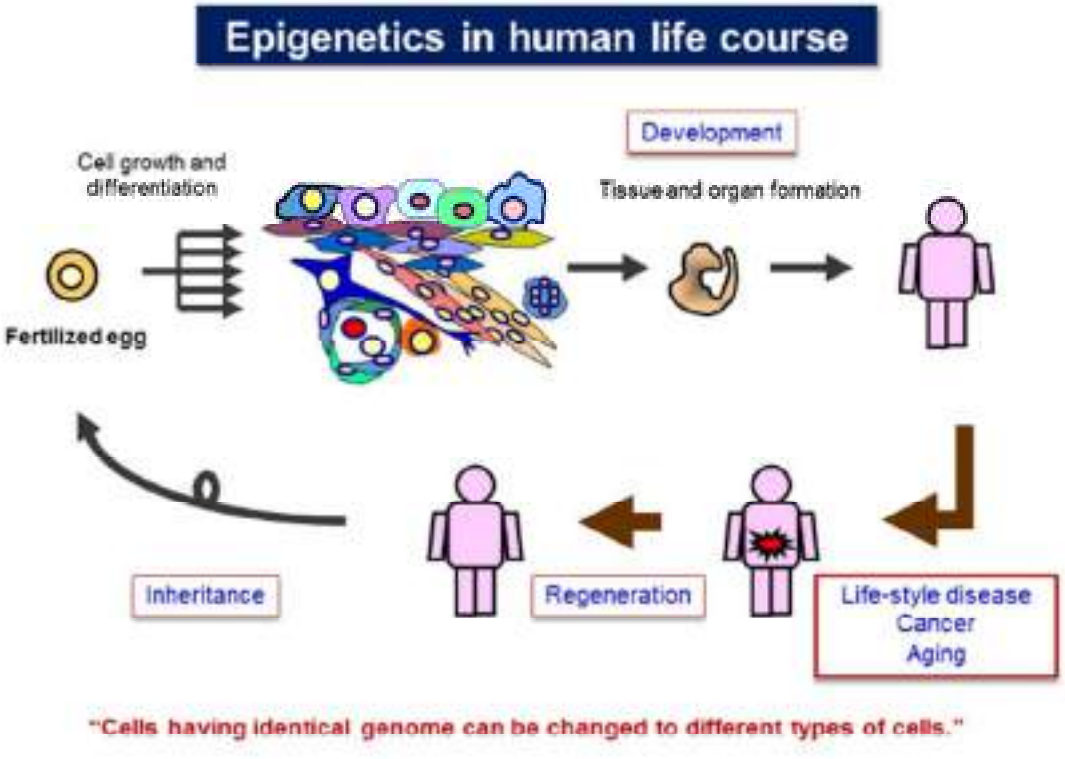
細胞医学分野
Department of Medical Cell Biology

エピジェネティクスの機構は、ゲノム上の全ての遺伝子の働き方を調節する仕組みであり、「生命のプログラム」を創出している。DNA のメチル化、ヒストンの修飾、クロマチンの形成で印付けられたゲノムをエピゲノムとよび、この印付けに従って、ゲノム上の遺伝子は選択的に活用されている。幹細胞の分化、iPS 細胞への初期化、老化、癌化では、それぞれ、エピジェネティックにリプログラムされている。さらに、エピゲノムは栄養や環境因子の影響を受けて、新たな印付けが記憶される。多くのヒト病気は、生命のプログラムの誤りと考えられる。エピジェネティクスの観点から、癌、生活習慣病、炎症、発生分化や老化の研究に挑戦する。そして、細分化した現代の医学・生命科学を統合的に理解することを目指す。

Our laboratory is studying the molecular basis of epigenetic cell regulation in development and human diseases. The term epigenetic is defined as “heritable changes in gene expression that occur without a change in DNA sequence”. Epigenetic regulation may include cytosine methylation, histone modification, chromatin formation, and nuclear structure.

We are studying how these epigenetic factors control gene expression and cellular function; 1) investigating the molecular basis of epigenome and gene control; 2) studying the epigenetics of energy metabolism; 3) identifying the mechanism involving in epigenetics of cancer and inflammation; 4) studying epigenome of cellular development and senescence; 5) detecting nuclear structure, function and dynamics; and 6) testing epigenetic technology useful for medical diagnosis and therapy.



ライフコースにおけるエピジェネティクスの役割

構成員　Staff (2018.3)

名前	職名	Name and Position
中尾　光善	教授	Mitsuyoshi Nakao, Professor
日野　信次朗	准教授	Shinjiro Hino, Associate Professor
古賀　友紹	助教	Tomoaki Koga, Assistant Professor
井形　朋香	研究員	Tomoka Igata, Research Fellow
阿南　浩太郎	研究員	Kotaro Anan, Research Fellow
田中　宏	研究員	Hiroshi Tanaka, Research Fellow
高瀬　隆太	大学院生	Ryuta Takase, Doctoral Student
安田　洋子	大学院生	Yoko Yasuda, Doctoral Student
興梠　健作	大学院生	Kensaku Kourogi, Doctoral Student
山本　達郎	大学院生	Tatsuro Yamamoto, Doctoral Student
荒木　裕貴	大学院生	Hirotaka Araki, Doctoral Student
田辺　やよい	技能補佐員	Yayoi Tanabe, Secretary Assistant
日野　裕子	技術支援者	Yuko Hino, Technical Assistant
坂本　智代美	技術支援者	Chiyomi Sakamoto, Technical Assistant
中津　有子	技術支援者	Yuko Nakatsu, Technical Assistant

元在籍者　Staff in the past (2012.4～2018.3)

名前	Name	在籍期間	在籍時職名	転出先
習　陽	Xi Yang	2009.4.1-2012.4.30	研究員	中国・宁波大学
徳永　和明	Kazuaki Tokunaga	2008.4.1-2012.9.30	研究員	ニコンインステック社
笹井　信広	Nobuhiro Sasai	2009.5.1-2013.3.31	研究員	慶應義塾大学メディアセンター
冨田　さおり	Saori Tomita	2008.10.1-2012.3.31	大学院生、特任助教	熊本大学
竹林　慎一郎	Shin-ichiro Takebayashi	2012.7.1-2014.5.31	研究員、特任助教	三重大学
長岡　克弥	Katsuya Nagaoka	2009.4.1-2015.3.31	大学院生、研究員	熊本大学（米国留学）
藤原　沙織	Saori Fujiwara	2012.4.1-2015.3.31	大学院生、研究員	熊本大学（米国留学）
王　雪莱	Xuelai Wang	2014.1.1-2015.6.30	研究員	中国・ハルビン医科大学
モハメド・オサマ・アリ・モハメド・アブダッラ	Mohamed Osama Ali Mohamed Abdalla	2010.10.1-2015.8.19	大学院生、研究員	エジプト・スエズ運河大学
坂元　顕久	Akihisa Sakamoto	2008.4.1-2016.3.30	研究員	鹿児島大学(米国留学)
中元　雅史	Masafumi Nakamoto	2011.4.1-2016.3.31	大学院生、研究員	熊本大学
松森　はるか	Haruka Matsumori	2011.4.1-2016.6.14	大学院生、技術支援者	ニコンインステック社
斉藤　典子	Noriko Saitoh	2002.4.1-2017.3.31	研究員、助教、准教授	がん研究所（部長）

