity of SWI/SNF function, LPS-inducible genes with CpG island-containing promoters in macrophages do not require SWI/SNF for transcriptional activation, as do most non-CpG promoters characterized by intrinsically accessible chromatin and relative nucleosome deficiency [34]. On the other hand, we show that other E2F/CpG-regulated promoters, e.g. of DNA repair-related *PARP1*, *BRCA1* and *XRCC1* genes, remain closed and transcriptionally inactive where SWI/SNF activity is deficient. Thus, the SWI/SNF requirement for nucleosome exclusion may vary between individual CpG islands in one cell. Since SWI/SNFs can likewise contribute to gene repression (in co-operation with HDAC1 or alone, e.g. both BRG1-RBs and BRG1-RBs-HDAC1 complexes have been detected at the promoters of E2F-dependent cell cycle-related cyclin A, cyclin E and cdc2 genes), we consider it to be potentially another key player, together with EP300-HDAC1, in the postulated “spring” model featuring CpG islands, where transcription decisions simultaneously at many promoters are pending and depend on the chromatin remodelling equilibria [8,35]. Although SWI/SNF subunits were found (among other proteins) bound to the E2F4-RBL2-HDAC1 repressive complex at the *PARP1* promoter in monocytes, inhibition of the chromatin remodelling complex did not unlock transcription [9]. Furthermore, restoration of histone acetylation with iHDAC did not affect the association of BRM and BRG1 with chromatin, suggesting that SWI/SNF occupies rather stably selected gene promoters and contributes to the defining density of histone, while taking part in a delicate steady-state equilibrium that can be swayed by differentiation signals for transcriptional activation. Even more options for the fine-tuning of the SWI/SNF-mediated regulation of gene transcription are indicated by the fact that BRM- and BRG1-containing complexes sometimes reveal functional synergism or antagonism, but their mutual interdependence has not be sufficiently explored [2]. The entire picture becomes even more intricate when we consider the existence of two parallel SWI/SNF complexes: BAF and PBAF, but it may also explain the mutual exclusion between BRM and BRG1 at some gene promoters, in specific cell types or conditions. Our study indicates that PBAF is recruited to *PARP1*, *BRCA1* and *XRCC1* promoters in response to macrophage differentiation, while in monocytes these promoters are deprived of PBRM1, or this protein occurs at considerably lower level. It also agrees with the role of BRM and BRG1 in controling gene transcription in monocytes and macrophages, since BRG1 was responsible for displacement of acetylated histones in PBAF enriched macrophage promoters, while BRM was responsible in monocytes where BAF complexes occurred (Fig. 3D and F, Fig. 4G and I, Supplementary Figs. 3H, 6I, 7I). Interestingly, PBRM1 association with the studied promoters correlated negatively with E2F4 and positively with cell cycle progression, suggesting that these two factors, which depend on each other and define local chromatin landscape by setting transcription activating and repressing complexes, may also define the affinity of BAF and PBAF complexes. In particular, E2F4 eviction is often associated with transcription activation and histone acetylation that tethers PBAF complexes to chromatin. Thus, based on premises described above, cell cycle-dependent genes could serve as easy and convenient targets to study BAF and PBAF mutual interdependence. Another thing that needs to be considered is the BAF and PBAF interaction with transcription factors and cofactors in a cell and tissue-specific manner that defines the composition of chromatin remodelling complexes at gene promoters and enhancers [36]. Also, some BAF and/or PBAF subunits (e.g. DPF3) reveal the ability to act as histone acetylation and methylation readers, thus determining the recruitment of SWI/SNF to the chromatin.

In conclusion, SWI/SNF chromatin remodelling complexes, together with EP300 and HDAC1, constitute a functional unit that controls transcription of functionally-linked genes, such as *PARP1*, *BRCA1* and *XRCC1*. In macrophages, SWI/SNF evicts histones acetylated by EP300 from E2F/CpG island-regulated promoters, thus activating the transcription of DNA repair enzymes repressed by HDAC1 in monocytes. The presence of EP300-HDAC1-SWI/SNF at the gene promoters controlled by E2F transcription factors allows the cells to adapt their gene transcription programme in a coordinated manner to the required (pheno)type of differentiating cells and to alterations in cell proliferation status.

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Transparency document

The Transparency document associated this article can be found, in online version.

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Author contribution statement

A.R. planned the project, performed the experiments, evaluated and analyzed the data and wrote the manuscript. J.P. performed experiments shown in Fig. 1A, B, D, E, H, Fig. 2G and J, Supplem. Fig. 1A (20%). T.P. analyzed gene expression shown in Fig. 4F and carried out statistical analysis show in Supplem. Table ANOVA1 (5%). Ł.P. evaluated and discussed the data and participated in writing the manuscript (5%).

All authors have read and approved the manuscript.

Competing financial interest statement

The authors declare no competing financial interests.

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Article

BRG1 Activates Proliferation and Transcription of Cell Cycle-Dependent Genes in Breast Cancer Cells

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Abstract: Cancer malignancy is usually characterized by unlimited self-renewal. In some types of advanced tumors that are rapidly dividing, gene expression profiles depict elevations in pro-proliferative genes accompanied by coordinately elevated transcription of factors responsible for removal of DNA lesions. In our studies, fast proliferating breast cancer cell lines (MDA-MB-231 and MCF7), BRG1, a component of the SWI/SNF complex, emerges as an activator of functionally-linked genes responsible for activities such as mitotic cell divisions and DNA repair. Products of at least some of them are considerably overrepresented in breast cancer cells and BRG1 facilitates growth of MCF7 and MDA-MB-231 cell lines. BRG1 occurs at the promoters of genes such as *CDK4*, *LIG1*, and *NEIL3*, which are transcriptionally controlled by cell cycle progression and highly acetylated by EP300 in proliferating cells. As previously documented, in dividing cells BRG1 directly activates gene transcription by evicting EP300 modified nucleosomes from the promoters and, thereby, relaxing chromatin. However, the deficiency of BRG1 or EP300 activity for 48 h leads to cell growth arrest and to chromatin compaction, but also to the assembly of RB1/HDAC1/EZH2 complexes at the studied cell cycle-dependent gene promoters. Epigenetic changes include histone deacetylation and accumulation of H3K27me trimethylation, both known to repress transcription. Cell cycle arrest in G1 by inhibition of CDK4/6 phenocopies the effect of the long-term BRG1 inhibition on the chromatin structure. These results suggest that BRG1 may control gene transcription also by promoting expression of genes responsible for cell cycle progression in the studied breast cancer cells. In the current study, we show that BRG1 binding occurs at the promoters of functionally linked genes in proliferating breast cancer cells, revealing a new mechanism by which BRG1 defines gene transcription.

Keywords: BRG1 (Brahma-Related Gene 1); EP300 (E1A Binding Protein P300); gene transcription; breast cancer epigenetics

1. Introduction

Genomic instability and deactivation of DNA repair genes are often associated with tumor-prone phenotypes and necessary for acquisition of tumor initiating mutations. However, from a certain moment of malignancy, the genetic stabilization and maintenance of genome integrity might be required in order to invade tissues and give rise to distant metastases, but the increased ability to repair DNA damage seems to depend on tumor type [1]. Transcription control and the epigenetic landscape that confers high expression of DNA repair genes in some types of malignant cells remain poorly explored. Relatively recent findings indicate that uncontrolled growth and cell division dominate tumor transcriptional programs, and that genes relevant to the removal of DNA damage correlate

with the proliferative status of the tumors [2]. Once again, direct molecular and mechanistical mutual interdependence has not been forthcoming.

Our recently published results provide evidence that transcription of some of DNA repair genes, such as *PARP1*, *BRCA1*, and *XRCC1*, is controlled by SWI/SNF in a cell cycle-dependent manner [3]. In our model of monocyte-to-macrophage differentiation promoters of DNA repair genes, characterized by the presence of CpG island and E2F motifs, were enriched in SWI/SNF-EP300-HDAC1 complexes. Macrophage differentiation that re-entered cell proliferation switched between BRM to BRG1 and HDAC1 to EP300 activity, thus it turned on gene transcription. In brief, in growth arrested monocytes EP300 remained inactive, while HDAC1 deacetylated gene promoters thereby preventing nucleosome eviction by BRM and leading to gene silencing. In proliferating macrophages, EP300 modified nucleosomes, while BRG1 served as acetylation reader and extruded acetylated histones. However, the interdependence of cell proliferation status, EP300 vs. HDAC1 and BRM vs. BRG1 activity remained unexplored.

With respect to other genes and cell types, the role of BRG1 remains ambiguous and a molecular mechanism that turns this enzyme into a gene activator or repressor has not been found. It might be linked with co-operating factors, nucleosome modifications or histone variants, which appear at a given place and time at chromatin [4–6]. Data collected for human monocytes and macrophages provided the first mechanistic insight into proliferation control of gene expression that was mediated by SWI/SNF-EP300-HDAC1 complexes assembled at E2F binding sites. E2F motifs emerged as genetic signatures of the occurrence of BRG1 in the genome. These findings prompted us to test if similar interdependence between BRG1 distribution in genome and gene overexpression occurs in cancer cells, since, in terms of cell cycle status, development of malignancies resembles differentiation of human proliferating macrophages from growth arrested monocytes.

E2F motifs mark proliferation-sensitive gene promoters, which respond to growth arrest by recruiting retinoblastoma-based repressive complexes comprising (depending on the biological circumstances) RB1, RBL1, and RBL2 as its basic components. These proteins co-occur with numerous chromatin writers and erasers, including HDACs, PRC2, DNMT1, SUV39H1, or HP1, all capable of suppressing gene transcription [7–9]. Since in the current study we observed that BRG1 promotes cell cycle progression in the breast cancer cells and that some of DNA repair genes are controlled by the cell proliferation status, we took into consideration a possible functional cross-talk between BRG1 activity, cell divisions and formation of RB1-based repressive complexes. A relatively older report noted BRG1 and RB1 co-operating physically and functionally in the human carcinoma cell line SW13 [10]; however, both proteins contributed to growth arrest. In the other study, BRG1-containing complexes were shown to control cellular proliferation by upregulation of p21 and, thereby, hypophosphorylation of pRB and repression of E2F target genes such as *CDK2*, *CCNE*, and *CCND* [11].

In the current study, we reveal additional complexity to how BRG1 can modulate E2F-dependent transcription. In our model, BRG1 occurs at E2F/CpG-positive, highly acetylated promoters of genes that are overexpressed in breast primary tumor, and two selected highly invasive breast cancer cell lines: MCF7 and MDA-MB-231. Among BRG1-enriched promoters we found genes encoding factors responsible for cancer cell proliferation and resistance to DNA damage. BRG1 dually activates their transcription: (a) directly by acting at the chromatin level and evicting acetylated nucleosomes from their promoters, and (b) indirectly by potentiating cell proliferation and preventing assembly of RB1-HDAC1-PRC2 repressive complexes at the gene promoters. The E2F binding motif at the promoters of some genes, which are functionally linked to cell proliferation and DNA repair in the studied breast cancer cells, allow BRG1-EP300 complexes to provide a common mechanism of gene transcription control.

2. Results

2.1. E2F/CpG Motifs at the Acetylated Gene Promoters Mark BRG1 Distribution in Genome of Breast Cancer Cells

To test if BRG1 may contribute to transcription regulation of genes in fast proliferating breast cancer cells, we investigated whether this enzyme co-occurs genome-wide with any particular histone mark that is known for its involvement in transcription control. For this analysis, we took publicly available data from ChIP-Seq experiments for BRG1 and selected histone modifications, and calculated Pearson correlation coefficient between their co-distribution in the genome of MDA-MB-231 cells. Genomic occurrence of BRG1 showed it was most strongly correlated with histone acetylation and H3K4me3, which are usually associated with gene promoters and active transcription (Figure 1A). Lack of reciprocity between enzyme and H3, as well as weak negative co-occurrence with H3K27me3, seem to further confirm a previously postulated mechanism, where BRG1 evicted histones from transcriptionally permissive promoters and enabled gene expression. In human macrophages, BRG1/H3K27ac-positive promoters are characterized by binding motif for E2F (indicative of likely gene dependence on cell cycle status) and/or the CpG island [3]. To test whether distribution of BRG1 is associated with similar chromatin and DNA features in proliferating breast cancer cells, MDA-MB-231, we looked for overlapping regions adjacent to TSS (±2 kbp), which are characterized by the occurrence of BRG1, H3K27ac, E2F motifs, and CpG islands. As shown in Figure 1B and Table S1, the great majority of BRG1-rich promoters was simultaneously acetylated and featured by CpG island, while to a lower extent by E2F motif. This analysis also supported the previously postulated mutual interdependence between occurrence of BRG1 and H3K27ac at the gene promoters.

Promoters characterized by all four considered features—BRG1/H3K27ac/E2F/CpG—were associated with genes involved in key cellular processes, among them DNA repair and mitotic divisions (Figure 1C, Table S2). Analysis of differential expression of genes belonging to the two mentioned GO groups showed that substantial part of these genes was overexpressed in primary tumor and 2 cancer cell lines compared to normal cells (Figure 1D). Genes that were suppressed in cancer cells were not further repressed by SWI/SNF inhibition, suggesting that BRG1 association with acetylated promoters is insufficient under certain chromatin circumstances to ensure a high transcription rate. Among the top 50 transcription factors and chromatin remodelers that appear at the promoters of genes overexpressed in cancer cells (Log2FC > 0.4), we identified enzymes EP300 and HDAC1, which we previously showed to create a functional unit with BRG1 at gene promoters in human macrophages (Figure 1E). The presence of TBP and POLR2A suggests that BRG1-based complexes may also contribute to polymerase release or pausing. POLR2A pre-initiation complexes are documented to often feature CpG-driven promoters. Since the overall majority of BRG1/H3K27ac promoters (>85%) carry CpG signature and only approximately half of them the motif for E2F, CpG islands might be considered a predictive hallmark of BRG1-dependent gene transcription. Nevertheless, chromatin-bound or signaling factors that suppress transcription from BRG1/H2K27ac promoters have yet to be identified.

To check if BRG1 creates a multi-enzyme complex with EP300 and HDAC1 in proliferating cancer cells, we searched for the latter two enzymes in BRG1 co-immunoprecipitates. As shown in Figure 1F, immunoprecipitation of BRG1 confirmed the physical interaction between the studied SWI/SNF component, acetylase and deacetylase. Furthermore, EP300 and BRG1 were also recently documented by us to create a functional and transcription regulating unit with PARP1 in MCF7 and MDA-MB-231 cells, where PARP1 poly-ADP-ribosylated and, thereby, activated EP300 [11].