研究概略　Projects

エピジェネティックな生命現象には、発生、再生、老化、遺伝、そして疾患が挙げられ、いずれも複雑な成り立ちではあるが、同一ゲノムをもつ細胞が異なる細胞に質的に変化する細胞リプログラミング（cellular reprogramming）および細胞記憶（cellular memory）を基礎としている。細胞の個性は遺伝子発現のパターンで概ね決まり、DNA のメチル化、ヒストンの修飾、クロマチンの形成で印付けられたエピゲノム（epigenome）が重要な役割を果たしている。エピゲノムは確立・維持・消去されることから、また印付けの組み合わせが多岐にあることから、細胞の恒常性と多様性を生み出している。

さらに、エピゲノムの高次の制御機構には、３次元のクロマチン・ループの形成、細胞核内のドメイン形成（染色体テリトリー、転写ファクトリー、ヘテロクロマチンなど）がある。エピジェネティクス機構の観点から、遺伝子の選択的活用、ゲノム機能の調節、エピジェネティックな細胞制御、そして、生命現象とヒト疾患の解明を目指している。

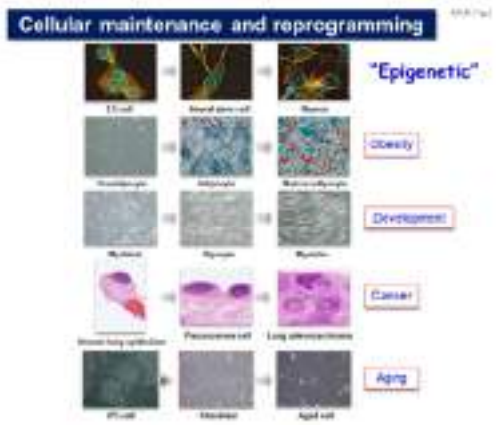


図１　エピゲノムによる細胞の維持･制御

ヒト疾患の多くは、ゲノムの遺伝因子および成育環境・生活習慣などの環境因子が相互作用する多因子疾患であり、健康な状態から発症、病期進行まで連続的な分布を呈している。エピゲノム制御が、遺伝子 ON/OFF の単なるスイッチではなく、多様な修飾基を用いることで、その発現の強度や時間幅というボリュームを連続的に調節できることに似ている。環境因子とエピゲノムの相関解析が始まり、環境因子がエピゲノムを変化させて、それが記憶されることが示唆されてきた（エピゲノム記憶）。発生期の環境因子が生涯の健康に影響するという DOHaD 説、生殖期の親が環境因子に暴露されると世代を超えて子孫に影響を与えるトランスジェネレーション遺伝も示唆されている。身近な環境因子として、栄養素、代謝物が挙げられるが、注目すべきは、これらがエピゲノムの修飾基や修飾酵素の補酵素になっている点である。代謝調節とエピゲノム制御が密接につながることを示唆しており、栄養･代謝のエピジェネティクスとして解析を進めている。

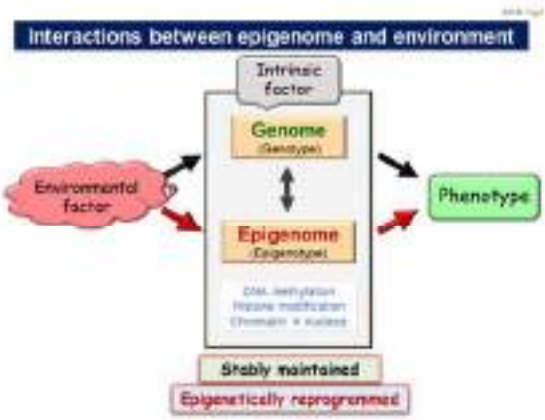


図２　エピゲノムと環境因子の作用

１．エピゲノムと遺伝子制御の分子基盤

DNA のメチル化は、哺乳類ゲノムの転写抑制とヘテロクロマチン形成を主に担っているが、その分子機序は不明であった。メチル化 DNA 結合タンパク質 **MBD1** がメチル化 DNA に結合する構造と機能、MBD1 がヒストン H3 の 9 番目リジン（H3K9）のメチル化酵素 **SETDB1** とその共役因子 **MCAF1**/ATF7IP の H3K9 トリメチル化複合体をリクルートして、転写抑制とヘテロクロマチン形成に働くことを報告した。DNA メチル化から H3K9 トリメチル化への経路を明らかにした（Cell, 2001; MCB, 2003）。

高次のクロマチンレベルの制御機構が、組織特異的、発生段階特異的、状況特異的に特定の遺伝子群が選択的に発現することを可能にしている。この機構には未知の点が多いが、遺伝子

のプロモーター、エンハンサー、インスレーターの時空間的な相互作用が重要な役割を果たすと考えられる。インスレーター結合因子 **CTCF**（CCCTC 結合因子）が染色体連結に関わるコヒーシン複合体（RAD21 など）とヒトゲノム上で共在することを明らかにした。また、ゲノム境界を形成するクロマチンインスレーターの分子機構として、CTCF がクロマチンリモデリング因子 **CHD8** と協働してインスレーター機能を果たすことを報告した（Mol Cell, 2006; Nature, 2008）。

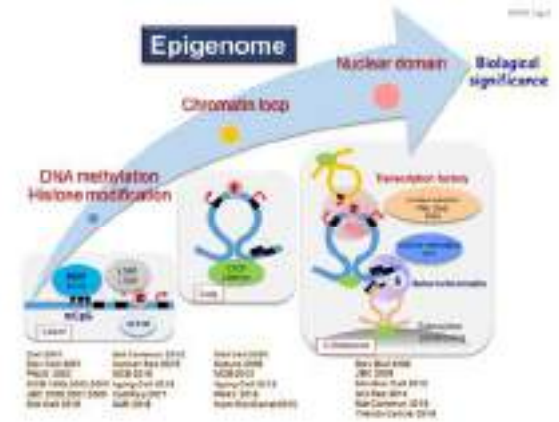


図３ 高次のエピゲノム機構

２．エネルギー代謝のエピジェネティクス

リジン特異的脱メチル化酵素 LSD1 の役割：

栄養摂取の状態がエネルギー代謝調節に関わる遺伝子群の発現に影響すると考えられるが、そのエピジェネティック制御は明らかではない。エネルギー代謝恒常性の機構に、フラビン（**FAD** : flavin adenosine dinucleotide）依存性のリジン特異的脱メチル化酵素 **LSD1** が重要な役割を果たすことを報告した。脂肪細胞および高脂肪食で誘導した肥満マウスにおいて、LSD1 複合体がエネルギー消費遺伝子群（ミトコンドリア呼吸、脂肪分解）の発現を抑制することを発見し、LSD1 阻害によるエネルギー代謝の向上が期待できることを報告した（Nat Commun, 2012）。

脂肪細胞の知見をもとに、肝臓、骨格筋、血液細胞、神経細胞におけるエネルギー代謝のエピジェネティック制御について明らかにした。また、LSD1 が多くの組織由来のがん細胞で高発現することから、好気的解糖（Warburg 効果）のがん代謝に関わることを報告した（Cancer Res, 2015）。もうひとつの **LSD2** について肝臓の脂肪代謝制御を明らかにした（MCB, 2015）。

骨格筋分化では、LSD1 が遅筋遺伝子群とミトコンドリア呼吸遺伝子群を抑制し、その結果、速筋分化を促進することを報告した（Nucleic Acids Res, 2018）。LSD1 の分解を促すグルココルチコイドと LSD1 阻害剤を組み合わせて、遅筋分化とミトコンドリア呼吸の増強が起こることを見出した。

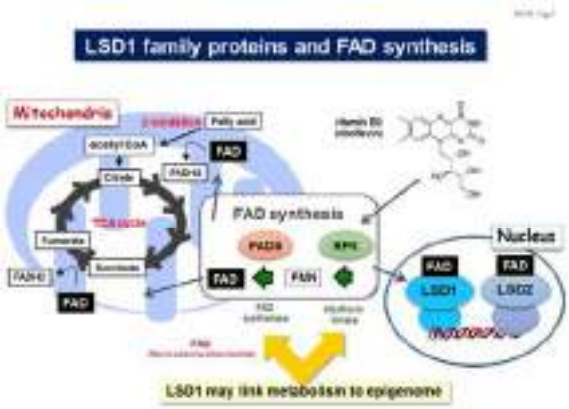


図４ LSD1 によるエネルギー代謝調節

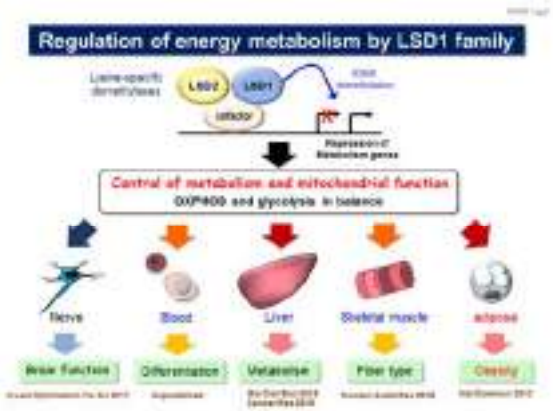


図５ 代謝臓器の機能制御

３．がんと炎症のエピジェネティクス

乳がんの治療抵抗性の機序と応用：

乳がんの約 70%はエストロゲンに依存して増殖するため、エストロゲンを阻害するホルモン療法が有効であるが、その後に治療抵抗性のがんが再発することが重大な課題である。**エストロゲン受容体 ER** をコードする ESR1 遺伝子が高発現することが要因のひとつであるが、その機序は不明であった。ヒト乳がん細胞株をエストロゲン枯渇下で 4 ヶ月以上に長期培養して、エストロゲン非依存的な増殖を獲得するという

培養系（LTED）を確立した。LTED 細胞と ER 陽性乳がん組織では、ESR1 遺伝子座から新規の長鎖非コード RNA 群「**エレノア（Eleanor）**」が高く発現し、その近傍に転写活性なドメインを形成することが分かった（Nat Commun, 2015）。乳がんのホルモン療法耐性化に関する臨床共同研究を通して、従来にない診断・予防・治療法の開発を目指している（Trends Cancer, 2018）。

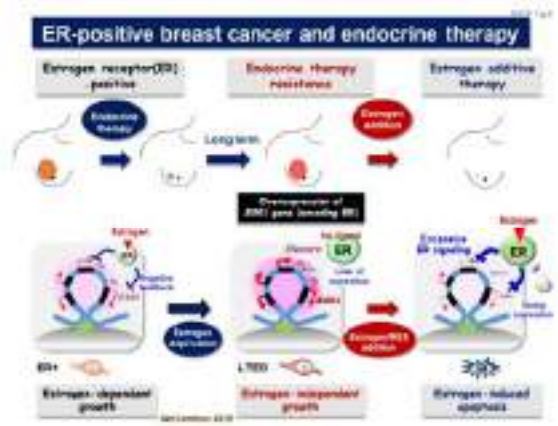


図６　ER 陽性乳がんとホルモン療法のモデル

炎症メモリーによる免疫応答：

病原体から自己を守ることは、健康の維持に欠かせない。その担い手には、病原体の構成成分を認識して直ぐに誘導される「自然免疫」、自然免疫の後に病原体を特異的に認識して長期に働く「獲得免疫」がある。獲得免疫の免疫記憶はよく知られているが、自然免疫もまた抗原に対する記憶をもっていると考えられる。代表的な２つの現象を紹介すると、１）病原体感染によって、1 回目よりも 2 回目に強く反応するという「訓練された自然免疫」、２）細菌由来の微量のエンドトキシンの前投与によって、その後のエンドトキシン投与に対する耐性を示すという「エンドトキシン・トレランス」がある。これらのエピゲノム基盤は不明であることから、分子レベルの解明を進めている。

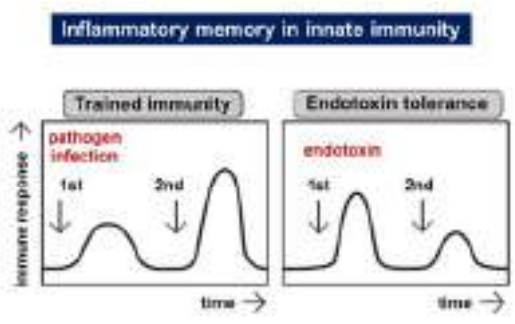


図７　炎症メモリーの形成

４．細胞分化と老化のエピジェネティクス

INK4/ARF 遺伝子群（p16, ARF をコード）は細胞老化で発現が誘導され、これらの発現制御に CTCF 依存性のクロマチン・ループの形成が必要であることを報告した（Aging Cell, 2012）。

核小体（エネルギー消費部位：リボソーム DNA 遺伝子群の発現およびタンパク質合成）、ミトコンドリア（エネルギー産生部位）に関わる未知のエピゲノム分子・経路を解析している。約 800 のエピゲノム因子に対する siRNA ライブラリーを用いて、核小体・ミトコンドリアの形態・機能変化を探索した。約 20 の有力因子を選定したところ、各々の単独阻害によってヒト線維芽細胞の細胞老化を誘導することが判明し、転写因子とヒストン修飾酵素の機能解析を進めている。メチル化酵素 **SETD8/PR-Set7**（ヒストン H4K20me1 のメチル化）の阻害による細胞老化、代謝リモデリングにおける役割が分かり、“細胞老化を防ぐ酵素”として報告した（Aging Cell, 2015; Cell Rep, 2017）。

図８　細胞老化とエピゲノム酵素

「**DoHaD 説**」（健康と病気の発生期起源説）では、胎児や新生児・乳児という発生期に、低栄養に曝された環境が基礎になっている。「即時の応答」として、蓄えた栄養分を消費し、身体の成長を抑えて、体内の器官を成熟させる（低出生体重）。また「予測の応答」として、将来の

飢餓に備えるために、栄養を蓄えるエピゲノムを形成する。即時の応答で生命を維持し、その後の飢餓に対して予測の応答で備える。

生後も低栄養の環境であれば、有利に働く。ところが、生後に十分な栄養を取れる場合、予測は外れて、環境に対して不適合になる。中性脂肪として皮下や内臓の脂肪組織に蓄積され、「肥満」「糖尿病」などの生活習慣病に罹りやすい。つまり、エピゲノムの記憶は、将来の環境に適合すれば有益であるが、一方、不適合になると不利益になる。