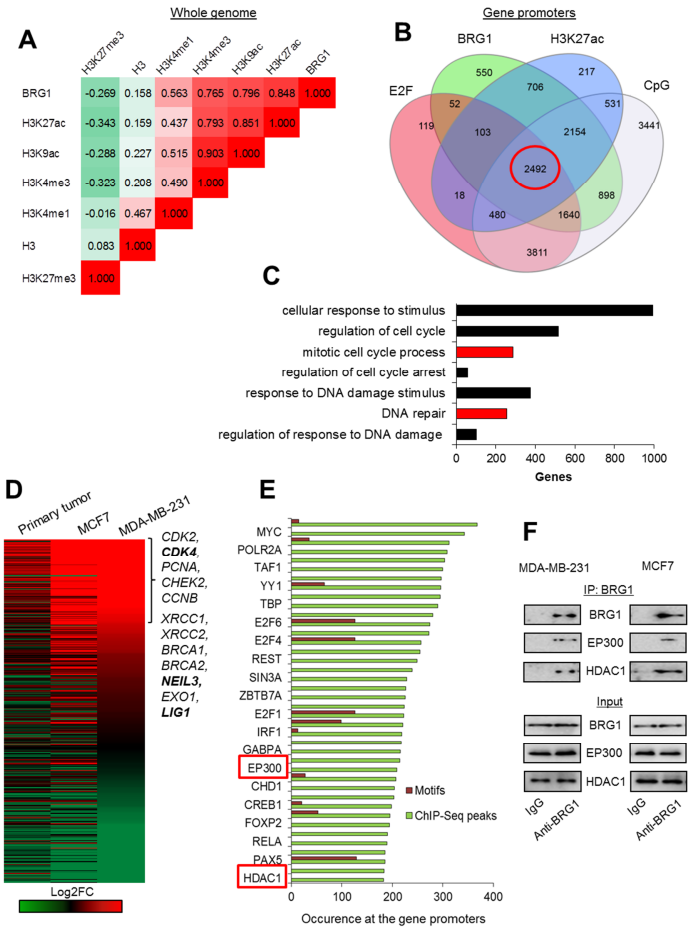

Figure 1. BRG1 occurs at the acetylated promoters of some highly transcribed genes, which control proliferation and DNA repair in breast cancer cells. (**A**) BRG1 co-distribution with histone H3 density and histone modifications in the genome of MDA-MB-231 is shown as Pearson's correlation coefficient. (**B**) Occurrence of BRG1 at the acetylated gene promoters characterized by E2F binding site and CpG island has been quantified on a Venn diagram and BRG1/H3K27ac/E2F/CpG promoters are marked in red circle. Green and blue circles represent gene promoters enriched in BRG1 and H3K27ac peaks according to MACS, while grey and red represent promoters featured by the presence of CpG islands according to cpgIslandExt and E2F binding motifs according to cpgIslandExt and wgEncodeRegTfbsClusteredV3, respectively. (**C**) Functional association of BRG1/H3K27ac/E2F/CpG gene promoters (marked in red circle in (**B**) leads to enrichment of intracellular processes that can define cancer physiology. Red bars represent biological processes, which are taken for further analysis in (**D**,**E**). (**D**) Analysis of differential gene expression from data derived from RNA-Seq confirms overexpression of genes functionally assigned to the mitotic cell cycle and to responses to stimuli of DNA damage in cancer cell lines versus normal breast cells. Genes marked in bold were taken as examples for further analysis in Figure 2A–D. (**E**) BRG1/H3K27ac/E2F/CpG promoters of genes overexpressed in cancer cells (**D**): Log2FC > 0.5 for at least 2 of the cell types used are characterized by common transcription factors and chromatin remodelers. Green columns correspond to the number of ChIP-Seq peak occurrences at the gene promoters according to UCSC wgEncodeRegTfbsClusteredV3, whereas red columns represent the occurrence of transcription factor binding motifs according to tfbsConsSites. Only every other transcription factor is labeled. (**F**) Immunoprecipitation of BRG1 allows to detect EP300 and HDAC1 by western blot and indicates the physical interaction between SWI/SNF component and the latter two enzymes. Pictures show cropped areas of western blots. Whole images are included in Figure S3.

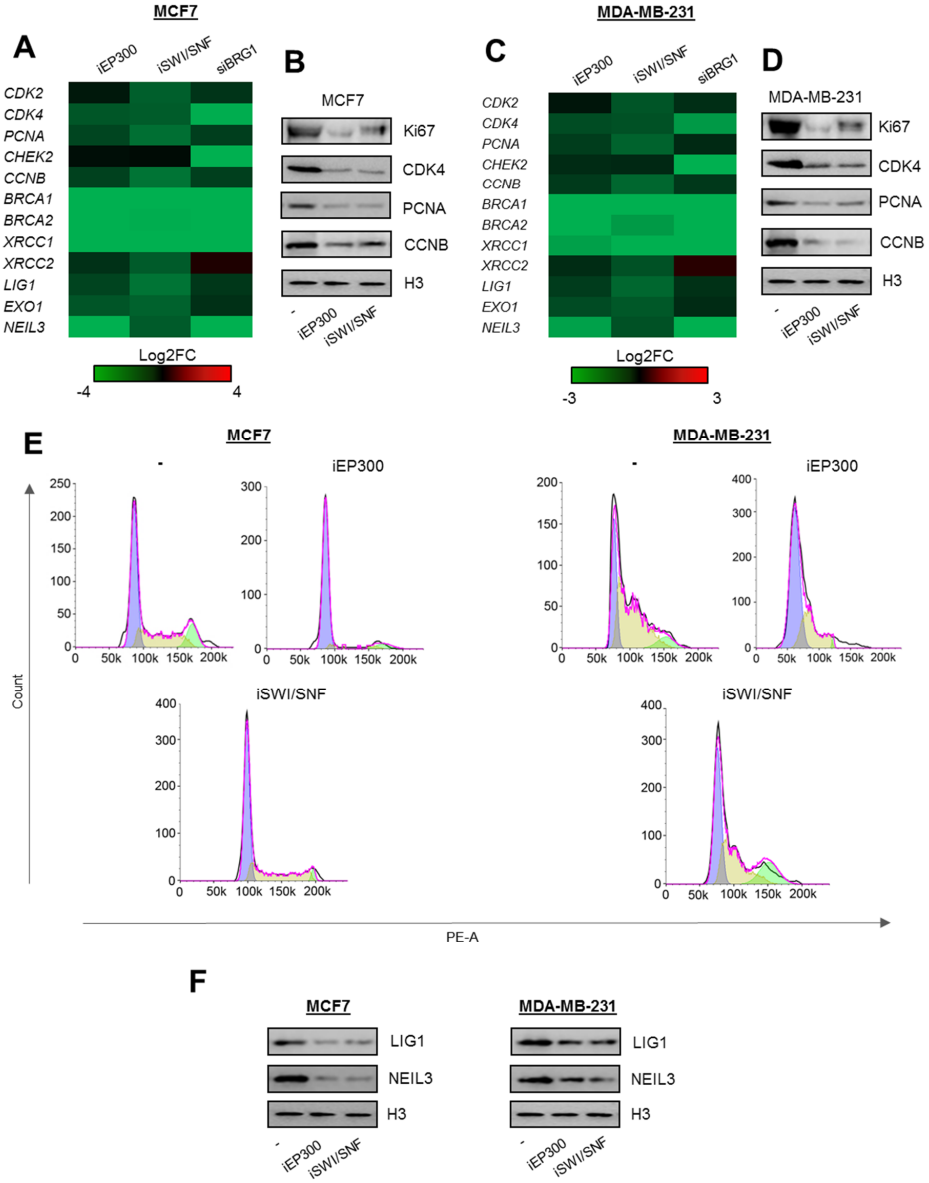

Figure 2. Proliferation and DNA repair-relevant gene promoters are controlled by BRG1 and EP300 activity in breast cancer cells. (**A**,**C**) Long-term (48 h) deficiency of BRG1 and EP300 activity limits transcription of genes encoding components of cell division and DNA repair mechanisms. The data present Log2FC of mRNA level measured by real-time PCR normalized to untreated control (iEP300 and iSWI/SNF) and non-template control (siBRG1). (**B**,**D**) Gene repression results in substantial loss of proliferation markers as seen from western blot, and by cell growth arrest in G1 phase (**E**). (**F**) Inhibition of BRG1 and EP300 similarly decreases the amount of proteins involved in removal of DNA damage. Heatmaps with long-term BRG1 and Ep300 deficiency (**A**,**C**) were prepared from four independent replicates for iEp300, and iSWI/SNF; from 3 for siBRG1. Detailed statistical analysis is presented in supplementary Table S3. Western blots in B, D, and F show representative images from three independent experiments. Pictures show cropped areas of western blots. Whole images are included in Figure S3. Analysis of the cell cycle was carried out twice in two technical replicates for each condition.

2.2. BRG1-EP300 Complexes Drive Transcription of Some Proliferation and DNA Repair Genes

To verify the possible contribution of BRG1-EP300 to transcription regulation of selected genes functionally linked to proliferation and removal of DNA damage, we treated MCF7 and MDA-MB-231

cells with inhibitors of EP300 (iEP300–C646–blocks acetylatransferase activity of EP300 and CBP), BRG1 (iSWI/SNF–PFI3 is a bromodomain inhibitor of BRG1 and BRM) and calculated Log2FC of mRNA level quantified by real-time PCR between inhibitor treated and control cells. A high transcription rate of at least some genes, which were found overexpressed in all cancer cell types according to Figure 1D, crucial for cell divisions (*CDK2*, *CDK4*, *PCNA*, *CHEK2*, *CCNB*) and removal of DNA damage (*BRCA1* and *BRCA2*, *XRCC1* and *XRCC2*, *LIG1*, *EXO1*, *NEIL3*—contribute to BER, NER, SSBR, MMR, and HR) [12,13], was simultaneously maintained by BRG1 and EP300 (Figure 2A,C). Inhibition of both enzymes led to substantial suppression of most genes studied in Figure 2A,C. Since the only commercially available inhibitor of SWI/SNF (PFI-3) also blocks the activity of BRM and BRG1, we targeted the latter enzyme with siRNA (Figure S1A–D). *XRCC2* was the only exception and was upregulated in BRG1 deficiency. Although iEP300 causes simultaneous inhibition of EP300 and CBP, the data for siEP300 (Silencer™ Select Pre-Designed siRNA ID:s534247) is missing due to the fact, that substantial EP300 silencing with siRNA induced dramatic cell death.

Repression of proliferation-relevant genes by the deficiency of BRG1 and EP300 activity led to a substantial reduction in protein proliferation markers (also others than BRG1 targets—Ki67) and to cell cycle arrest in G1 (Figure 2B,D,E). Similarly, DNA repair gene repression was followed by loss of LIG1 and NEIL3 protein upon EP300 and SWI/SNF inhibition (Figure 2F).

2.3. Cell Cycle-Dependent Chromatin Composition Controls Transcription of Proliferation and DNA Repair Genes

To study in detail the molecular mechanism of BRG1 contribution to regulation of transcription and possible functional interdependence with the cell cycle progression, we focused on three promoters of genes, which are crucial for cell proliferation—*CDK4* and effective removal of DNA lesions—*LIG1* and *NEIL3*. As shown in Figures 1B and 2A–F), all three genes are transcriptionally controlled by BRG1 and EP300. Their promoters are featured by the presence of BRG1, EP300, CpG island, and E2F motif. Especially, due to the last feature, they are likely to respond to alterations in cell proliferation status and to assemble RB1-based repressive complexes. Therefore, these promoters were chosen to study the considered cross-talk between BRG1 activity, chromatin remodeling, and cell cycle status. Furthermore, CDK4 deficiency caused by BRG1-dependent gene repression, that in cell cultures results in cell growth arrest, can be easily phenocopied by the use of CDK4/6 inhibitors, which equally arrest cells in G1. Thus, from this point the following hypotheses were tested: (a) BRG1/H3K27ac/CpG/E2F promoters of proliferation and DNA repair genes respond to G1 arrest with decreased gene transcription and formation of RB1-based repressive complexes; (b) long-term inhibition of BRG1-EP300 complexes that results in G1 arrest also leads to RB1-based chromatin remodeling at the studied, cell cycle-dependent promoters.

First, to test hypothesis quoted in (a) we checked whether the arrest of cell cycle progression triggered by the inhibition of cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) that blocks cells in G1 (Figure 3A; the same as iSWI/SNF and iEP300—Figure 2E) suppresses gene expression (Figure 3B), thereby also reducing the protein level (Figure 3C). This led to recruitment of RB1-based repressive complexes to promoters of representative genes: *CDK4*, *LIG1*, and *NEIL3* (the DNA fragment analyzed overlapped the E2F motif within the CpG island) (Figure 3D). All three regulatory regions shared two common features, an increase in H3 density and a decrease in histone acetylation that likely resulted from the switch between EP300 and HDAC1 activity. These phenocopy previously described mechanism that drives proliferation-dependent and BRG1-EP300-HDAC1-mediated chromatin transition from permissive to a compacted structure, where gain of HDAC1 activity in growth arrested cells prevents nucleosome extrusion by BRG1 due to loss of histone acetylation. Unexpectedly, G1 arrest enhances BRG1 association with 2 out of 3 studied gene promoters. However, also inhibition of THP-1 proliferation with mimosine resulted in the massive recruitment of both SWI/SNF ATPases to the promoter of *PARP1* as described in our previous study [14]. As currently we observed such an effect only for two genes, it seems to be promoter or transcription co-factor dependent, but may represent

still unknown mechanism of SWI/SNF contribution to repression of cell cycle responsive promoters under certain conditions as documented in Dunaief et al [10]. BRG1 seems to occur and act as activator of transcription by displacing acetylated histones in proliferating cells, but at some gene promoters gets further and strongly enriched upon the growth inhibition.

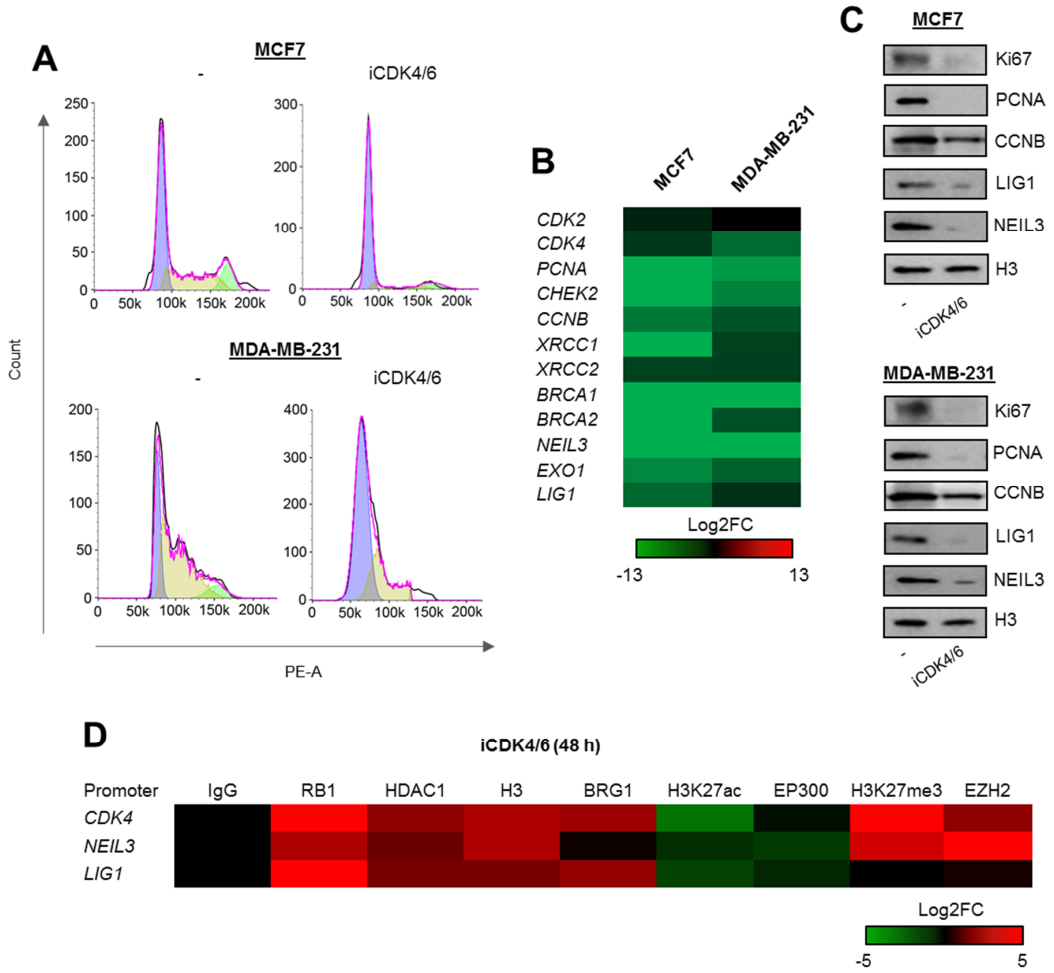

Figure 3. DNA repair genes are transcriptionally controlled by the cell cycle progression. (**A**) Deficiency of CDK4/6 activity results in complete cell cycle blockade in G1. (**B**,**C**) Inhibition of CDK4/6 for 48 h leads to repression of genes that drive cell cycle progression and genome resistance to damage. mRNA ((**B**) shows Log2FC versus untreated) and protein (**C**) was monitored by real-time PCR and western blot in breast cancer cells exposed to iCDK4/6 for 48 h. Pictures in (**C**) show cropped areas of western blots. Whole images are included in Figure S3. (**D**) G1 arrest induces considerable chromatin remodeling and adjustment of protein composition to gene repressive conditions at the promoters of *CDK4*, *NEIL3*, and *LIG1* (ChIP/real-time PCR) 48 h after administration of iCDK4/6 in culture of MCF7 cells. Analysis of the cell cycle progression (**A**) was monitored twice in two technical replicates for each condition, quantification of gene expression in response to iCDK4/6 (**B**) and ChIP/real-time PCR analysis of *CDK4*, *NEIL3* and *LIG1* promoters (**D**) were carried out 4 times, while western blots were repeated 3 times and representative images are shown in (**C**). Detailed statistical data can be found in Table S3.