図９　DOHaD 説モデル

５．細胞核の構造・機能と細胞診断

細胞核内には多種多様な核内ドメインが存在しており、これらのドメインは時空間・状況に応じてダイナミックに集合・離散する分子集合体である。染色体領域と染色体間領域に分けられ、さらに、転写の場である転写ファクトリー、転写が不活化されるヘテロクロマチン、核小体、核スペックル（クロマチン間顆粒群）、PML ボディー、ポリコームボディーなどが挙げられる。

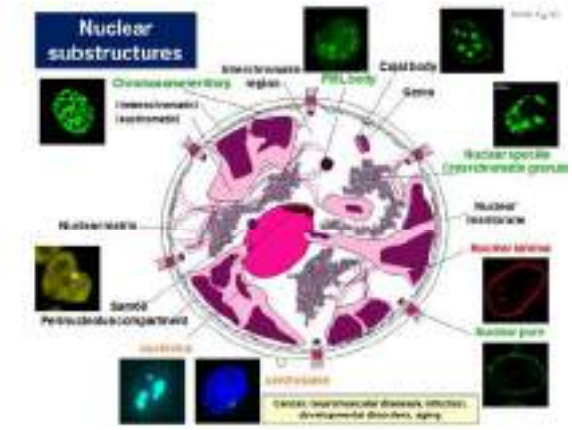


図１０　細胞核内構造体の形成

血清刺激下の PML ボディーの形成、ポリコームによるヘテロクロマチンの形成、核スペックルにおける選択的スプライシング、核小体の形成機構、RNA クラウドなどについて明らかにしてきた（JBC, 2008; Mol Biol Cell, 2012）。

Job Dekker 博士らが開発した染色体コンフォメーション捕捉法（chromosome conformation capture：**3C**）を用いて、細胞核内における特定のゲノム部位間の相互作用（相対的な距離）を検出できる。この 3C 技術を用いて、ヒト遺伝子座（アポリポタンパク質 *APO A1/C3/A4/A5*、サイクリン依存性キナーゼ阻害因子 *INK4/ARF*、炎症性サイトカイン *TNF/LT*、体節形成のマスター因子 *HOXA*）の高次エピゲノム解析を行い、これらの遺伝子座のエンハンサー（E）、プロモーター（P）、インスレーター（I）等の相互作用を明らかにした。この相互作用を E-P-I インターアクションと呼んでいる（EMBO J, 2009; MCB, 2012; Aging Cell, 2012; Hum Mol Genet, 2016）。

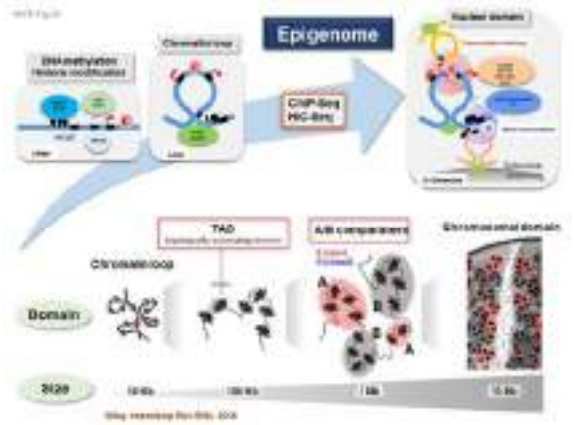


図１１　高次エピゲノムの形成

エピゲノムと細胞核の解析、RNA・タンパク質の発現解析、イメージング技術およびパターン認識・分類ソフトウエア wndchrm 解析（weighted neighbour distances using a compound hierarchy of algorithms representing morphology）、エネルギー代謝の解析、マウス個体レベルの表現型解析など、各種の手法を組み合わせて、基礎研究から臨床橋渡し研究に繋がる工夫を重ねている。

６．エピゲノム研究による応用・社会貢献

以上の研究成果を社会に還元するために、下記のような特許出願・登録を行った。

発明の名称：ミトコンドリア機能向上剤
(MITOCHONDRIAL FUNCTION-IMPROVING AGENT)
登録番号／登録日:EP2417985／5/10/2016（EU）
登録番号／登録日：特許第 5685764 号／2015/01/30（日本）
登録番号／登録日：US8,637,480／2014/1/28（米国）
出願人：国立大学法人 熊本大学
発明者：中尾光善；日野信次朗

発明の名称：神経変性疾患治療剤
(THERAPEUTIC AGENT FOR NEURODEGENERATIVE DISEASES)
出願番号／出願日：PCT/JP2017/002244／2017/1/24
出願人：国立大学法人　熊本大学
発明者：谷原秀信；岩尾圭一郎；中尾光善；林秀樹；日野信次朗；堤孝之

発明の名称：誘導多能性幹細胞の識別方法
(METHOD FOR DISCRIMINATING INDUCTION MULTIPOTENT STEM CELL)
登録番号／登録日：特許第 5800312 号／2015/09/04
特許公報発行日：2015/09/04
出願人：国立大学法人　熊本大学; オリンパス株式会社
発明者：中尾光善；徳永和明；斉藤典子；小林民代

発明の名称：細胞核を構成する構造体の解析方法、及び細胞核の形態の解析方法
(METHOD OF ANALYZING STRUCTURE COMPOSING CELL NUCLEUS AND METHOD OF ANALYZING FORM OF CELL NUCLEUS)
出願番号／出願日：2009-223587／2009/09/29
公開番号／公開日：2011-75278／2011/04/14
登録番号／登録日：特許第 5482057 号／2014/2/28
出願人：熊本大学、オリンパス株式会社
発明者：中尾光善；齊藤典子；徳永和明；村瀬八重子；小林民代；天川玄太

図１２　エピジェネティクスの解説

Our laboratory is studying the molecular basis of epigenetic cell regulation in development and human diseases. The term epigenetic is defined as "heritable changes in gene expression that occur without a change in DNA sequence". Epigenetic regulation may include cytosine methylation, histone modification, chromatin formation, and nuclear structure.

We are studying how these epigenetic factors control gene expression and cellular function; 1) investigating the molecular basis of epigenome and gene control; 2) studying the epigenetics of energy metabolism; 3) identifying the mechanism involving in epigenetics of cancer and inflammation; 4) studying epigenome of cellular development and senescence; 5) detecting nuclear structure, function and dynamics; and 6) testing epigenetic technology useful for medical diagnosis and therapy.

1. Molecular basis of epigenome and gene control

Methylated DNA is specifically recognized by a set of proteins called methylated DNA-binding proteins, which belong to three different families in mammals: the MBD proteins, the zinc finger (Kaiso) proteins, and the SRA (UHRF) domain proteins. Once bound to methylated DNA, these proteins translate the DNA methylation signal into appropriate functional states, through interactions with various partners.

We investigated the function of MBD1 and found MBD1-containing chromatin-associated factor (MCAF), also known as ATFa-associated modulator (AM) and activating transcription factor 7-interacting protein (ATF7IP), which mediates gene regulation (Cell, 2001; MCB, 2003).

Long-range regulatory elements and higher-order chromatin structure coordinate the expression of multiple genes in cluster, and CTCF/cohesin-mediated chromatin insulator may be a key in this regulation, together with CTCF/CHD8 chromatin remodeling (Mol Cell, 2006; Nature, 2008). The human *apolipoprotein* (*APO*) *A1/C3/A4/A5* gene region, whose alterations increase the risk of dyslipidemia and atherosclerosis, is partitioned at least by three CTCF-enriched sites and three cohesin protein RAD21-enriched sites, resulting in formation of two transcribed chromatin loops by interactions between insulators (EMBO J, 2009).

2. Epigenetics of energy metabolism

Environmental factors such as nutritional state may act on the epigenome which consequently contributes to the metabolic adaptation of cells and the organisms. The lysine demethylase LSD1 is a unique nuclear protein that utilizes flavin adenine dinucleotide (FAD) as a cofactor. We showed that LSD1 epigenetically regulates energy expenditure genes in adipocytes depending on the cellular FAD availability (Nat Commun, 2012). We also revealed that LSD1 is an integrative regulator of aerobic glycolysis in cancer (Cancer Res, 2015; Int J Cancer, 2016), and is a determinant of metabolism-fiber type switch in muscle cells (Nucleic Acids Res, 2018). In addition, we reported that another FAD-dependent demethylase, LSD2, protects hepatic cells from lipotoxicity by repressing lipid transport genes (MCB, 2015). Our data shed light on an essential mechanism of energy utilization which might explain how cells determine their energy strategy depending on nutritional availability.

3. Epigenetics of cancer and inflammation

Estrogen receptor-α (ER)-positive breast cancer cells undergo hormone-independent proliferation after deprivation of estrogen, leading to endocrine therapy resistance. Up-regulation of the ER gene (*ESR1*) is critical for this process, but the underlying mechanisms remain unclear. Recently, the combination of transcriptome and fluorescence *in situ* hybridization analyses revealed that estrogen deprivation induced a cluster of non-coding RNAs that defined a large chromatin domain containing the *ESR1* locus. We named these RNAs as *Eleanors* (*ESR1 locus enhancing and activating non-coding RNAs*) (Nat Commun, 2012).

Eleanors were present in ER-positive breast cancer tissues and localized at the transcriptionally active *ESR1* locus to form characteristic RNA foci. Depletion of one of *Eleanors*, *upstream* (*u*)-*Eleanor*, impaired cell growth and transcription of intragenic *Eleanors* and *ESR1* mRNA, indicating that *Eleanors* *cis*-activate the *ESR1* gene. Furthermore, resveratrol, a kind of polyphenol, was found to repress these RNAs and inhibit the proliferative activity of breast cancer cells which had acquired estrogen-independent growth. Thus, *Eleanors*-mediated gene activation plays an essential role in the adaptation of breast cancer cells. We clinically discuss diagnostic and therapeutic targets for endocrine therapy-resistant breast cancer (Trends Cancer, 2018).

We further investigated the effects of tumor necrosis factor (TNF) signaling on spatiotemporal enhancer-promoter interactions in the human *tumor necrosis factor* (*TNF*)/*lymphotoxin* (*LT*) gene locus, mediated by CTCF-dependent chromatin insulators (MCB, 2012). The cytokine genes *LT*α, *TNF*, and *LT*β are differentially regulated by NF-κB signaling

in inflammatory and oncogenic responses. We identified at least four CTCF-enriched sites with enhancer-blocking activities and a TNF-responsive TE2 enhancer in the *TNF/LT* locus. The insulators mediate the spatiotemporal control of enhancer-promoter associations in the *TNF/LT* gene cluster.

In addition, we are investigating the epigenetics of inflammation memory. Immunological memory in vertebrates is generally thought to be controlled by memory T and B cells. However, recent studies in invertebrates including mosquito and drosophila that lack adaptive immune systems can also exhibit inflammation memory, which means second infection is exclusively affected by first infection without lymphocytes (T and B cells). Inflammation memory is also reported in vertebrates such as adaptive immune system-deficient *Rag1* knockout mice. However, it is still unknown how initial inflammation affects second infection and how inflammation memory is maintained. We thus investigate whether epigenetic changes by first inflammation contributes to the innate immune responses against second inflammation with respect to various epigenetic status including DNA methylation, histone methylation, histone acetylation, chromatin loop, nuclear compartments including euchromatin and heterochromatin.

4. Epigenetics of development and senescence

The *INK4/ARF* locus encodes $p15^{INK4b}$, *ARF*, and $p16^{INK4a}$ genes in human chromosome 9p21, the products of which are known as common key reprogramming regulators. Compared with growing fibroblasts, CTCF is remarkably up-regulated in iPS cells with silencing of the three genes in the locus and is reversely down-regulated in senescent cells with high expression of $p15^{INK4b}$ and $p16^{INK4a}$ genes. There are at least three CTCF-enriched sites in the *INK4/ARF* locus, which possess chromatin loop-forming activities. These results suggest that senescent cells have distinct higher-order chromatin signature in the *INK4/ARF* locus (Aging Cell, 2012).

We revealed that metabolic reprogramming is essential for the progression of cellular senescence, and that histone methyltransferase SETD8/PR-Set7 prevents cellular senescence and senescence-associated metabolic remodeling through regulating specific gene expression (Aging Cell, 2015; Cell Rep, 2017).

5. Nuclear structure, function and dynamics

The nucleus is the origin of cellular function, because it can govern biological information within it. Transcription, RNA dynamics, DNA replication, DNA damage responses, and recombination can be regulated by accumulation of key molecules and their complexes at the respective unique domains in the nucleus. The nuclear domains are actively formed and dispersed in response to the cell environments. During the cell division, the nuclear architectures and domains are broken up and then re-established. Because many of the etiologies in cancer, autoimmune diseases and neurological disorders target the components of the nucleus, we investigated the dynamic structure and function of the nucleus, from the physiological and pathological aspects (JBC, 2008; Mol Biol Cell, 2012).

6. Epigenetic technology useful for medical diagnosis and therapy

Based on our recent progresses, MCAF1 can be used for diagnostic and therapeutic agents in human cancers, since this protein is highly expressed in a variety of cancers.

We then found that the loss of LSD1 function either by siRNAs or by selective inhibitors in adipocytes induces a number of regulators of energy expenditure and mitochondrial metabolism resulting in the activation of mitochondrial respiration. In the adipose tissues from high fat diet-fed mice, expression of LSD1-target genes was reduced compared with that in normal-diet mice, which can be reverted by suppressing LSD1 function.

Further, we established methods to quantitatively assess the imaging data of nuclear domains or substructures such as PML bodies and nucleolus using the pattern recognition software wndchrm (weighted neighbour distances using a compound hierarchy of algorithms representing morphology) (Sci Rep, 2014).

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LSD1 mediates metabolic reprogramming by glucocorticoids during myogenic differentiation

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ABSTRACT

The metabolic properties of cells are formed under the influence of environmental factors such as nutrients and hormones. Although such a metabolic program is likely initiated through epigenetic mechanisms, the direct links between metabolic cues and activities of chromatin modifiers remain largely unknown. In this study, we show that lysine-specific demethylase-1 (LSD1) controls the metabolic program in myogenic differentiation, under the action of catabolic hormone, glucocorticoids. By using transcriptomic and epigenomic approaches, we revealed that LSD1 bound to oxidative metabolism and slow-twitch myosin genes, and repressed their expression. Consistent with this, loss of LSD1 activity during differentiation enhanced the oxidative capacity of myotubes. By testing the effects of various hormones, we found that LSD1 levels were decreased by treatment with the glucocorticoid dexamethasone (Dex) in cultured myoblasts and in skeletal muscle from mice. Mechanistically, glucocorticoid signaling induced expression of a ubiquitin E3 ligase, JADE-2, which was responsible for proteasomal degradation of LSD1. Consequently, in differentiating myoblasts, chemical inhibition of LSD1, in combination with Dex treatment, synergistically de-repressed oxidative metabolism genes, concomitant with increased histone H3 lysine 4 methylation at these loci. These findings demonstrated that LSD1 serves as an epigenetic regulator linking glucocorticoid action to metabolic programming during myogenic differentiation.