Increase in BRG1 association with gene promoters induced by G1 arrest was followed by displacement of EP300, which may (in addition to recruitment and activation of HDAC1) further explain reduced nucleosome acetylation. The assembled repressive complex was supported by polycomb repressive complex 2 (PRC2) at the promoters of *CDK4* and *LIG1*. The enzymatically active subunit of PRC2–EZH2 catalyzed methylation of lysine 27 of histone H3, that is known to cause gene suppression. Of note, PRC2 was found only at two of these gene promoters, and did not correlate with

enrichment or extrusion of other possible components of the repressive complex. This may indicate that PRC2 targets chromatin in a promoter rather than in a RB1-dependent manner, and that the presence of RB1 is a prerequisite, but not essential, for PRC2 contribution to gene silencing.

2.4. BRG1 Couples Cell Divisions with Transcription of DNA Repair Genes

To search for a molecular mechanism that links BRG1/EP300 activity with cell cycle-dependent gene transcription, we analyzed the impact of BRG1 and EP300 on chromatin structure at the chosen gene promoters (Figure 4A). Long-term inhibition of both EP300 and SWI/SNF shown to arrest proliferating cells in G1 (Figure 2E) results in substantial chromatin remodeling, with recruitment of RB1, HDAC1, and an increase in nucleosome density. This phenomenon phenocopied the effect of iCDK4/6 inhibition on promoter composition (Figure 3D) and provides evidence that repression of genes, which potentiate cell division, leads to growth arrest-dependent modification of chromatin architecture. As expected, iEP300 reduced acetylation of histones, whereas iSWI/SNF prevented displacement of acetylated nucleosomes, thereby confirming a direct action of BRG1 associated to gene promoters. As for EP300 and BRG1, their occurrence seemed to be controlled by the type of enzyme inhibited (cell treatment with iBRG1 or iEP300) and in a promoter-dependent fashion, but the tendency for EP300 to be displaced from chromatin regardless of the inducer of cell growth arrest was also noted. Consistently to the effect of iCDK4/6, however, EZH2 was not enriched at the *LIG1* promoter, whereas it faintly methylated lysine residues at the promoters of *CDK4* and *NEIL3*.

To confirm that long-term effect of deficiency of BRG1 activity on gene transcription is mediated by BRG1 gene targets which drive cancer cell proliferation (e.g., *CDK4*, *PCNA*, *CCNB*), we checked their transcription rate and chromatin features which are representative of BRG1/EP300 proximate effects (H3 density and nucleosome acetylation), and also looked for cell cycle-related chromatin remodeling (RB1) after short-term MCF7 cell treatment with inhibitors (Figure 4B,C). Loss of BRG1 and EP300 activity immediately reduced gene transcription (Figure 4B) and induced chromatin compaction without a considerable recruitment of RB1 (Figure 4C). In a similar way to long-term deficiency in enzyme activity, decreased acetylation was only found with EP300 inhibition clearly indicates that BRG1 defines chromatin composition dually—by evicting acetylated histones (which ensures transcriptionally permissive and an open chromatin state), and by favoring cell proliferation (precludes assembly of RB1-based repressive complexes).

To check which of the two described BRG1-dependent modes of chromatin rearrangements is crucial in growth arrested cells, we restored promoter acetylation by treating growth inhibited cells with iHDAC (Figure S2). Reinstatement of nucleosome acetylation (represented by H3K27ac) was insufficient to open chromatin and re-initiate gene transcription in the presence of RB1 that remains associated with gene promoters (Figure 4D). This suggests that cell cycle status overrides the BRG1 activating effect on gene transcription during growth arrest. BRG1-driven expression of proliferation-relevant genes, which potentiates cell divisions, impacts chromatin structure in addition to direct, nucleosome eviction-relevant chromatin opening. Thus, the final chromatin composition at the studied BRG1-driven promoters is defined by cell cycle-dependent and independent aspects, where first corresponds to synthesis of drivers of mitotic divisions, while the latter to acting as a transcriptional co-factor (Figure 4E).

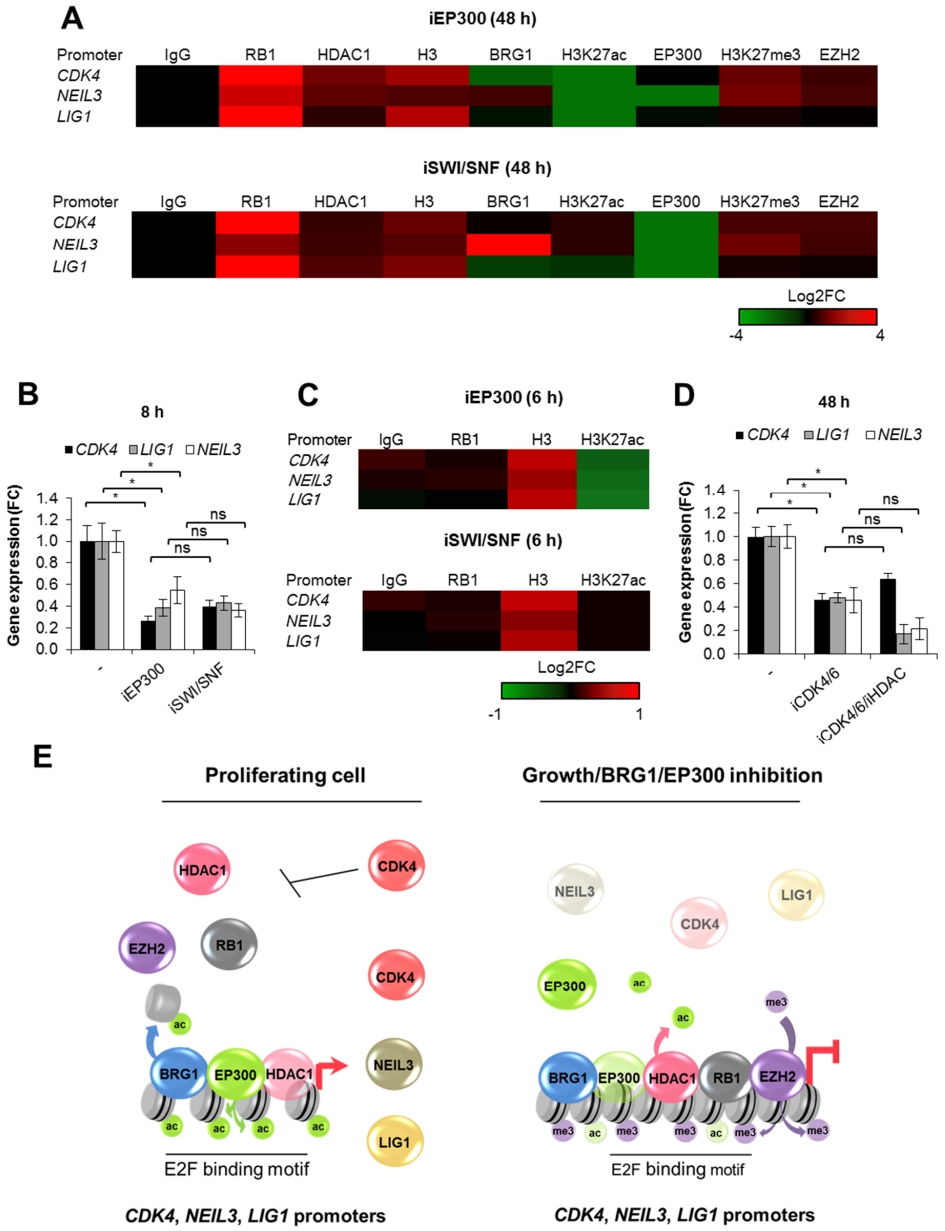

Figure 4. BRG1-dependent cell cycle progression defines chromatin composition at the promoters of DNA repair genes. (**A**) Long-term (48 h) deficiency of BRG1 and EP300 activity induces substantial changes in the chromatin structure, and recruitment of cell cycle-dependent repressors to the promoters of genes involved in cell proliferation and response to DNA damage (ChIP/real-time PCR). (**B**) Short-term (8 h) inhibition of BRG1, EP300, but not CDK4/6, results in transcription restraint (nascent RNA quantification by Click-iT chemistry), and (**C**) immediate chromatin closure without RB1 association with the studied gene promoters (ChIP/real-time PCR) of MCF7 cells. (**D**) Gene transcription (mRNA level; real-time PCR) was measured 48 h after MCF7 cell treatment with iCDK4/6. iHDAC was added 24 h prior to RNA isolation to restore chromatin acetylation. (**E**) Graphic representation of immediate and late chromatin response to cell growth arrest, BRG1 and EP300 inhibition. In the presented model, BRG1 and EP300 stimulate cell proliferation and, therefore, activate transcription of genes, which are transcriptionally driven by cell cycle progression. Data presented in figures (**A**–**D**) are presented as mean of four independent replicates (n = 4). Detailed statistical data can be found in Table S3.

3. Discussion

The SWI/SNF chromatin remodeling complex has attracted increasing attention, particularly in cancer biology due to mutations, deletions and insertions in BRG1, and in some of the SWI/SNF core subunits (e.g., SNF5, ARID1A) in ~20% of human tumors. Mutations associated with gain and loss of function, as well as fluctuation in the expression of SWI/SNF components, are linked to the occurrence of cancer and its progression in several ways [15,16]. BRG1 was initially considered as a tumor suppressor, based on, for example, premises from mouse model of primary cells, where inactivation of Brg1 and Snf5 leads to an overall decrease in nucleosome occupancy at a large number of promoters, products of which potentiate cell proliferation [17]. However, more recent data provides evidence that overexpression of some SWI/SNF subunits apparently lacking mutations can be seen as an alternative mechanism by which cellular transformation occurs [18]. As for breast cancer, analysis of the genomic data from TCGA database showed <2% mutation frequency in invasive breast carcinomas [19], whereas elevated expression of BRG1 occurred in 35% to nearly 100% of analyzed primary tumors and is responsible for the high proliferation rate; it also served as a predictive marker for patients at high risk of developing metastases [20,21]. A BRG1-dependent increase in cancer cell division was assigned to (a) upregulated expression of enzymes responsible for fatty acid and lipid biosynthesis by BRG1, which binds at the loci encoding these genes; (b) induction of ABC transporter expression, particularly in response to drug treatment; and (c) association with ER and ER-mediated transcriptional activation [18,22]. Our study also shows the genomic aspect that explains BRG1's role in breast cancer progression, where the activity of this enzyme maintains a high transcription rate of genes encoding direct drivers of cell proliferation that can act at different cellular levels—signaling cascades (*CDK2*, *CDK4*, *CCNB*—cell cycle phase progression), checkpoints (*CHEK2* responses to genome integrity assessment), DNA replication (*PCNA*—DNA clamp and scaffold for DNA polymerase) and many others listed in Table S2. BRG1 was enriched at >2500 acetylated promoters in MDA-MB-231 cells (Figure 1B); thus their functional association goes beyond proliferation and DNA repair, and comprises inter alia cellular metabolism, response to stress, and regulation of transcription from POLR2A (Figure 1C), i.e., all three mentioned aspects related to BRG1's role in defining the potential of cancer cells to proliferate. Another crucial feature in cancer biology that renders these cells resistant to anticancer treatment includes the abovementioned presence of ABC transporters in the membrane and DNA repair enzymes in the cytoplasm/nucleoplasm. The latter genes have been documented by us in the current paper to be transcriptionally controlled by BRG1/EP300 complexes in the breast tumors. Thus, a deficiency in BRG1 activity may make cancer cells vulnerable to drugs in two ways, by inhibiting drug efflux, and impairing the removal of DNA damage. An attempt based on BRG1 inhibition might be of particular importance in the treatment of double- or triple-negative breast cancer characterized by the absence of the estrogen receptor (ER), the progesterone receptor (PR) and low to normal levels of HER2, notably where therapeutic approaches targeting these proteins is impossible and patients can benefit only from less specifically targeted cytotoxic drugs. BRG1 functional co-operation with some nuclear receptors were already documented in relatively old reports. Human BRM/hSNF2 alpha and BRG-1/hSNF2 beta, counterparts of yeast SWI2/SNF2 and the *Drosophila* brahma, were identified as activators of estrogen and the retinoic acid receptors at the gene promoters which respond to nuclear receptors [23]. A later mechanistic study revealed that ER targets BRG-1 to the promoters of estrogen-responsive genes in a manner that occurs simultaneously to histone acetylation [24]. BRG1-mediated structural remodeling of chromatin that led to hormone-dependent transcriptional activation by nuclear receptors, was shown to operate in a collaborative manner with histone acetyltransferases such as CREB-binding protein (CBP), p300, and PCAF; this result agrees with our model of co-operation between BRG1 and EP300. These findings also pay attention to the possible use of BRG1 inhibitors or antagonists (once discovered) in the treatment of ER positive cancers in future. In another study, BRG1 suppressed prostate specific antigen (*PSA*) that is transcriptionally controlled by androgen receptor [25]. However, in that model, BRG1-dependent gene repression occurred under the androgen antagonists'-induced growth arrest, where prohibitin recruited BRG1 to

PSA promoter and caused eviction of EP300. Also, our study shows that enrichment of BRG1 at some gene promoters was associated with displacement of EP300 from chromatin in response to inhibition of cell proliferation with iCDK4/6, iEP300 and iBRG1. Thus, BRG1 impact on *PSA* expression should be also checked in dividing cells. BRG1 may act as gene activator or suppressor at the target gene promoter depending on the cell cycle status, which can possibly switch the BRG1 role from extrusion of acetylated nucleosome to insertion of their deacetylated form. Again, the molecular trigger (e.g., co-factor, mutual interdependence with BRM, posttranslational modification, element upstream of specific proximal promoter [26]) that can change the influence of the SWI/SNF complex on target gene activation remains unknown.

In the context of possible use of BRG1 inhibitors in anticancer therapies, of particular importance is our finding regarding BRG1's co-operation with EP300, since only one non-specific inhibitor of BRG1 is commercially available for scientific purposes. Due to a similar degree of repression of BRG1-dependent genes by EP300 inhibition (Figure 2A–D), such an option extends the possibility of gene targeting by a wider range of compounds. Notably, BRG1 (and perhaps EP300) inhibition might be considered as a therapeutic strategy in other cancer types because BRG1 is overexpressed in other tumor types, although limited insights into how different SWI/SNF subunits drive the development of tumors and complex nature of contribution to defining specific oncogenic pathways clearly requires further investigation [15].

Even at the genomic level, chromatin features or DNA sequence that guide BRG1 distribution and association with particular gene promoters or regulatory regions remain poorly defined. It is well acknowledged that this enzyme marks actively transcribed genes and correlates with nucleosome acetylation and trimethylation of H3K4me3, but spatiotemporal mutual interdependence between BRG1's occurrence and covalent modifications of nucleosomes are also unknown. Since in our study's SWI/SNF complexes turned out to be readers of nucleosome acetylation governed by EP300-HDAC1 balance intwo distinct cell types (human macrophages and two breast cancer cell lines), one might think that BRG1 confers active gene transcription in other cancer types characterized by BRG1 overexpression.