INTRODUCTION

Environmental factors exert profound influences on the epigenome, leading to phenotypic variations in cells and organisms (1). In particular, nutritional conditions, such as malnutrition and high fat feeding, induce genome-scale rearrangement of epigenomic signatures, including DNA methylation and histone modifications (2,3). However, direct mechanistic links between environmental cues and the activities of individual chromatin modifiers are poorly understood.

Lysine-specific demethylase-1 (LSD1 or KDM1A) is a member of the amine oxidase family that catalyzes demethylation of methylated lysine side chains within proteins, including histone H3 lysine 4 (H3K4) (4,5). LSD1 contributes to a variety of cellular processes, such as stem cell maintenance, development and carcinogenesis (6–8). We previously demonstrated that LSD1 epigenetically suppressed oxidative phosphorylation (OXPHOS) through H3K4 demethylation in adipogenic and cancer cells (9,10). Notably, metabolic regulation by LSD1 depended on environmental conditions such as energetic state, hormones and neurological stimuli (9,11), indicating that LSD1 reacts to the nutritional and metabolic conditions of cells and modulates their metabolic properties.

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non-transformed cells. Increasing B7-H3 signaling in the recipient cells by exosomes could activate downstream signaling pathways, for example the aforementioned JAK/STAT and PI3K/Akt/mTOR pathways, and thus induce proliferation, metastasis, or resistance to therapy. Similarly, soluble B7-H3 isoforms can also contribute to increased invasion and metastasis capacity.

B7-H3 also promotes resistance to cancer drugs. A growing number of studies show that inhibition or reduced expression of B7-H3 increases the response of tumor cells to drugs that target DNA replication, alkylating agents, and inhibitors of PI3K/Akt/mTOR and Ras/Raf/MEK signaling [4,6,9,10]. This further supports B7-H3 as a target in anticancer therapy, alone or in combination with other existing therapeutic modalities.

Concluding Remarks

The differential expression of B7-H3 in tumors versus healthy tissues makes targeting of B7-H3 particularly attractive, potentially with limited side effects. The efficacy of inhibiting B7-H3 activity was evaluated in preclinical studies with short hairpin RNAs, RNA interference, or anti-B7-H3 monoclonal antibodies. Inhibition or reduction of B7-H3 protein expression in tumor cells decreased proliferation and glycolysis, and increased drug sensitivity. Currently, several clinical trials are targeting B7-H3 (Table 1). The first results from a clinical trial with enoblituzumab (MGA271; anti-B7-H3 antibody) indicate antitumor properties and increased T cell repertoire, with no dose-limiting toxicity and no severe immune-related side effects [12]. The outcome of clinical studies combining anti-B7-H3 antibodies with chemotherapy, small-molecule inhibitors of PI3K/Akt/mTOR and Ras/Raf/MEK pathways, or immune checkpoint inhibitors may establish targeting B7-H3 in cancer as a synergistic treatment approach.

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Forum

Cancer Navigation Strategy for Endocrine Therapy-Resistant Breast Tumors

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Estrogen receptor (ER) α-positive breast cancers frequently acquire resistance to endocrine therapy. However, recent studies found that a fraction of these tumors overexpress ER, and that estrogen treatment induces apoptosis. We propose a 'cancer navigation' strategy to systematically lead resistant cells to growth arrest and apoptosis.

Many tumors initially respond to therapy, but later become insensitive. Therapy resistance is one of the most unfortunate complications in cancer that directly impacts survival [1]. Breast cancer is the most common cause of death in women with cancer, and is classified according to the presence of estrogen receptor (ER) α and/or progesterone receptor (PR), or amplification of human epidermal growth factor receptor (HER2). ER-positive breast cancer represents approximately 70% of cases, and depends on estrogen for tumor growth [1,2]. These tumors typically respond to endocrine blockade therapies using anti-estrogen drugs, such as tamoxifen or aromatase inhibitors, which confer significant improvement in prognosis and survival [2]. In spite of side effects, such as gynecologic symptoms, osteoporosis, or arthralgias, these drugs are well tolerated even in older patients. However, primary resistance to endocrine therapy occurs in ~30% of patients and acquired resistance

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RESEARCH ARTICLE

Ki-67 and condensins support the integrity of mitotic chromosomes through distinct mechanisms

Masatoshi Takagi[1,*], Takao Ono[2], Toyoaki Natsume[3], Chiyomi Sakamoto[4], Mitsuyoshi Nakao[4], Noriko Saitoh[4,5], Masato T. Kanemaki[3], Tatsuya Hirano[2] and Naoko Imamoto[1]

ABSTRACT

Although condensins play essential roles in mitotic chromosome assembly, Ki-67 (also known as MKI67), a protein localizing to the periphery of mitotic chromosomes, had also been shown to make a contribution to the process. To examine their respective roles, we generated a set of HCT116-based cell lines expressing Ki-67 and/or condensin subunits that were fused with an auxin-inducible degron for their conditional degradation. Both the localization and the dynamic behavior of Ki-67 on mitotic chromosomes were not largely affected upon depletion of condensin subunits, and vice versa. When both Ki-67 and SMC2 (a core subunit of condensins) were depleted, ball-like chromosome clusters with no sign of discernible thread-like structures were observed. This severe defective phenotype was distinct from that observed in cells depleted of either Ki-67 or SMC2 alone. Our results show that Ki-67 and condensins, which localize to the external surface and the central axis of mitotic chromosomes, respectively, have independent yet cooperative functions in supporting the structural integrity of mitotic chromosomes.

KEY WORDS: Ki-67, Condensin, Mitotic chromosome, Auxin-inducible degron, AID

INTRODUCTION

During the mitosis of animal cells, the nuclear envelope breaks down and chromatin surrounded by the nuclear envelope is packaged into a discrete set of rod-shaped structures, known as mitotic chromosomes. This process enables different chromosomes to untangle, duplicated chromatids to resolve and sister kinetochores to properly attach to the mitotic spindle, thereby ensuring the faithful segregation of genetic materials into daughter cells. Extensive studies during the past two decades have established that a class of multiprotein complexes, condensins, play central roles in mitotic chromosome assembly and segregation (Hirano, 2016; Uhlmann, 2016). Most eukaryote species have two different types of condensin complexes (condensins I and II). The two complexes share the same pair of structural maintenance of chromosome (SMC) ATPase subunits (SMC2 and SMC4), and have distinct sets of non-SMC regulatory proteins [CAP-H, -D2 and -G for condensin I (also known as NCAPH, NCAPD2 and NCAPG, respectively), and CAP-H2, -D3 and -G2 for condensin II (also known as NCAPH2, NCAPD3 and NCAPG2, respectively)]. A recent study has shown that structures reminiscent of mitotic chromosomes can be reconstituted *in vitro* using a limited number of purified factors, including core histones, three histone chaperones, topoisomerase IIα (topo IIα) and condensin I (Shintomi et al., 2015). It is clear, however, that this list represents a minimum set of proteins required for building mitotic chromosomes, and that additional proteins must cooperate to provide them with physical and physicochemical properties that support and promote their own segregation. Candidates for such proteins include linker histones (Maresca et al., 2005), the chromokinesin KIF4 (Mazumdar et al., 2006; Samejima et al., 2012; Takahashi et al., 2016) and Ki-67 (also known as MKI67) (Booth et al., 2016; Takagi et al., 2016).