Apart from the documented BRG1 role at gene promoters, cancer cell divisions that are transcriptionally controlled by this enzyme regulate expression of numerous functionally-related genes, which may set the phenotype of breast cancer cells. Their link with proliferation is primarily through the presence of the E2F motif, which responds to cell cycle status by recruiting or releasing retinoblastoma-based complexes. However, only ~60% of BRG1/H3K27ac/CpG/E2F-positive genes are overexpressed in fast dividing cells (Figure 1E), suggesting that promoter acetylation in proliferating cells is insufficient to activate transcription, and that unidentified co-factors of BRG1 must operate on or upstream of chromatin. In addition to promoting transcription of genes that potentiate mitotic division, BRG1 has been documented to regulate cell proliferation by co-operating with p53. This tumor suppressor, which displays growth and transformation inhibition functions, adds another link to controlling some of E2F promoters by BRG1. hSNF5 or BRG1 inhibit p53-mediated cell growth arrest and apoptosis by enhancing p53 binding to the promoter of p21—cyclin-dependent kinase inhibitor [27,28]. Such a mode of BRG1 contribution to p53-mediated cell cycle control was observed in MCF7 cells treated with doxorubicin. Recently, BRG1 was found as an activator of p53 transcription in mice embryo-derived P19 cells [29]. These findings make the entire picture of BRG1-proliferation-transcription of cell cycle-controlled genes even more complex, since it depicts BRG1 as a pathway-dependent player. In mitotic dividing cells it promotes proliferation and expression of proliferation-dependent genes, whereas upon cellular stress and p53 activation it contributes to cell growth arrest, where it was shown to repress i.a. nuclear receptor-induced gene transcription [25].

As long as the abundance of DNA repair enzymes might not be of particular importance or even antagonize cancer development, the described functional dependence of cancer cell growth on transcription of enzymes capable of removing DNA lesion might be of key importance, not only for cell protection from anticancer therapeutics, but for the maintenance of cancer cell viability and metastases since production of proliferation-associated reactive oxygen species challenges genome integrity and

might limit tumor progression. Therefore, BRG1 turns out to be considerable regulator of breast cancer cell physiology. Malignancy-relevant cell cycle re-entrance from a differentiated cell state may globally activate the desired gene expression in a unified mode due to the placement of BRG1-EP300-HDAC1 complexes at promoters that have CpG islands (>85% of BRG1/H3K27ac peaks are associated with CGIs; Figure 1B). However, such a hypothesis requires further experimental confirmation. CpG islands were thought to indicate SWI/SNF independence attributed to the assembly of CpG islands into unstable nucleosomes, but occurrence of BRG1 at the such promoters strongly suggests the possible function of this enzyme in transcription regulation of genes driven by CpG promoters. However, the molecular mechanisms that regulate transcription initiation and elongation from the paused POLR2A pre-initiation complex has not been disclosed [30].

Our study shows that BRG1 makes use of BRG1-activated proliferation promoting gene products and shifts chromatin composition towards a transcriptionally permissive state by allowing nucleosome acetylation. Modified nucleosomes can be further displaced by promoter-bound BRG1. Although BRG1 was found at repressed gene promoters together with RB1, HDAC1, and PRC2, its presence may allow immediate activation of gene transcription upon cell cycle re-entrance [7,31]. Such an idea has been postulated in the past, where BRG1's association with the promoter of osteocalcin was considered to be a mechanism that ensures re-activation of gene transcription after removal of the proliferation inhibiting factor(s) [32]. However, that paper and others lack information on any mechanistic link between particular chromatin-bound components. Our study explains at least some BRG1-relevant controversies regarding its occurrence, especially at proliferation-driven gene promoters. In dividing cells, BRG1 occurs at the E2F motifs of some cell cycle-dependent genes and, by potentiating expression of genes that promote mitotic cell divisions, BRG1 prevents silencing of their transcription.

4. Materials and Methods

4.1. Materials

MCF7 and MDA-MB-231 cell lines were purchased from ATCC and Sigma Aldrich, respectively. DMEM High Glucose w/L-Glutamine w/Sodium Pyruvate, fetal bovine serum and antibiotics (penicillin and streptomycin) were from Biowest (CytoGen, Zgierz, Poland), L15 Medium, iEP300 (C646), iSWI/SNF (PFI-3), iCDK4/6 (PD0332991, palbociclib, Imbrance); anti-rabbit IgG (A0545) and anti-mouse IgG (A4416) (whole molecule)–peroxidase antibody produced in goat, BLUeye prestained protein ladder (#94964), oligonucleotides for real-time PCR were from Sigma Aldrich (Poznan, Poland). Anti-HDAC1 (PA1-860), anti-EP300 (PA1848), Lipofectamine RNAiMAX, OptiMem, Dynabeads™ Protein G, High-Capacity cDNA Reverse Transcription Kit, Click-iT™ Nascent RNA Capture Kit for gene expression analysis, SuperSignal™ West Pico Chemiluminescent Substrate, TRI Reagent™, Silencer™ Select Pre-Designed siRNA ID:s13141 (BRG1), RNase A were from Thermofisher Scientific (Thermofisher Scientific, Warsaw, Poland), while iCDK4/6 (PD0332991) was from Cayman Chemical (Biokom, Janki/Warsaw, Poland).

KAPA HiFi™ HotStart ReadyMix (2X) from KapaBiosystems and Takyon™No ROX SYBR Core Kit blue dTTP from Eurogentec were purchased from Polgen (Łódź, Poland). EvaGreen® Dye, 20X in water was purchased from Biotium (Corporate Place, Hayward, CA, USA). WB antibodies: anti-BRG1 (sc-17796), anti-DNA Ligase I (sc-271678), anti-CDK4 (sc-23896), anti-NEIL3 (sc-393703), anti-PCNA (sc-56), anti-Ki-67 (sc-23900), and anti-cyclin B (CCNB; sc-166210) were purchased from were Santa Cruz Biotechnology (AMX, Lodz, Poland). ChIP grade antibodies: normal rabbit IgG (#2729), BRG1 (#49360), H3K27ac (#4353), anti-histone H3 (#4620), anti-RB1 (#9313), anti-H3K27me3 (#9733), anti-EZH2 (#5246) were purchased from Cell Signaling Technology (LabJOT, Warsaw, Poland).

4.2. Cell Culture and Treatment with Inhibitors

MCF7 were cultured in DMEM supplemented with 10% FBS, penicillin/streptomycin (50 U/mL and 50 µg/mL, respectively) in 5% CO_2, whereas MDA-MB-231 in F15 medium supplemented with

15% FBS, penicillin/streptomycin (50 U/mL and 50 μg/mL, respectively) without CO_2 equilibration. Both cell lines were maintained in a logarithmic growth phase in a culture and prior to the treatment with any compound. iEP300 (10 μM; C646), iSWI/SNF (10 μM; PFI-3) and iCDK4/6 (1 μM; PD0332991) were added to cells 48 h prior to analysis. Concentration of the studied inhibitors and the time required to induce enzyme inhibition were chosen based on our and other reports. iEP300 and iCDK4/6 at the doses higher than tested induced dramatic cell death in response to longer incubation periods.

4.3. Quantification of Gene Expression

For mRNA quantification the total RNA was extracted with TRI Reagent™, reverse transcribed with High-Capacity cDNA Reverse Transcription Kit and selected cDNA fragments were amplified in real-time PCR (Takyon, Eurogentec; CFX96 C1000 Touch, BioRad Warsaw, Poland) using the following primer pairs: *CDK2*, 5′-CAGGATGTGACCAAGCCAGT-3′ (forward) and 5′-TGAGTCCAAATAGCCCAAGG-3′ (reverse); CDK4, 5′-CTGGTGTTTGAGCATGTAGACC-3′ (forward) and 5′-AAACTGGCGCATCAGATCCTT-3′ (reverse), XRCC2, 5′-TCGCCTGGTTCTTTTTGCA-3′ (forward) and 5′-TCTGATGAGCTCGAGGCTTTC-3′ (reverse), BRCA2, 5′-CTTGCCCCTTTCGTCTATTTG-3′ (forward) and 5′-TACGGCCCTGAAGTACAGTCT-3′ (reverse), LIG1, 5′-CAGAGGGCGAGTTTGTCTTC-3′ (forward) and 5′-AGCCAGTTGTGCGATCTCTT-3′ (reverse), EXO1, 5′-AAACCTGAATGTGGCCGTGT-3′ (forward) and 5′-CCTCA TTCCCAAACAGGGACT-3′ (reverse), NEIL3, 5′-GGTCTCCACCCAGCTGTTAAAG-3′ (forward) and 5′-CACGTATCATTTTCATGAGGTGATG-3′ (reverse), PCNA, 5′-TCTGAGGGCTTCGACACCTA-3′ (forward) and 5′-TTCTCCTGGTTTGGTGCTTCA-3′ (reverse); CHEK2, 5′-CAGGTTCTAGCCCAGCCTTC-3′ (forward) and 5′-ACGGAGTTCACAACACAGCA-3′ (reverse); CCNB1, 5′-TGGAGAGGTTGATGTCGAGC-3′ (forward) and 5′-AAGCAAAAAGCTCCTGCTGC-3′ (reverse); BRG1, 5′-AAGAAGACTGAGCCCCGACATTC-3′ (forward) and 5′-CCGTTACTGCTAAGGCCTATGC-3′ (reverse), BRCA1, 5′-TGCCCACAGATCAACTGGAA-3′ (forward) and 5′-CACAGGTGCCTCACACATCT-3′ (reverse); XRCC1, 5′-CGGCGGAAACTCATCCGATA-3′ (forward) and 5′-CCATCAGGGCCTCCTCAAAG-3′ (reverse); ACTB, 5′-TGGCACCCAGCACAATGAA-3′ (forward) and 5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′ (reverse). GAPDH and B2M were from Human Toll-like Receptor Signaling Primer Library (HTLR-I). ACTB, GAPDH, and B2M (HSKG) were used for normalization. Data in figures are shown as Log2FC with respect to untreated control.

Nascent *CDK4*, *NEIL3*, and *LIG1* mRNA were measured 8 h after cell treatment with iEP300 and iSWI/SNF using Click-iT® Nascent RNA Capture Kit as described previously and was normalized to *ACTB* [33].

For protein detection cell lysates were separated with SDS-PAGE, transferred to nitrocellulose membranes and stained overnight with primary antibodies (1.5:10,000) at 4 °C. After staining with HRP-conjugated secondary antibodies (1:10,000; room temperature; 2 h), the signal was developed using SuperSignal™ West Pico Chemiluminescent Substrate and acquired with ChemiDoc-IT2 (UVP, Meranco, Poznan, Poland).

4.4. Evaluation of Cell Proliferation

Cell cycle progression in MCF7 and MDA-MB-231 cells treated with iCDK4/6, iEP300 and iSWI/SNF for 48 h was analyzed by flow cytometry as described previously [3]. Additionally, protein level of Ki67, PCNA and CCNB was monitored in cell lysates by western blot.

4.5. Chromatin Immunoprecipitation

Chromatin immunoprecipitation was carried out according to the protocol previously described [12,19]. Fragments spanning BRG1/H3K27ac/E2F/CpG sites in selected gene promoters were amplified using KAPA HiFi™ HotStart ReadyMix supplemented with EvaGreen® Dye and 4% DMSO and the following primers: *CDK4* prom, 5′-ATAACCAGCTCGCGAAACGA-3′ and 5′-AGAGCAATGTCAAGCGGTCA-3′, *LIG1* prom, 5′-AACACACTCAGATCCGCCAG-3′

and 5′-GCTTCCACCGATTCCTCCTC-3′, *NEIL3* prom, 5′-GTAGGGAGCGACCTCAACAG-3′ and 5′-AGTACAGCCTGGTCCTTCCA-3′.

4.6. Transient Gene Silencing

For BRG1 silencing MCF7 and MCD-MB-23 were seeded at the density of 100,000 cells per well and transfected on the following day with RNAiMAX-siRNA complexes prepared in OptiMem according to the following ratio: 20 nmol siRNA and 3 µL of transfection reagent. BRG1 silencing versus non-template control siRNA was confirmed by real-time PCR and western blot 48 h after cell transfection.

4.7. ChIP-Seq Analysis in Galaxy Version 19.05.dev

Publically available, generated by other groups and deposited in the PubMed Central Database data from MDA-MB-231 cells were taken for ChIP-Seq analysis in Galaxy Version 19.05.dev [34]: BRG1—GSM1856026 (SRR2171350), GSM1856027 (SRR2171351) and GSM1856028 (SRR2171352), H3K27ac—GSM1855991 (SRR2171311) and GSM1855992 (SRR2171312); H3K4me3—GSM1700392 (SRR2044734), H3K4me1—GSM2036932 (SRR3096750 and SRR3096751), H3K27me3—GSM949581 (SRR513994), H3K9ac—GSM1619765 (SRR1820123 and SRR1820124), H3—GSM2531568 (SRR5332805), Input—GSM1964894 (SRR2976843). FASTQ quality formats were unified to sanger formatted with FASTQ Groomer [35]. Reads were aligned to Human Genome version 19 using Map with Bowtie for Illumina and unmapped reads were filtered out [36]. ChIP-seq peaks were called in MACS with P value cutoff for peak detection set at 10^{-3} [14].

Co-distribution of BRG1 and selected histone modifications in the whole genome was studied by MulitBamSummary/plotCorrelation [37]. In brief, bam files with mapped reads for BRG1 and all studied histone modifications were taken as samples and the genome coverage was computed for equally sized bins (bin size in bp = 1000). For the heatmap in Figure 1A matrix file was generated from the multiBamSummary tool and Pearson correlation was calculated.

Gene promoters enriched in BRG1, H3K27ac, CpG islands, and E2F were identified by returning intersects of recalled peaks and genomic regions ±2000 bp centered on TSS (overlapping intervals of both datasets) [38]. Genomic intervals for E2F1, E2F4, and CpG Islands were taken from UCSC Main tables wgEncodeRegTfbsClusteredV3 and cpgIslandExt, respectively. Venn diagrams were created in http://www.interactivenn.net/ from gene lists. Annotation of differentially expressed genes to gene ontology terms was carried out in GOrilla (using two unranked lists of genes and complete list of genes expressed in MDA-MB-231 as a background).

The following data from normal breast, primary tumor, MCF7 and MDA-MB-231 cells were taken for RNA-Seq analysis: normal breast—GSM1695870 (SRR2040339), GSM1695872 (SRR2040341), GSM1695873 (SRR2040342), GSM1695874 (SRR2040343), GSM1695877 (SRR2040346), GSM1695878 (SRR2040347); primary tumor—GSM1695891 (SRR2040360), GSM1695898 (SRR2040367), GSM1695899 (SRR2040368), GSM1695882 (SRR2040351), GSM1695890 (SRR2040359), GSM1695894 (SRR2040363); MCF7—GSM2422725 (SRR5094305), GSM2422726 (SRR5094306), GSM2422727 (SRR5094307), GSM2422728 (SRR5094308), GSM2422729 (SRR5094309), GSM2422730 (SRR5094310); MDA-MB-231 - GSM2422731 (SRR5094311), GSM2422732 (SRR5094312), GSM2422733 (SRR5094313), GSM2422734 (SRR5094314), GSM2422735 (SRR5094315), GSM2422736 (SRR5094316).