Ki-67 is a nucleolar protein widely appreciated as a cell proliferation marker (Scholzen and Gerdes, 2000). During mitosis, Ki-67 is localized around mitotic chromosomes and constitutes a perichromosomal layer to which many nucleolar proteins are targeted (Booth et al., 2014; Takagi et al., 2014). To assess the mitotic function of Ki-67, we have recently generated HCT116-based cell lines in which endogenous Ki-67 can be degraded conditionally and acutely via an auxin-inducible degron (AID) (Takagi et al., 2016). By using these cell lines, we have demonstrated that Ki-67 aids the completion of mitotic chromosome assembly and the maintenance of rod-shaped chromosome structures (Takagi et al., 2016). Another recent study has demonstrated that Ki-67 may act as a biological 'surfactant' to prevent the coalescence of mitotic chromosomes by using its positively charged, extended conformation that orients perpendicular to the surface of mitotic chromosomes (Cuylen et al., 2016). Despite these intriguing observations, it remains unclear how the perichromosomally localized proteins, such as Ki-67, might functionally cooperate with the axially localized proteins, such as condensins, to build individual chromosomes and to support their segregation during mitosis.

In the current study, we aimed to address this question by conditionally depleting Ki-67 and condensin subunits individually or simultaneously from mitotic cells. To this end, we generated a panel of HCT116-based cell lines expressing Ki-67 and/or condensin subunits that were fused with an AID for their conditional degradation and with fluorescent proteins for imaging. Remarkably, ball-like chromosome clusters with no sign of discernible thread-like structures were observed in mitotic cells depleted of both Ki-67 and SMC2. To further assess this unprecedented 'rice cake' phenotype, we performed a quantitative analysis with a supervised machine-learning algorithm, wndchrm (Ono et al., 2017; Orlov et al., 2008). We also present evidence that aberrant kinetochore–microtubule attachments accompany the formation of the rice cake. The observations presented here argue that Ki-67 and condensins, which localize to the external surface and the central axis of mitotic chromosomes, respectively, have independent yet cooperative functions in supporting the structural integrity of mitotic chromosomes in mammalian cells.

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Research paper

Maternal undernutrition during early pregnancy inhibits postnatal growth of the tibia in the female offspring of rats by alteration of chondrogenesis

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ABSTRACT

Epidemiological research has suggested that birth weights are correlated with adult leg lengths. However, the relationship between prenatal undernutrition (UN) and postnatal leg growth remains controversial. We investigated the effects of UN during early pregnancy on postnatal hindlimb growth and determined whether early embryonic malnutrition affects the functions of postnatal chondrocytes in rats.

Undernourished Wistar dams were fed 40% of the daily intake of rats in the control groups from gestational days 5.5–11.5, and femurs, tibias, and trunks or spinal columns were morphologically measured at birth and at 16 weeks of age in control and undernourished offspring of both sexes. We evaluated cell proliferation and differentiation of cultured chondrocytes derived from neonatal tibias of female offspring and determined chondrocyte-related gene expression levels in neonatal epiphysis and embryonic limb buds.

Tibial lengths of undernourished female, but not male, offspring were longer at birth and shorter at 16 weeks of age ($p < .05$) compared with those of control rats. In chondrocyte culture studies, stimulating effects of IGF-1 on cell proliferation ($p < .01$) were significantly decreased and levels of type II collagen were lower in female undernourished offspring ($p < .05$). These phenomena were accompanied by decreased expression levels of *Col2a1* and *Igf1r* and increased expression levels of *Fgfr3* ($p < .05$), which might be attributable to the decreased expression of specificity protein 1 ($p < .05$), a key transactivator of *Col2a1* and *Igf1r*.

In conclusion, UN stress during early pregnancy reduces postnatal tibial growth in female offspring by altering the function of chondrocytes, likely reflecting altered expression of gene transactivators.

1. Introduction

Maternal nutrition during pregnancy and lactation plays a pivotal role in the health of human offspring. Previous epidemiological studies show that maternal undernutrition (UN) during early gestation increases the risk of obesity, cardiovascular disease, and mental illness, whereas UN during mid and late gestation is correlated with reduced glucose tolerance and obstructive airway disease, respectively (Roseboom et al., 2001; Schulz, 2010). Moreover, related mechanisms have been demonstrated in line with "fetal origins hypothesis" and "*in utero* programming" concepts (Barker and Osmond, 1986; Lucas, 1998; Roseboom et al., 2011; Sayer and Cooper, 2005; Victora et al., 2008). Maternal dietary components also have strong effects on epigenetic processes during specific periods of fetal and early postnatal development.

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ORIGINAL PAPER

Nrf2 promotes oesophageal cancer cell proliferation via metabolic reprogramming and detoxification of reactive oxygen species

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Abstract

Cancer cells consume a large amount of energy and maintain high levels of anabolism to promote cell proliferation via metabolic reprogramming. Nuclear factor erythroid 2-related factor 2 (Nrf2; NFE2L2) is a master transcription regulator of stress responses and promotes metabolic reprogramming to support cell proliferation in various types of cancer. As oesophageal cancer is one of the most aggressive gastrointestinal cancers, we aimed to clarify the effect of Nrf2 on metabolic reprogramming in oesophageal cancer. The relationship between Nrf2 expression and clinical outcome was evaluated using a database comprising 201 oesophageal cancers. Using *in vitro* assays and metabolome analysis, we examined the mechanism by which Nrf2 affects malignant phenotype. High-level immunohistochemical expression of Nrf2 was significantly associated with poor recurrence-free survival (HR = 2.67, $p = 0.0004$) and overall survival (HR = 2.90, $p < 0.0001$) in oesophageal cancer patients. In an *in vitro* assay with siRNA in TE-11 cells, which showed high Nrf2 expression, Nrf2 depletion significantly decreased cell growth and enhanced G1 cell cycle arrest and apoptosis. In addition, reactive oxygen species (ROS) were not removed by detoxification via the Nrf2 pathway, with concomitant induction of the p38 mitogen-activated protein kinase pathway. The metabolome analysis showed that Nrf2 strongly promoted metabolic reprogramming to glutathione metabolism, which synthesizes the essential fuels for cancer progression. Furthermore, metabolome analysis using oesophageal cancer specimens confirmed that samples displaying high Nrf2 expression promoted glutathione synthesis. Metabolic reprogramming to glutathione metabolism, and ROS detoxification by activation of Nrf2, enhanced cancer progression and led to a poor clinical outcome in oesophageal cancer patients.

Keywords: Nrf2; NFE2L2; glutathione metabolism; ROS detoxification; oesophageal cancer

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No conflicts of interest were declared.

Introduction

Oesophageal cancer is the sixth most common cause of cancer-related death worldwide, resulting in approximately 400 000 deaths per year [1]. Despite recent advances in multimodality therapies including chemotherapy, radiotherapy, and chemoradiotherapy, the prognosis of patients, even those who have undergone curative resection, remains poor [2–4]. Further studies are therefore needed to clarify the pathogenesis and biology of oesophageal cancer and to explore new diagnostic and therapeutic possibilities.