Having FASTQ quality formats unified to sanger formatted with FASTQ Groomer reads were mapped to Human Genome version 19 using TopHat [39]. Transcripts were assembled with Cufflinks (using UCSC Known Gene as a reference annotation) and merged with Cuffmerge [40]. Differential gene expression in cancer versus normal breast cells was calculated with Cuffdiff and shown as heatmap for two selected GOs (mitotic cell cycle process and DNA repair). Frequencies of transcription factors and chromatin remodelers occurrence at the promoters of genes that were overexpressed in all three cancer cell types (Log2FC > 0.5) were scored based on results of bedtools Intersect intervals

(gene promoters spanning 2 kbp from TSS and UCSC Main tables wgEncodeRegTfbsClusteredV3 and tfbsConsSites) [38].

4.8. Statistical Analysis

Data in Table S3 are shown as mean ± standard deviation of the mean (SEM). Student's *t*-test was used to determine statistically significant differences between two means (marked with * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$), while one-way analysis of variance (ANOVA) was carried out in GraphPad Prism 5 to compare means in several groups (marked with * when $p < 0.05$, ** when $p < 0.01$, *** when $p < 0.001$).

5. Conclusions

Summarizing, activity of one chromatin-associated enzyme defines the expression profile of numerous genes in breast cancer cells by acting directly on chromatin, and by promoting cell cycle progression. BRG1 removes acetylated nucleosomes, thereby facilitating transcription and preventing recruitment of retinoblastoma-based repressive complexes to E2F-driven promoters. Thus, BRG1 defines key breast cancer features in the cell lines we have investigated. Inhibitors of BRG1 and EP300 can be considered as future anticancer drugs that can arrest cell growth and/or render them sensitive to DNA damaging agents.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/12/2/349/s1, Figure S1: Efficiency of BRG1 silencing; Figure S2: Effect of HDAC inhibitor on gene promoters in G1 arrested cells; Figure S3: Whole western blot images; Table S1: BRG1 and H3K27ac distribution at the E2F/CpG positive gene promoters; Table S2: Gene ontology; Table S3: Statistical analysis.

Author Contributions: Conceptualization: A.R.; Project administration: A.R.; Supervision: A.R.; Resources: A.R.; Investigation: M.S., J.P., T.P. and A.R.; Data curation: M.S., J.P., T.P. and A.R.; Methodology: A.R.; Validation: T.P.; Writing—original draft: A.R.; Writing—review and editing: M.S., J.P. and T.P. All authors have read and agreed to the published version of the manuscript.

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PARP Traps Rescue the Pro-Inflammatory Response of Human Macrophages in the In Vitro Model of LPS-Induced Tolerance

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Abstract: Secondary infections cause sepsis that lead to patient disability or death. Contact of macrophages with bacterial components (such as lipopolysaccharide—LPS) activates the intracellular signaling pathway downstream of Toll-like receptors (TLR), which initiate an immune proinflammatory response. However, the expression of nuclear factor-kappa B (NF-κB)-dependent proinflammatory cytokines significantly decreases after single high or multiple LPS stimulations. Knowing that poly(ADP-ribose) polymerase-1 (PARP1) serves as a cofactor of NF-κB, we aimed to verify a hypothesis of the possible contribution of PARP1 to the development of LPS-induced tolerance in human macrophages. Using *TNF-α* mRNA expression as a readout, we demonstrate that PARP1 interaction with the *TNF-α* promoter, controls macrophage immunoparalysis. We confirm that PARP1 is extruded from the gene promoter, whereas cell pretreatment with Olaparib maintains macrophage responsiveness to another LPS treatment. Furthermore, cell pretreatment with proteasome inhibitor MG132 completely abrogates the effect of Olaparib, suggesting that PARP1 acts with NF-κB in the same regulatory pathway, which controls pro-inflammatory cytokine transcription. Mechanistically, PARP1 trapping allows for the re-rebinding of p65 to the *TNF-α* promoter in LPS-stimulated cells. In conclusion, PARP traps prevent PARP1 extrusion from the *TNF-α* promoter upon macrophage stimulation, thereby maintaining chromatin responsiveness of TLR activation, allowing for the re-binding of p65 and *TNF-α* transcription.

Keywords: immunoparalysis; lipopolysaccharide tolerance; macrophages; sepsis; poly(ADP-ribose) polymerase-1 (PARP1)

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1. Introduction

Sepsis-induced immunoparalysis is a complication of secondary infections that compromises the immune response [1]. It is a life-threatening dysfunction caused by a dysregulation of the host response to infection. It leads to death or disability of patients and costs to the healthcare system [2,3]. Immune response in sepsis is characterized by the excessive pro-inflammatory response to pathogen-associated molecules, such as endotoxin (e.g., lipopolysaccharide (LPS) from *Escherichia coli*), with a simultaneous decreasing anti-inflammatory mechanism. Many models, including in vitro macrophage cultures, reveal that multiple LPS stimulation induces attenuation of pro-inflammatory response. This phenomenon is known as "endotoxin tolerance" [2,4]. The contact of bacterial LPS with the Toll-like receptor 4 (TLR4) on the macrophage cell surface induces secretion of numerous proinflammatory cytokines and factors, such as tumor necrosis factor-α (TNF-α); interleukins IL-1β, IL-6, and IL-8; macrophage inflammatory protein 2 (MIP2); and cyclooxygenase-2 (COX2) [5,6].

Stimulation of TLR4 activates the nuclear factor-kappa B (NF-κB)/mitogen signaling pathway, which involves several adaptor proteins, including myeloid differentiation factor 88 (MyD88), IL-1 receptor-associated kinase (IRAK), and tumor necrosis factor-alpha receptor-associated factor 6 (TRAF6), and thus leads to transcription of NF-κB-dependent

pro-inflammatory cytokines [7,8]. NF-κB transcription factor consists of few subunits—p65 (RelA), RelB, c-ReL, p50 (NF-κB1), and p52 (NF-κB2)—that form homo- or heterodimers in the cytoplasm. The phosphorylation and degradation of inhibitor of κB proteins (IκB) in the canonical allow for the translocation of the NF-κB dimers into the nucleus, where they activate transcription as heterodimers (p50–p65) or repress transcription as homodimers. Moreover, p50 homodimers repress the inflammation by recruitment of co-repressors, such as histone deacetylases (HDACs), which remove acetyl groups from histones, thereby leading to chromatin condensation and transcription repression [9].

Transcription activation by canonical NF-κB heterodimers often requires cofactors, such as poly(ADP-ribose) polymerase-1 (PARP1). The enzyme belongs to the PARP family that comprises 17 members, which vary in the mode of protein mono- and poly-ADP-ribosylation, subcellular location, as well as in cellular function. A growing number of processes has been shown to be regulated by PARP1, which covalently modifies its targets by utilizing nicotinamide adenine dinucleotide (NAD) to synthesize poly-ADP-ribose polymers as well as physically interacts with various proteins and DNA, thereby tuning their activity, structure, or biological function [10]. By the interaction with p300 acetyltransferase, PARP1 coactivates NF-κB-dependent gene expression in response to single LPS stimulation. It also promotes the formation of the preinitiation complex at the gene promoters in the transcription-factor-binding regions [11,12]. This enzyme was postulated to contribute to the development of inflammatory disorders, such as rheumatoid arthritis, diabetes, neurodegenerative disorders (including Parkinson's and Alzheimer's), infarction–reperfusion, and septic shock. Numerous recent scientific reports suggest that PARP inhibition serves as a promising strategy to treat immune diseases [13,14]. Some PARP inhibitors, including olaparib, act as PARP1–DNA traps at the sites off single-strand breaks, and prevent the repair of (and enhance) the generation of the double-strand breaks, which are particularly detrimental for breast cancer 1/2 (BRCA1/2)-deficient tumors [15]. In this study, we demonstrate that PARP1 trapping on the chromatin with olaparib in LPS-stimulated cells may have a new application in maintaining an NF-κB-dependent pro-inflammatory response.

2. Results

2.1. PARP Trapping Prior to Macrophage Activation Maintains Their Pro-Inflammatory Response

To test the possible contribution of PARP1 to the development of endotoxin-induced tolerance of human macrophages, we first optimized the doses of LPS, which paralyze the pro-inflammatory response of considered phagocytes. We used transcription of *TNF-α* (mRNA) as a readout and set a 24 h window between two consecutive cell treatments with LPS (Figure 1a). As shown in Figure 1b, two higher concentrations of LPS substantially blocked the expression of TNF-α, thereby indicating the development of macrophage tolerance to endotoxin. For these two concentrations, we checked the immunomodulatory potential of olaparib, which acts as a PARP1–DNA trap, inhibits PARP activity, and prevents protein ADP-ribosylation. Macrophage pretreatment with 1 μM olaparib for 1 h prior to tolerance induction maintained the pro-inflammatory response of phagocytes (Figure 1b). Due to the observed higher variation of TNF-α expression in macrophages stimulated with 10 ng/mL LPS, and its lower biological relevance, we chose 1 ng/mL LPS for the induction of immune paralysis in the following experiments. In contrast to macrophages, human monocytes that are PARP1-deficient did not respond to olaparib and showed tolerance to bacterial endotoxin regardless of their pretreatment with the PARP inhibitor (Supplementary Figure S1) [16].

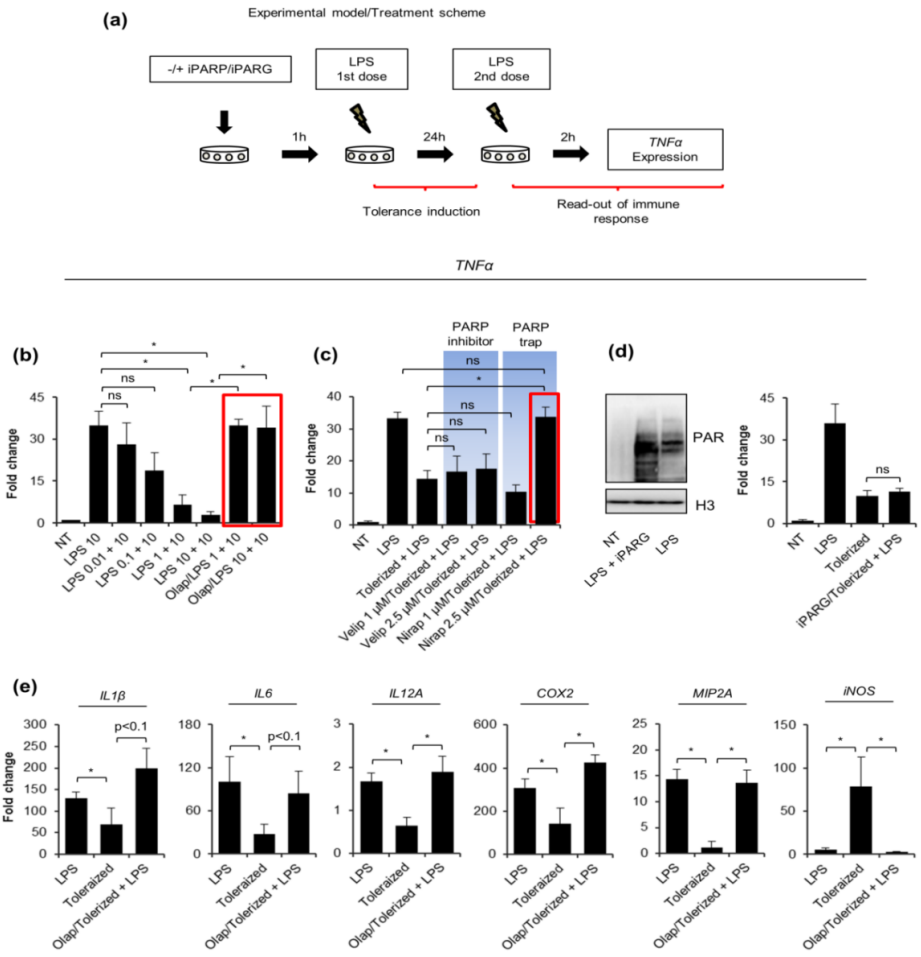

Figure 1. PARP traps maintain proinflammatory response in human macrophages activated with tolerance-inducing LPS doses. (**a**) The scheme of tolerance induction presents an experimental approach and cell treatments: the first dose of LPS aims to induce cell paralysis or priming within 24 h, the second serves to check macrophage pro-inflammatory response using mRNA of TNF-α as a readout after cell stimulation for 2 h. To test a possible effect of the key enzymes involved in the ADP-ribosylation metabolism (PARP inhibitors: olaparib, veliparib, niraparib, and PARG inhibitor: ADP–HPD) on the tolerance development, the corresponding compounds were added for 1 h prior to the tolerance-inducing (first) dose of bacterial endotoxin. Expression of TNF-α was quantified by real-time PCR, normalized to median of ACTB and GAPDH, and shown as a fold change with respect to control cells (LPS untreated = 1). (**b**) Three doses of LPS were tested for macrophage paralysis and the immunomodulatory effect of PARP inhibitor—olaparib (1 μM) was estimated based on the TNF-α transcription. The red rectangular indicates the couple: olaparib-LPS that was chosen for further experiments. (**c**) The other two PARP inhibitors, which differ in PARP-DNA binding ability—niraparib (MK-4827) and veliparib (ABT-888), as well as (**d**–**e**) PARG inhibitor—ADP–HPD (10 μM) were analyzed for their possible effect on the induction of tolerance by LPS. (**d**) The increased accumulation of ADP-ribosylated proteins caused by ADP–HPD pretreatment of LPS-induced macrophages was confirmed by western blot. H3 was used as a loading control. Full-length western blot images are included in the Supplementary Figure S3. (**e**) The possible modulatory role of PARP1 in the paralysis of macrophage pro-inflammatory phenotype was tested also for other cytokine genes, such as IL-1β, IL-6, IL-12, MIP2A, COX2, and iNOS using real-time PCR for the measurement of mRNA levels. Bars in the figures represent mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA1) was carried out in GraphPad Prism 5 to compare means in several groups. Once the significance was detected, ANOVA1 was followed by the Tukey post-hoc test and significant differences between the two considered means are marked with * when significant at $p < 0.05$, ns—non-significant at $p > 0.05$. Abbreviations: iPARP—poly(ADP-ribose) polymerase (PARP) inhibitor(s), iPARG—poly(ADP-ribose) glycohydrolase (PARG) inhibitor, LPS—lipopolysaccharide, Olap—olaparib, Velip—veliparib, Nirap—niraparib, TNF—tumor necrosis factor, IL1β—interleukin 1 beta, IL6—interleukin 6, IL-12A—interleukin 12 subunit alpha, COX2—cyclooxygenase-2, MIP2A—macrophage inflammatory protein 2 subunit alpha, iNOS—inducible nitric oxide synthase, ACTB—actin beta, GAPDH—glyceraldehyde 3-phosphate dehydrogenase.

To distinguish between the possible effect of ADP-ribosylation and PARP trapping on the tolerance development, we involved another two PARP inhibitors that differ in the DNA-binding potential. Olaparib and niraparib are far more effective in PARP trapping than veliparib at the concentrations where they fully inhibit PARylation [14,17]. Moreover, we tested two concentrations for each of the two compounds. None of the studied PARP inhibitors increased the *TNF-α* transcription under the LPS-free condition (Supplementary Figure S2). Results in Figure 1c indicate the anti-paralyzing potential of Niraparib, which was particularly prominent at the higher dose. The lack of impact of veliparib on *TNF-α* transcription in tolerized cells provides further evidence that the interaction of some PARP family members (PARP1, PARP2, PARP3) with the chromatin, rather than ADP-ribosylation, somehow contributes to macrophage paralysis. To further support such a hypothesis and confirm that ADP-ribosylation does not promote the tolerance to endotoxin, the mRNA of *TNF-α* was quantified in cells deficient in the activity of poly-ADP-ribose-degrading enzyme poly-(ADP-ribose) glycohydrolase (PARG). As shown in Figure 1d,e, the accumulation of poly-ADP-ribose polymers due to the pretreatment of macrophages with PARG inhibitor ADP–HPD did not alter *TNF-α* transcription.

Since pro-inflammatory response involves numerous other cytokines in addition to TNF-α, we checked the profile of *IL1β*, *IL6*, *IL12A*, *MIP2A*, *COX2*, *iNOS* transcription under the same experimental conditions in differentiated human macrophages (Figure 1e). Most of them, but *iNOS*, showed considerable decline in responsiveness after the first stimulation with 1 ng/mL LPS. As same as for *TNF-α*, the applied PARP trap maintained or even increased the cytokine transcription after the second dose of bacterial endotoxin when compared to 10 ng/mL LPS alone.

These results all indicate that the PARP occurrence on chromatin during the first challenge with LPS rescues the pro-inflammatory phenotype of human macrophages.

2.2. PARP1 Level Serves as a Key Determinant of Tolerance Development

Since, among other PARP family members, PARP1 has been most frequently linked to canonical NF-κB signaling and documented as inflammatory-relevant factor, we first paid attention to this enzyme. Due to the lack of commercially available and specific PARP1 inhibitors, we decided to make use of both silencing and overexpression of the enzyme. Bearing in mind that mRNA targeting in phagocytes is of a considerable challenge and that cationic transfection reagents uncouple LPS binding by preventing CD14–TLR4 interactions (we failed to induce tolerance to LPS in chemically transfected human macrophages), we changed macrophages to THP1 human monocytic cell line to generate stable PARP1 knockdowns [18].

First, we tested these cells as a model to study the principles of tolerance induction to LPS and checked whether PARP trapping also protects these cells from LPS-induced tolerance. Results in Figure 2a indicate that THP1 cells develop resistance to LPS and stop responding to the consequent dose of bacterial endotoxin. Similar to macrophages, olaparib protects *TNF-α* from LPS-triggered repression. Knowing that PARP1 is assigned to the synthesis of over 80% of ADP-ribose polymers and is frequently documented as a transcription coregulator, we first followed the PARP1 occurrence at the *TNF-α* promoter in intact cells and cells challenged with bacterial endotoxin. We searched for PARP1 in the region overlapping the NF-κB-binding site (Figure 2b) since this enzyme was documented in various contexts as a cofactor of NF-κB-dependent transcription of cytokines in macrophages. The results of ChIP-qPCR confirmed the occurrence of PARP1 at the *TNF-α* promoter and the extrusion of the enzyme, as soon as 1 h after LPS treatment (Figure 2c). Olaparib, which is of relatively high trapping potency, maintained PARP1 on the chromatin. In summary, Olaparib prevents PARP1 extrusion from *TNF-α* promoter in response to LPS, and maintains TNF-α responsiveness to bacterial endotoxin.

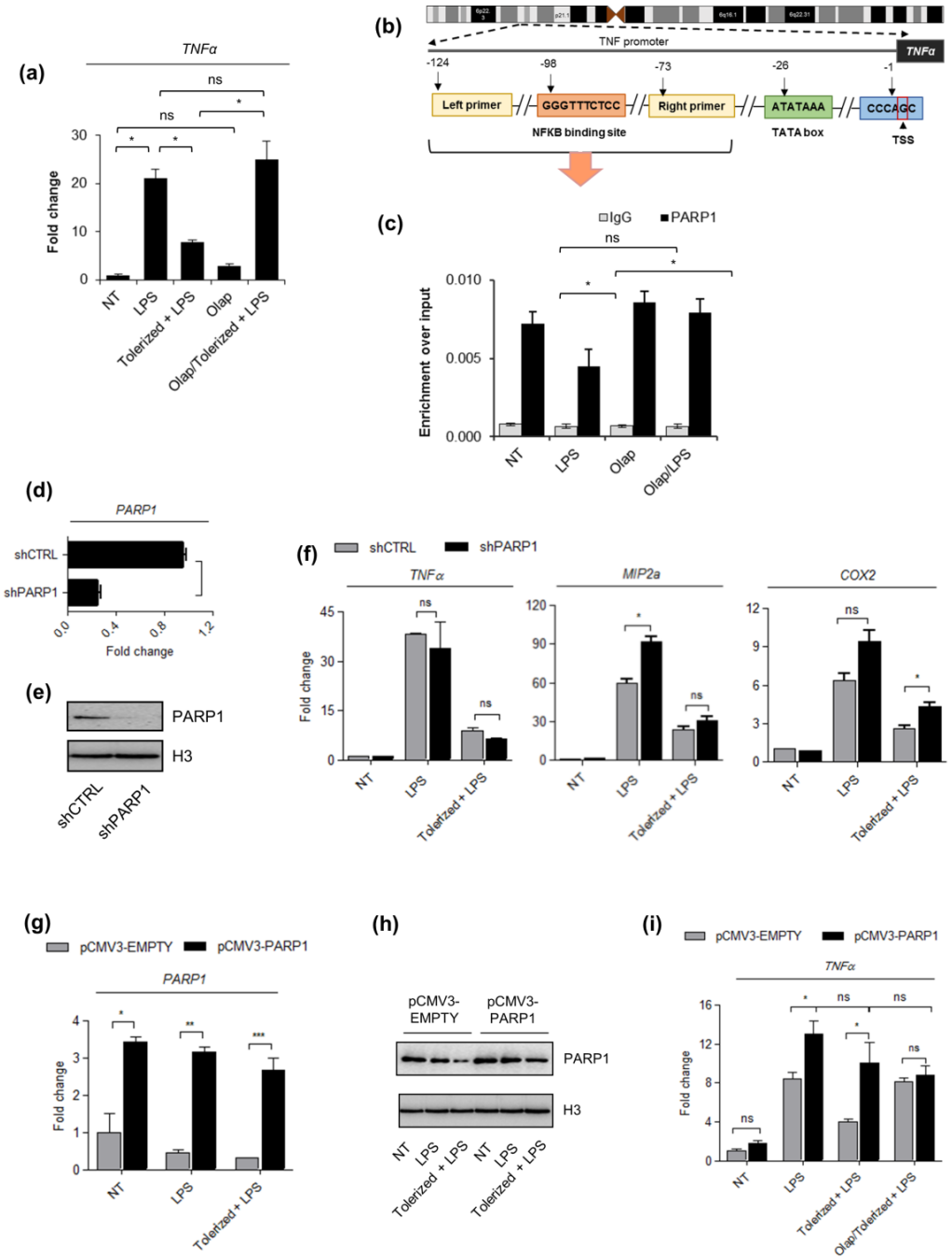

Figure 2. The PARP1 level drives LPS-induced immune paralysis in THP1 cells. (**a**) The induction of tolerance in acute leukemia cells was evaluated based on the *TNF-α* expression that was quantified by real-time PCR, normalized as median of *ACTB* and *GAPDH*, and shown as a fold change with respect to untreated cells. (**b**) The illustration in outlines the human proximal *TNF-α* promoter and the position of NF-κB (−98) as well as primer (forward: −124, reverse: −73) binding sites with respect to transcriptional start site (TSS). The fragment between the two indicated primers was amplified to quantify the immunoprecipitated DNA by real-time PCR. (**c**) The impact of the single LPS dose and olaparib on PARP1 occurrence at the *TNF-α* promoter was estimated with ChIP-qPCR. (**d**–**e**) The silencing efficiency of PARP1 in THP1 cells stably transfected with PARP-1 shRNA Plasmid was determined by real-time PCR and western blot, respectively versus corresponding control cells (shCTRL; control shRNA Plasmid-A). *PARP1* mRNA was normalized to the median of *ACTB* and *GAPDH*, and shown as a fold change with respect to control cells with the normal PARP1 expression. In western blot, H3 was used as a loading control. Full-length western blot images are available in the Supplementary Figure S4. (**f**) The mRNA level of *TNF-α*, *MIP2a*,

and *COX2*, which served to assess the development of immune tolerance in response to LPS, was measured with real-time PCR as described above. (**g**–**h**) PARP1 overexpression in pCMV3–PARP1 versus pCMV3–EMPTY-transfected cells was confirmed by real-time PCR and western blot, respectively. Full-length western blot images are included in the Supplementary Figure S5. mRNA level of PARP1 was normalized to *ACTB* and *GAPDH* and is presented as a fold change of PARP1 knock-in with respect to control cells. (**i**) The effect of PARP poison (olaparib) on the development of endotoxin tolerance in pCMV3–EMPTY and pCMV3–PARP1 cells was estimated on the basis of *TNF-α* transcription (mRNA), which was set as a readout. Bars in the figures represent mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA1) was carried out in GraphPad Prism 5 to compare means in several groups. Once the significance was detected, ANOVA1 was followed by the Tukey post-hoc test and significant differences between the two considered means are marked with * when significant at $p < 0.05$, ** when significant at $p < 0.01$, *** when significant at $p < 0.001$, ns—non-significant at $p > 0.05$. Abbreviations: THP1—human monocytic cell line, LPS—lipopolysaccharide, Olap—Olaparib, TNF—tumor necrosis factor, ACTB—actin beta, GAPDH—glyceraldehyde 3-phosphate dehydrogenase, shCTRL—THP1 cell transfected with Control shRNA Plasmid-A, shPARP1—THP1 cell transfected with PARP-1 shRNA Plasmid, pCMV3–PARP1—THP1 cells transfected with pCMV3—PARP1 plasmid, pCMV3–EMPTY—THP1 cells transfected with pCMV3-EMPTY plasmid, IgG—immunoglobulin G.

If PARP1 extrusion from the *TNF-α* promoter contributes to the development of endotoxin tolerance, then PARP1 deficiency was expected to have no effect on or enhance *TNF-α* suppression. To verify this hypothesis, we generated stably silenced PARP1 knockdowns by THP1 transfection with shPARP1-carrying versus shCTRL vector and selection with puromycin (Figure 2d,e). As expected, the considerable decrease in PARP1 abundance did not interfere with macrophage paralysis (Figure 2f). The analysis extended to two other pro-inflammatory cytokines, *COX2* and *MIP2a*, LPS-induced suppression of which was also prevented by olaparib in human macrophages, led to the same conclusion.

In such a case, we took a reverse approach and transiently overexpressed PARP1 in THP1 cells by their transfection with PARP1-overexpressing vector. We made use of ViaFect Transfection Reagent and an optimized transfection protocol, which allowed us to induce tolerance in THP1 transfected cells. As documented in our previous study [19], LPS caused repression and cleavage of PARP1, whereas overexpression of the enzyme maintained high PARP1 abundance regardless of LPS treatment (Figure 2g,h). PARP1 overexpression protected cells from *TNF-α* repression induced by bacterial endotoxin. This all confirms that LPS-induced PARP1 deficiency at the *TNF-α* promoter allows for gene silencing and inhibits gene responsiveness to another cell stimulation with LPS (Figure 2i).

2.3. PARP1 Extrusion form TNF-α Promoter Hampers p65 Rebinding after Tolerance Induction

To confirm that PARP1 operates on chromatin in the same regulatory circuit with canonical NF-κB (p65-p50 heterodimers), we first estimated the degree of NF-κB contribution to macrophage response to LPS. Cell pretreatment with proteasome inhibitor MG132, which protects IκB (NF-κB inhibitor) from ubiquitination and proteasomal degradation, therefore precludes NF-κB binding to the target gene promoters, completely blocked LPS-induced transcription of *TNF-α* (Figure 3A). The effect of PARP inhibitor olaparib was abrogated in the presence of MG132, suggesting that PARP1 affects NF-κB-dependent transcription of pro-inflammatory cytokine.

Analysis of the occurrence of p65 at the *TNF-α* promoter revealed the immediate binding of this transcription factor after cell stimulation with LPS and its complete loss in tolerized cells (Figure 3B). It suggests that the initial wave of p65 recruitment to chromatin is transient, followed by the extrusion of the protein from the studied gene promoter. Stimulation of tolerized cells with another dose of LPS did not result in p65 rebinding. Importantly, PARP1 trapping at the promoter of *TNF-α* maintained chromatin responsiveness and allowed for p65 recruitment in tolerized cells. Notably, the extent of p65 enrichment in cells tolerized in the presence of olaparib was comparable to that of intact cells challenged for the first time with bacterial endotoxin. These results suggest that PARP1 extrusion from the gene promoter during the first contact of cells with LPS allows for chromatin remodeling, which impedes further recruitment of p65.

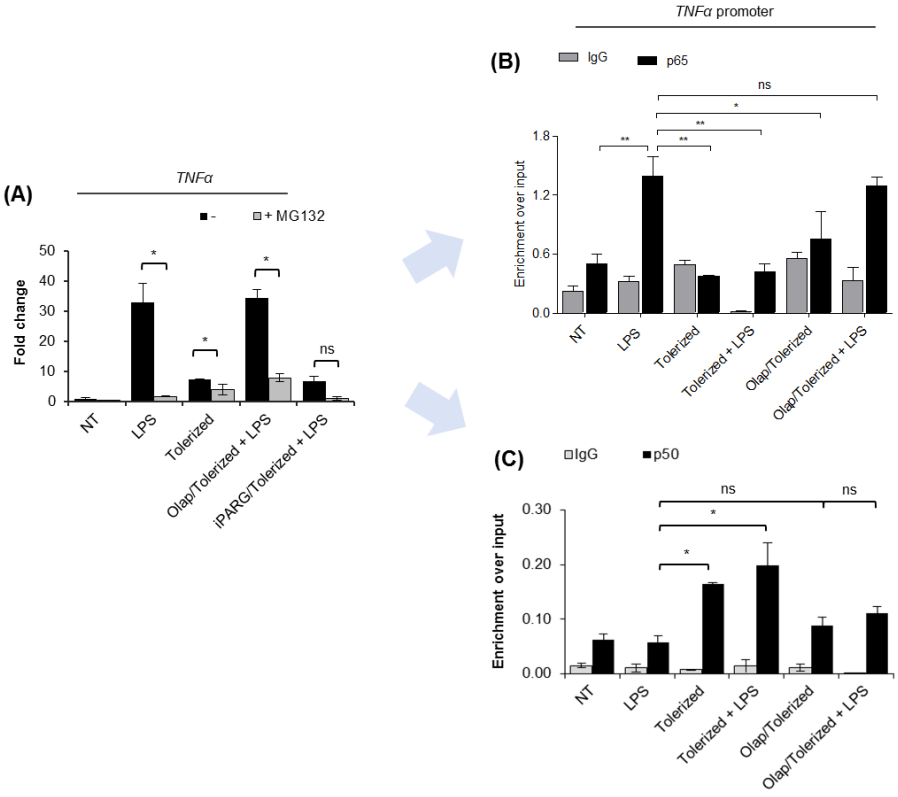

Figure 3. PARP1 eviction from the *TNF-α* promoter prevents p65 re-binding after LPS re-stimulation. (**A**) The contribution of canonical NF-κB to macrophage response to LPS was estimated by comparing *TNF-α* transcription in control and cells pre-treated with proteasome inhibitor (MG132; 1 μM). mRNA level of the cytokine was quantified by real-time PCR, normalized to median of *ACTB* and *GAPDH* and presented as a fold change with respect to untreated cells. (**B**) p65 and (**C**) p50 occurrence at the *TNF-α* promoter was analyzed by ChIP-real-time PCR. Bars in the figures represent mean ± standard error of the mean (SEM). One-way analysis of variance (ANOVA1) was carried out in GraphPad Prism 5 to compare means in several groups. Once the significance was detected, ANOVA1 was followed by the Tukey post-hoc test and significant differences between the two considered means are marked with * when significant at $p < 0.05$, ** when significant at $p < 0.01$, ns—non-significant at $p > 0.05$. Abbreviations: LPS—lipopolysaccharide, Olap—olaparib, TNF—tumor necrosis factor, ACTB—actin beta, GAPDH—glyceraldehyde 3-phosphate dehydrogenase, IgG—immunoglobulin G, p65—transcription factor p65 (RelA), p50—nuclear factor NF-kappa-B p105 subunit.

Knowing that p50 forms transcription-promoting heterodimers with p65 and that enrichment of p50 in certain circumstances leads to gene suppression due to the assembly of repressive complexes with chromatin remodeling enzymes such as HDAC1, we tested the occurrence of p50 at the promoter of *TNF-α* in intact and tolerized cells. As shown in Figure 3C, the single and lower dose of LPS (10 ng/mL) did not trigger a considerable enrichment of p50 at the promoter of *TNF-α*. It might be surprising in the light of well-approved concept of p65–p50 heterodimer contribution to transcriptional gene activation, and it may indicate the preexistence of certain pool of p50 at the NF-κB-dependent gene promoters that is necessary for immediate gene response after recruitment of p65. Certainly, such an aspect needs to be taken into further consideration. Anyway, the substantial enrichment of p50 was observed 24 h after tolerance induction and remained unchanged after the second dose of LPS. PARP1 trapping on chromatin with olaparib prior to LPS prevented p50 enrichment in tolerized cells. This indicates that PARP1 extrusion from the promoter of *TNF-α* in response to the first contact of cell with LPS allows for p50 interaction with the gene promoter, which might possibly preclude the binding of p65 in tolerized

cells by recruiting other repressors and, thereby, chromatin remodeling [9]. Again, such a hypothesis requires further and mechanistic examination.

3. Discussion

Sepsis is the outcome of the invalid coordination of inflammation against pathogens driven by monocytes and macrophages as well as other granulocytes, which belong to the first line of immune defense [20]. Long-term or high doses of lipopolysaccharide exposure of these cells can lead to endotoxin tolerance, which is seen in patients with sepsis. LPS paralyzes the proper immune response and reprograms it by reducing the production of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and others [21,22]. Many studies have described the impact of LPS-induced tolerance on the development of sepsis. Nonetheless, the correlation between the immunoparalysis and epigenetic modifications of cytokine regulatory elements is still under investigation. High-dose LPS stimulation of monocytic cells contributes to the decreased nuclear level of p65 and its binding to DNA, with simultaneous activation of p50 homodimers and their recruitment to chromatin. This can represent an adaptive response to prevent the harmful consequences of excessive production and release of cytokines such as TNF-α [23,24]. Stimulation of macrophage with bacterial ligand of TLRs triggers gene reprogramming, mainly by epigenetic changes, such as nucleosome and chromatin remodeling, histone modifications, and reduction of transcription factor recruitment [25]. For example, NF-κB can recruit nuclear receptor co-repressor 1–histone deacetylase 3–p50 (NCOR-HDAC3-p50) repressive complex or methyltransferase G9a to promoters, thereby leading to epigenetic silencing [26,27]. Particularly, in the described murine genome, p50 emerged essential for assembling the repressosome and LPS-induced tolerance. In that experimental model, the enrichment of HDAC1, HDAC3, and NcoR negatively correlated with the occurrence of p65 at the promoter of *TNF-α* [26]. The deacetylation of nucleosomes by HDAC1 and/or HDAC3 possibly prevent the recruitment of p65. Earlier study on NF-κB p65 subunits provided evidence on their binding to genes characterized higher levels of histone acetylation and that increase in aforementioned, transcription promoting modification precedes the p65 interaction with DNA [28]. Moreover, p50 homodimers, which bind nucleosomal κB sites in vitro, were also observed in the nucleus of unstimulated cells, and were displaced by activating dimers (p65 or c-Rel) in stimulated cells, possibly due to the chromatin remodeling. Therefore, the observed enrichment of p50 at the promoter of *TNF-α* after the first macrophage stimulation with LPS may be associated with formation of repressive complexes, which deacetylate nucleosome, thereby making chromatin inaccessible for p65. LPS-tolerized macrophages were shown unable to deposit active histone marks at promoters in response to LPS restimulation. Moreover, LPS-treated macrophages maintain the epigenetic profile of chromatin similar to monocytes, which is low in H3K4me1 and high in H3K27me3 occurrence [29,30]. A previously published report indicates that the dimer composition of the NF-κB complex, which is recruited or occupies the gene promoters, varies in LPS-responsive and LPS-tolerized cells [31]. As described in Porta et al., the tolerance is followed by the extensive occurrence of p50 homodimer rather than p65–p50 heterodimers [32]. A similar situation was also found in tumor-associated macrophages (TAMs). This agrees with our study, where LPS-trained macrophages were characterized by the considerable enrichment of p50 at the promoter of *TNF-α*. Moreover, our results provide the first evidence that the PARP1 enrichment at the gene promoter of *TNF-α* prevents LPS-induced switching from transcription-promoting p65–p50 to repressive p50–50 dimers. The brake on the NF-κB-dependent inflammatory gene transcription may be in part mediated by an interaction of p50 with the epigenetic repressor protein histone deacetylase 1 (HDAC1), which is capable of removing nucleosome acetylation, and by the lack a transcription activation domain in p50 [33].

In macrophages, PARP1 facilitates inflammatory cytokine expression by promoting NF-κB accessibility at regulatory sequences [22]. According to our findings, macrophage response to higher doses of LPS leads to a substantial decrease in NF-κB-dependent expression of TNF-α. The product of this gene is considered as a marker of inflammatory

macrophage response and, thus, also of endotoxin tolerance. The macrophage pretreatment with olaparib prior to LPS stimulation maintains a pro-inflammatory response. It confirms the role of PARP1 in cytokine expression control that has already been published by other groups [31,34]. Furthermore, the involvement of PARP inhibitors that differ in the DNA binding potential showed that PARP1 extrusion from the chromatin allows for chromatin remodeling, which prevents p65 rebinding upon the following macrophage stimulation with LPS. PARP1 occurrence at the *TNF-α* promoter prevents enrichment of p50, which was observed upon macrophage primary stimulation with LPS. Importantly, PARP trapping at the gene promoter, but not inhibition of ADP-ribosylation, turned out to protect macrophages from endotoxin-induced tolerance. Further investigation of mechanical aspects of the negative correlation between PARP1 and p50 upon activation of TLR-downstream signaling pathways is needed to explain the observed phenomenon.

PARP1 extrusion from the chromatin is induced by the activation of TLR receptors. TLR activation is essential for innate response against pathogens; nonetheless, the same group of receptors may contribute to the development of the chronic inflammatory state that is characteristic of sepsis [35,36]. Therefore, PARP trapping may serve as an alternative to TLR antagonists, which have been tested in clinical trials. These include Lipid A derivatives (glycolipids) and anti-TLR4 antibody or TLR4 ligand-J KB-122, which has been approved by the Food and Drug Administration (FDA) for the treatment of autoimmune hepatitis (AIH) [37]. TLR downstream targets include adaptor molecules and kinases (such as MyD88, IRAK, or TRAF6) and NF-κB transcription factor, which have been documented as active players in the development of endotoxin tolerance [38]. Therefore, inhibition of poly-ADP-ribosylation may also inhibit NF-κB pathways at these steps, which require PARP1 activity. ADP-ribose molecules were shown to be crucial for the formation of ATM-PIASy-IKKg that led to IKK proteasomal degradation and NF-κB activation upon DNA damage, which also occurs during cell stimulation with LPS [39]. This suggests that interference with the ADP-ribosylation can possibly modulate the NF-κB pathway responsible for the development of endotoxin tolerance at more than one-step.

Due to the limited possibility of genetic manipulation in macrophages, we performed some experiments on a THP1 human monocytic cell line. Although cancer cells differ from primary macrophages in inter alia expression of LPS receptor CD14, they responded similarly to LPS also in terms of PARP1 contribution to the induction of endotoxin tolerance [40]. This indicates that the PARP1 role in LPS-triggered *TNF-α* transcription is similar among various (myeloid) cell types [41]. It is also of particular importance for anti-cancer therapies, which involve PARP inhibitors, since these compounds may help to prevent macrophage reprogramming to a trained phenotype that resembles M2 macrophages. On the other hand, the M2d macrophage subtype that has been linked with the tumor progression is characterized by the extensive production of TNF-α, and PARP poisons may facilitate tumor progression by promoting the activity of tumor-associated macrophages (TAMs) [42]. In any case, further in vivo validation of PARP traps is necessary to confirm the immunomodulatory effects of PARP1 binding to DNA.

Sepsis is a highly lethal disease entity that, according to a recent scientific publication in 2017, had 48.9 million cases and 11 million sepsis-related deaths worldwide, which constituted almost 20% of all global deaths [43]. The success of treatment depends on two factors considered in the medical environment: an early and appropriate antibiotic, as well as all-purpose supporting treatment [44]. In recent years, numerous studies have been provided, which described the approaches of sepsis patient treatment. These include TNF-α neutralizing antibodies, bactericidal/permeability-increasing protein or platelet-activating factor, with a positive result in intensive care units [45]. The ongoing clinical trials have been testing anti-CD14 antibodies or lipid A analogs as antiendotoxin agents [46]. Our study describes another option to prevent the development of sepsis: the use of PARP traps, which are capable of binding PARP1 to chromatin, to maintain the macrophage pro-inflammatory phenotype upon succeeding stimulation of phagocytes with LPS. Due to the contribution of PARP1 to DNA repair, PARP inhibitors have been extensively tested,

and some of them (as well as PARP traps, including olaparib and talazoparib) have been approved for the monotherapies of patients with breast or ovarian cancer characterized by DNA repair deficiencies such as *BRCA1* and *BRCA2* mutations [47]. The undisputed advantage of the possible reuse of PARP inhibitors in other clinical settings is their well-characterized and favorable safety profiles in clinical trials, as well as the known side effects in the body [48]. Numerous open questions remain, particularly for the efficiency of prevention of LPS tolerance induction in in vivo and optimization of the optimal moment for PARP trap application. Currently, several models of septic shock induction have been described, however none of them are adequate to satisfy in sepsis research [49]. For example LPS injection, which has been used for nearly 100 years of sepsis studies, mimics most of the physiology of severe sepsis, but the doses of LPS to induce murine response are dissimilar from humans, which can be connected with different values of the median lethal dose (LD50) for LPS [50,51]. Explanation of those differences can be related to expression of various protective proteins among species or the use of LPS featured by different purity [52]. Other methods of sepsis induction in mice are also taken into consideration. They include the injection of bacteria or cecal contents from a donor rodent, the wildly used Cecal Ligation and Puncture (CLP) model, or colon ascendens stent peritonitis (CASP). These new methods in the murine model more adequately mimic dysfunction of organs observed in human sepsis [50]. Nevertheless, due to the existing differences among species, the physiological and immunological consequences of sepsis differ and limit the credibility of the conclusions, which drive the tested compounds or approaches to clinical trials [49].

In conclusion, the interaction of PARP1 with the promoter of *TNF-α* determines chromatin structure and its responsiveness to p65 recruitment upon activation of canonical NF-κB pathway with LPS. PARP traps emerge as efficient inhibitors of development of LPS-induced tolerance in the in vitro culture of human macrophages.

4. Materials and Methods

4.1. Materials

THP1 cells and human monocytes were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA) and from healthy donors from the blood bank in Lodz, respectively. RosetteSep™ Monocyte Enrichment Cocktail was purchased from STEMCELL Technologies (Grenoble, France); cell culture media were from Biowest (CytoGen, Zgierz, Poland); granulocyte–macrophage colony-stimulating factor (GM-CSF) from PeproTech (London, UK); BLUeye prestained protein ladder (#94964), oligonucleotides for real-time PCR were from Sigma Aldrich (Poznan, Poland); 5x HOT FIREPol EvaGreen qPCR Mix Plus (no ROX) (CytoGen, Zgierz Poland), ChiP grade antibodies anti-histone H3 (#4620), anti-PARP1 (#9532) and normal rabbit IgG (#2729) were from Cell Signaling Technology (Lab-JOT, Warsaw, Poland); TRI Reagent, anti-rabbit IgG (A0545) (whole molecule)–peroxidase antibody produced in goat were from Sigma Aldrich (Poznan, Poland); Lipofectamine RNAiMAX, Dynabeads™ Protein G, High-Capacity cDNA Reverse Transcription Kit, SuperSignal™ West Pico Chemiluminescent Substrate, OptiMem were from Thermo Fisher Scientific (Warsaw, Poland). Lipopolysaccharide (LPS), PARG inhibitor—ADP-HPD (Dihydrate, Ammonium Salt–Calbiochem) were from Sigma Aldrich (Poznan, Poland), Olaparib, Niraparib (MK-4827) and Veliparib (ABT-888), MG-132 and iPARG were from Cayman Chemical Biokom, (Warsaw, Poland). ViaFect™ Transfection Reagent was purchased from Promega (Warsaw, Poland). The human PARP1 Gene cDNA Clone (full-length ORF Clone), expression-ready, untagged (HG11040-UT; pCMV3-PARP1), and pCMV3-untagged Negative Control Vector (CV011; pCMV3-EMPTY) were purchased from Hölzel Diagnostika Handels GmbH (Köln, Germany). Puromycin dihydrochloride, PARP-1 shRNA Plasmid (h) (sc-29437-SH) and Control shRNA Plasmid-A(sc-108060), antibodies: anti-p50 (sc-1190), anti-p65 (sc-372) were from Santa Cruz Biotechnology (Lodz, Poland).

4.2. Monocyte Isolation, Differentiation of Macrophages and Cell Culture

Human monocytes were isolated from buffy coats from healthy donors using RosetteSep Monocyte Enrichment Cocktail, as described previously [16]. After attachment to the plate, monocytes were differentiated with GM-CSF (5 ng/mL) for 7 days in RPMI with 10% FBS, penicillin/streptomycin (50 U/mL, and 50 μg/mL, respectively).

THP1 cells (monocytic leukemia/premonocytes) were cultured under the same conditions.

4.3. Induction of Immune Paralysis

To initiate the development of immune tolerance to bacterial endotoxin, differentiated macrophages were stimulated with a single dose of LPS for 24 h. The macrophage activating bacterial compound was present in the cell culture medium during the entire period of the tolerance induction and was not washed out before another round of macrophage treatment with LPS. The second dose aimed to test cell pro-inflammatory response and the mRNA level of pro-inflammatory cytokines was measured 2 h after cell activation.

To initiate the development of immune paralysis, human macrophages were subjected to the following doses of the TLR4 ligand: 0.01 ng/mL, 0.1 ng/mL, 1 ng/mL, and 10 ng/mL for 24 h, whereas for testing responsiveness, cells were stimulated with 10 ng/mL LPS for 2 h. After initial evaluation of the paralyzing potential, one combination of LPS concentrations (1 ng/mL for tolerance induction + 10 ng/mL for the second activation) was chosen and applied to experimental procedures.

The scheme of THP1 cells remained the same, but the first dose of LPS was set as 50 ng/mL.

To estimate the impact of PARP, PARG and NF-κB inhibitors on the tolerance development, these compounds were added to cells for 1 h prior to first cell stimulation with bacterial endotoxin. PARP inhibitors were used in the following concentrations: olaparib (1 μM), MK-4827 and ABT-888 (0.5 μM, 2.5 μM), whereas a PARG inhibitor, ADP–HPD (Dihydrate, Ammonium Salt—Calbiochem), and an inhibitor of the proteasome/NF-κB pathway, MG132, at concentrations of 10 μM and 1 μM, respectively.

4.4. Quantification of the Gene Expression

For mRNA quantification, total RNA was extracted with TRI Reagent™ and reverse-transcribed with High-Capacity cDNA Reverse Transcription Kit, and cDNA fragments were amplified by real-time PCR with 5x HOT FIREPol EvaGreen qPCR Mix Plus (no ROX). Primer pairs used for cDNA amplification are listed in Table 1. Analysis of gene expression was performed on the basis of the fold-change and *p*-values, and expression levels of ACTB and GAPDH were taken for normalization.

Table 1. Sequences (5′–3′) of primers used.

GENE	FORWARD	REVERSE
TNF-α	GGAGAAGGGTGACCGACTCA	TGCCCAGACTCGGCAAAG
ACTB	TGGCACCCAGCACAATGAA	CTAAGTCATAGTCCGCCTAGAAGCA
GAPDH	TTCTTTTGCGTCGCCAGCCGA	GTGACCAGGCGCCCAATACGA
COX2	GAATCATTCACCAGGCAAATTG	TGGAAGCCTGTGATACTTTCTGTACT
MIP2a	CGCCCAAACCGAAGTCAT	GATTTGCCATTTTTCACATCTTT
PARP1	AAGCCCTAAAGGCTCAGAACG	ACCATGCCATCAGCTACTCGGT
IL6	GGCACTGGCAGAAAACAACC	GCAAGTCTCCTCATTGAATCC
IL12a	CTCCTGGACCACCTCAGTTTG	GGTGAAGGCATGGGAACATT
iNOS	GTTCTCAAGGCACAGGTCTC	GCAGGTCACTTATGTCACTTATC

4.5. Western Blot

For PARP1 protein detection by western blot, cell lysates were separated by SDS-PAGE, transferred to nitrocellulose membranes, blocked with skimmed milk and stained overnight at 4 °C with primary rabbit anti-PARP1 antibody (1:1000), and next day by 1 h

with secondary goat anti-rabbit HRP-conjugated antibody (1:10,000). The HRP-derived signal was developed using SuperSignal™ West Pico Chemiluminescent Substrate. Pictures were acquired with ChemiDoc-IT2 (UVP, Meranco, Poznan, Poland). Tubulin was used as a loading control.

4.6. Chromatin Immunoprecipitation (CHIP) Assay

The PARP1 occurrence at the *TNF-α* promotor was analyzed in THP1 cells stimulated with the single dose (50 ng/mL) of LPS for 1 h. In brief, the cells were fixed with 1% formaldehyde, quenched with 125 mM glycine and rinsed 3x with cold PBS. After centrifugation, the acquired pellet was lysed by lysis buffers with protease inhibitor cocktail and sonicated with the ultrasonic homogenizer Bandelin Sonopuls (HD 2070). Lysates were added to 1% Triton-X100 and centrifuged, and supernatants were incubated with antibody-conjugated magnetic beads (Dynabeads™ Protein G) at 4 °C overnight. The next day, the immunoprecipitated chromatin was washed and de-crosslinked overnight at 65 °C. The DNA was isolated with phenol/chloroform/isoamyl alcohol and analyzed via real-time PCR.

To identify the NF-κB binding site, we searched through the binding motifs for p65 and p50 in the proximal promoter of TNF-α. The window for analysis was set at ±1 kbp. The sequence derived from University of California, Santa Cruz (UCSC), Santa Curz, CA, USA. Genome Browser was processed with TFBind, and the following binding site was identified with parameters: p50: $p = 0.789871$, in position 28 on input sequence (+); p65: $p = 0.783234$, in position 27 on input sequence (+). Therefore, primers to TNF-α promoter at −305/−254 from the CDS region designed as follows: 5′-ACTACCGCTTCCTCCAGATGA-3′ (forward) and 5′-GGGAAAGAATCATTCAACCAGCGG-3′ (reverse).

4.7. Permanent Gene Silencing and Transient Overexpression

PARP1 stable knockdowns and corresponding controls were generated using Amaxa® Nucleofector® Technology in the Laboratory of Transcriptional Regulation, Institute for Medical Biology, PAS, Lodz, and with the kind help of prof. Łukasz Pułaski. In brief, 0.5 μg of PARP1 shRNA Plasmid and Control shRNA Plasmid-A were mixed with THP1 cells suspended in Nucleofector Solution, subjected to electroporation with Amaxa® Nucleofector® according to the manufacturer's instructions, and immediately diluted with warm RPMI with 10% FBS. After 24 h in culture, cells were selected with puromycin (5 μg/mL) for a month. After selection, puromycin was added to cells every second week.

The transient PARP1 overexpression was carried out as described previously [53]. In brief, THP1 cells at a density of 1,000,000/mL were treated with the complexes of pCMV3-EMPTY or pCMV3-PARP1 vectors and transfection reagent ViaFect. After 24 h, cells were cultured for 24 h as described in 2.2 and then subjected to LPS treatment (±olaparib).

4.8. Statistical Analysis

Data are shown as mean ± standard deviation of the mean (SEM). One-way analysis of variance (ANOVA) was carried out in GraphPad Prism 5 to compare means in several groups and followed by the Tukey test to detect statistically significant differences between means (marked with * when $p < 0.05$).

Supplementary Materials: The following are available online at https://www.mdpi.com/1424-8247/14/2/170/s1, Figure S1: The effect of human monocyte treatment with 1μM Olaparib for 1 h prior to induction of tolerance on the transcription of TNFα that was measured by real-time PCR, Figure S2: The effect of human macrophage treatment with PARP1 inhibitors 1 h on the tran-scription of TNFα that was measured by real-time PCR; Abbreviations: Olap—Olaparib, Velip—Veliparib, Nirap—Niraparib, LPS—lipopolysaccharide, Figure S3: The representative full length western blot images of ADP-ribosylated proteins and H3; cropped, red rectangular indicate picture areas that are included in the main Figures, Figure S4: The representative full length western blot images of PARP1 and H3 in the PARP1 sta-ble knock-downs; cropped, red rectangular indicate picture areas that are included in the main Figures, Figure S5: The representative full length western blot images of PARP1

and H3 in cells transiently transfected with the PARP1 expressing vector; cropped, red rectangular indicate picture areas that are included in the main Figures.

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Oświadczenie

Oświadczam, że w pracy:

Pietrzak J, Spickett CM, Płoszaj T, Virág L, Robaszkiewicz A. PARP1 promoter links cell cycle progression with adaptation to oxidative environment. Redox Biol. 2018 Sep;18:1-5. doi: 10.1016/j.redox.2018.05.017, mój wkład w powstanie tej pracy polegał przygotowaniu tekstu publikacji w rozdziałach 4 i 5 oraz rysunków 3 i 4. Mój udział procentowy szacuję na 40%.

Pietrzak J, Płoszaj T, Pułaski Ł, Robaszkiewicz A. EP300-HDAC1-SWI/SNF functional unit defines transcription of some DNA repair enzymes during differentiation of human macrophages. Biochim Biophys Acta Gene Regul Mech. 2019 Feb;1862(2):198-208. doi: 10.1016/j.bbagrm.2018.10.019, mój wkład w powstanie tej pracy polegał na wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników, oraz w przygotowaniu manuskryptu. Mój udział w pracy wynosi 20%.

Sobczak M, Pietrzak J, Płoszaj T, Robaszkiewicz A. BRG1 Activates Proliferation and Transcription of Cell Cycle-Dependent Genes in Breast Cancer Cells. Cancers (Basel). 2020 Feb;12(2):349. doi: 10.3390/cancers12020349, mój wkład w powstanie tej pracy polegał na wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników, oraz w przygotowaniu manuskryptu. Mój udział procentowy szacuję na 30%.

Pietrzak J, Gronkiewicz K, Robaszkiewicz A. PARP Traps Rescue the Pro-Inflammatory Response of Human Macrophages in the In Vitro Model of LPS-Induced Tolerance. Pharmaceuticals. 2021 Feb; *14*(2), 170; doi: 10.3390/ph14020170, , mój wkład w powstanie tej pracy polegał na częściowym opracowaniu koncepcji publikacji, wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników, oraz w przygotowaniu manuskryptu. Mój udział procentowy szacuję na 60%.

Julita Pietrzak

(podpis)

Łódź 03.03.2021

(miejsce, data)

Dr hab. Agnieszka Robaszkiewicz, profesor UŁ
Katedra Biofizyki Ogólnej,
Instytut Biofizyki, Uniwersytet Łódzki,
Pomorska 141/143, 90-236 Łódź

Oświadczenie

Oświadczam, że w pracy:

Pietrzak J, Spickett CM, Płoszaj T, Virág L, Robaszkiewicz A. PARP1 promoter links cell cycle progression with adaptation to oxidative environment. Redox Biol. 2018 Sep;18:1-5. doi: 10.1016/j.redox.2018.05.017, mój wkład w powstanie tej pracy polegał na opracowaniu koncepcji publikacji, wybraniu opisywanych zagadnień, przygotowaniu tekstu publikacji oraz rysunków 1 i 2, koordynowaniu pracy pozostałych współautorów, przygotowaniu i wysłaniu manuskryptu. Mój udział procentowy szacuję na 55%.

Pietrzak J, Płoszaj T, Pułaski Ł, Robaszkiewicz A. EP300-HDAC1-SWI/SNF functional unit defines transcription of some DNA repair enzymes during differentiation of human macrophages. Biochim Biophys Acta Gene Regul Mech. 2019 Feb;1862(2):198-208. doi: 10.1016/j.bbagrm.2018.10.019, mój wkład w powstanie tej pracy polegał na opracowaniu koncepcji badań, zdobyciu środków finansowych na ich wykonanie, przeprowadzeniu eksperymentów i opracowaniu danych, koordynowaniu pracy pozostałych współautorów, przygotowaniu i wysłaniu manuskryptu. Mój udział procentowy szacuję na 70%.

Sobczak M, Pietrzak J, Płoszaj T, Robaszkiewicz A. BRG1 Activates Proliferation and Transcription of Cell Cycle-Dependent Genes in Breast Cancer Cells. Cancers (Basel). 2020 Feb;12(2):349. doi: 10.3390/cancers12020349, mój wkład w powstanie tej pracy polegał na opracowaniu koncepcji badań, zdobyciu środków finansowych na ich wykonanie, przeprowadzeniu eksperymentów i opracowaniu danych, koordynowaniu pracy pozostałych współautorów, przygotowaniu i wysłaniu manuskryptu. Mój udział procentowy szacuję na 29%.

Pietrzak J, Gronkiewicz K, Robaszkiewicz A. PARP Traps Rescue the Pro-Inflammatory Response of Human Macrophages in the In Vitro Model of LPS-Induced Tolerance. Pharmaceuticals. 2021 Feb; *14*(2), 170; doi: 10.3390/ph14020170, mój wkład w powstanie tej pracy polegał na opracowaniu koncepcji badań, zdobyciu środków finansowych na ich wykonanie, przeprowadzeniu eksperymentów i opracowaniu danych, koordynowaniu pracy pozostałych współautorów, przygotowaniu i wysłaniu manuskryptu. Mój udział procentowy szacuję na 39%.

Agnieszka Robaszkiewicz

(podpis)

Łódź, 02.03.2021 r.

(miejsce, data)

Karolina Gronkowska
Katedra Biofizyki Ogólnej,
Instytut Biofizyki, Uniwersytet Łódzki,
Pomorska 141/143, 90-236 Łódź

OŚWIADCZENIE

Oświadczam, że w pracy:

Pietrzak J, Gronkowska K, Robaszkiewicz A. PARP Traps Rescue the Pro-Inflammatory Response of Human Macrophages in the In Vitro Model of LPS-Induced Tolerance. Pharmaceuticals. 2021 Feb; *14*(2), 170; doi: 10.3390/ph14020170 mój wkład w powstanie tej pracy polegał na wykonaniu części doświadczeń, Mój udział procentowy szacuję na 1%.

Karolina Gronkowska

(podpis)

Łódź, 04.03.2021.....

(miejsce, data)

Dr hab. Łukasz Pułaski, prof. nadzw. UŁ
Katedra Biofizyki Molekularnej,
Uniwersytet Łódzki,
Pomorska 141/143, 90-236 Łódź

Oświadczenie

Oświadczam, że w pracy:

Pietrzak J, Płoszaj T, Pułaski Ł, Robaszkiewicz A. EP300-HDAC1-SWI/SNF functional unit definestranscription of some DNA repairenzymesduringdifferentiation of humanmacrophages. BiochimBiophys Acta GeneRegul Mech. 2019 Feb;1862(2):198-208. doi: 10.1016/j.bbagrm.2018.10.019.
mój wkład w powstanie tej pracy polegał na ocenie uzyskanych wyników i udziale w przygotowaniu manuskryptu. Mój udział procentowy szacuje na 5%.

Ł. Pułaski

(podpis)

Łódź 5.03.2021

(miejsce, data)

Mgr Maciej Sobczak
Katedra Biofizyki Ogólnej,
Instytut Biofizyki, Uniwersytet Łódzki,
Pomorska 141/143, 90-236 Łódź

OŚWIADCZENIE

Oświadczam, że w pracy:

Sobczak M, Pietrzak J, Płoszaj T, Robaszkiewicz A. BRG1 Activates Proliferation and Transcription of Cell Cycle-Dependent Genes in Breast Cancer Cells. Cancers (Basel). 2020 Feb;12(2):349. doi: 10.3390/cancers12020349, mój wkład w powstanie tej pracy polegał na wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników, oraz w przygotowaniu manuskryptu. Mój udział procentowy szacuję na 40%.

Maciej Sobczak

(podpis)

Łódź, 03.05.2021

(miejsce, data)

Dr n.med. Tomasz Płoszaj
Zakład Genetyki Klinicznej,
Wydział Lekarski, Uniwersytet Medyczny,
Pomorska 251, 92-213 Łódź

Oświadczenie

Oświadczam, że w pracy:

Pietrzak J, Spickett CM, Płoszaj T, Virág L, Robaszkiewicz A. PARP1 promoter links cell cycle progression with adaptation to oxidative environment. Redox Biol. 2018 Sep;18:1-5. doi: 10.1016/j.redox.2018.05.017, mój wkład polegał na przygotowaniu abstraktu graficznego oraz korekty tekstu. Mój udział w pracy wynosił 1%.

Pietrzak J, Płoszaj T, Pułaski Ł, Robaszkiewicz A. EP300-HDAC1-SWI/SNF functional unit defines transcription of some DNA repair enzymes during differentiation of human macrophages. Biochim Biophys Acta Gene Regul Mech. 2019 Feb;1862(2):198-208. doi: 10.1016/j.bbagrm.2018.10.019, mój wkład w powstanie tej pracy polegał na mój udział polegał na planowaniu i wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników). Mój udział procentowy szacuję na 5%.

Sobczak M, Pietrzak J, Płoszaj T, Robaszkiewicz A. BRG1 Activates Proliferation and Transcription of Cell Cycle-Dependent Genes in Breast Cancer Cells. Cancers (Basel). 2020 Feb;12(2):349. doi: 10.3390/cancers12020349, mój wkład w powstanie tej pracy polegał na mój udział polegał na planowaniu i wykonaniu części doświadczeń, częściowej analizie i interpretacji wyników. Mój udział procentowy szacuję na 1%.

Tomasz Płoszaj

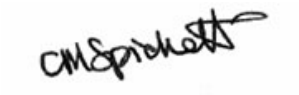
(podpis)

Prof. Corinne M Spickett
School of Life & Health Sciences,
Aston University,
Aston Triangle, Birmingham, B4 7ET, UK
e-mail: c.m.spickett@aston.ac.uk

Birmingham, October 29, 2018

STATEMENT

I hereby declare that my contribution to the considered paper entitled “PARP1 promoter links cell cycle progression with adaptation to oxidative environment” by Julita Pietrzak, Corinne M Spickett, Tomasz Płoszaj, Lászlo Virág and Agnieszka Robaszkiewicz published in Redox Biology in 2018 (18:1-5; DOI: 10.1016/j.redox.2018.05.017) consisted of manuscript proofreading and language correction. My contribution is valued as 3%.



Prof. Corinne Spickett

Prof. László Virág
Department of Medical Chemistry,
University of Debrecen
e-mail: lvirag@med.unideb.hu

Debrecen, September 25th, 2018

STATEMENT

I hereby declare that my contribution to the considered paper entitled "*PARP1* promoter links cell cycle progression with adaptation to oxidative environment" by Julita Pietrzak, Corinne Spickett, Tomasz Płoszaj, **László Virág** and Agnieszka Robaszkiewicz published in Redox Biology in 2018 (18:1–5, DOI: 10.1016/j.redox.2018.05.017) consisted of manuscript proofreading. My contribution is valued as 1%.

Prof. László Virág