Metabolic activities in proliferating cells are fundamentally different from those in quiescent cells. Proliferating cells shunt their metabolites into anabolic pathways, which consume large quantities of nutrients [5]. Utilizing such characteristics of proliferating cells, detection of the glucose analogue 2-[^{18}F]fluoro-2-deoxy-D-glucose by positron emission tomography is by far the most commonly used imaging technique in clinical situations including oesophageal cancer [6–8]. Recent studies have revealed that oncogenic pathways directly promote the metabolism of glucose and glutamine [9–11]. Furthermore, considering that such aberrant activated metabolism in proliferating cells promotes the accumulation of reactive oxygen species (ROS), the efficient detoxification of ROS is a requisite for cancer cell proliferation [12–16]. To counteract the detrimental effects of ROS, cancer cells provide two of the most abundant antioxidants – reduced glutathione (GSH) and reduced nicotinamide adenine dinucleotide phosphate – by genetic changes and metabolic adaptations [16,17].

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Article

The SETD8/PR-Set7 Methyltransferase Functions as a Barrier to Prevent Senescence-Associated Metabolic Remodeling

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SUMMARY

Cellular senescence is an irreversible growth arrest that contributes to development, tumor suppression, and age-related conditions. Senescent cells show active metabolism compared with proliferating cells, but the underlying mechanisms remain unclear. Here we show that the SETD8/PR-Set7 methyltransferase, which catalyzes mono-methylation of histone H4 at lysine 20 (H4K20me1), suppresses nucleolar and mitochondrial activities to prevent cellular senescence. SETD8 protein was selectively downregulated in both oncogene-induced and replicative senescence. Inhibition of SETD8 alone was sufficient to trigger senescence. Under these states, the expression of genes encoding ribosomal proteins (*RP*s) and ribosomal RNAs as well as the cyclin-dependent kinase (CDK) inhibitor *p16^{INK4A}* was increased, with a corresponding reduction of H4K20me1 at each locus. As a result, the loss of SETD8 concurrently stimulated nucleolar function and retinoblastoma protein-mediated mitochondrial metabolism. In conclusion, our data demonstrate that SETD8 acts as a barrier to prevent cellular senescence through chromatin-mediated regulation of senescence-associated metabolic remodeling.

INTRODUCTION

Cellular senescence is induced by various cellular stresses such as oncogene expression, telomere attrition, and genome-scale perturbation of chromatin. It is characterized by irreversible cell cycle arrest, senescence-associated β-galactosidase (SA-β-Gal) activity, and the senescence-associated secretory phenotype (SASP), as well as alterations of gene expression and chromatin (Benayoun et al., 2015; Campisi and d'Adda di Fagagna, 2007; Kuilman et al., 2010). These changes in senescent cells contribute to tumor suppression, tissue repair, and developmental processes, as well as age-related deterioration of tissue functions in vivo (Baker et al., 2016; Muñoz-Espín and Serrano, 2014; van Deursen, 2014). The senescent cells also undergo metabolic remodeling as indicated by enlarged cell size and increased protein content. Various metabolic pathways, including protein synthesis and degradation, autophagy, glycolysis, and mitochondrial oxidative phosphorylation (OXPHOS), are essential for the establishment of senescence (Salama et al., 2014; Wiley and Campisi, 2016). However, it is not clear how senescent cells remodel their metabolic status in combination with other senescence-associated features.

Chromatin-modifying factors play a fundamental role in gene regulation and are involved in DNA methylation, histone modification, and the formation of higher-order chromatin structures. The epigenomic landscapes in senescent cells differ from those of proliferating cells (Chandra et al., 2012; Chicas et al., 2012; Criscione et al., 2016; Cruickshanks et al., 2013; Hirosue et al., 2012; O'Sullivan et al., 2010; Shah et al., 2013), indicating that chromatin regulators play an important role in establishing and maintaining the senescent state. Growing evidence suggests that there is a reciprocal relationship between epigenetic regulation and cellular metabolism (Gut and Verdin, 2013; Hino et al., 2012, 2013). Most chromatin-modifying enzymes use substrates or cofactors derived from various metabolites (such as S-adenosylmethionine for many methyltransferases), while biochemical reactions depend on coordinated expression of many enzyme-encoding genes in metabolic pathways (Desvergne et al., 2006). So far, we do not understand how epigenetic and metabolic mechanisms cooperate to establish cellular senescence.

SETD8, also known as PR-Set7 or SET8, is a nucleosome-specific methyltransferase that is responsible for mono-methylation of histone H4 lysine 20 (H4K20me1) (Nishioka et al., 2002). SETD8 is involved in various genomic functions including DNA replication, mitosis, DNA repair, and gene expression via H4K20 methylation (Beck et al., 2012; Jørgensen et al., 2013). The protein levels of SETD8 are precisely controlled through proteasomal degradation, resulting in the lowest level during

RESEARCH ARTICLE

The Glucocorticoid Receptor Regulates the *ANGPTL4* Gene in a CTCF-Mediated Chromatin Context in Human Hepatic Cells

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Abstract

Glucocorticoid signaling through the glucocorticoid receptor (GR) plays essential roles in the response to stress and in energy metabolism. This hormonal action is integrated to the transcriptional control of GR-target genes in a cell type-specific and condition-dependent manner. In the present study, we found that the GR regulates the *angiopoietin-like 4* gene (*ANGPTL4*) in a CCCTC-binding factor (CTCF)-mediated chromatin context in the human hepatic HepG2 cells. There are at least four CTCF-enriched sites and two GR-binding sites within the *ANGPTL4* locus. Among them, the major CTCF-enriched site is positioned near the *ANGPTL4* enhancer that binds GR. We showed that CTCF is required for induction and subsequent silencing of *ANGPTL4* expression in response to dexamethasone (Dex) and that transcription is diminished after long-term treatment with Dex. Although the *ANGPTL4* locus maintains a stable higher-order chromatin conformation in the presence and absence of Dex, the Dex-bound GR activated transcription of *ANGPTL4* but not that of the neighboring three genes through interactions among the *ANGPTL4* enhancer, promoter, and CTCF sites. These results reveal that liganded GR spatiotemporally controls *ANGPTL4* transcription in a chromosomal context.

Introduction

The glucocorticoid receptor (GR) is a member of a family of transcription factors that regulate biological processes, such as basal and stress-associated homeostasis, energy metabolism, and the immune response in a cell-type and condition-dependent manner [1, 2]. In the absence of ligand, GR is present in the cytoplasm in a complex with chaperons such as heat-shock proteins. Upon ligand-induced activation, GR dissociates from the complex and translocates to the nucleus, typically by binding to the glucocorticoid response elements (GREs) to activate or repress transcription of target genes. After the gene control, GR dissociates from its ligand or is degraded [2].

Condensin II plays an essential role in reversible assembly of mitotic chromosomes in situ

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ABSTRACT **Condensins I and II are multisubunit complexes that play a central role in mitotic chromosome assembly. Although both complexes become concentrated along the axial region of each chromatid by metaphase, it remains unclear exactly how such axes might assemble and contribute to chromosome shaping. To address these questions from a physicochemical point of view, we have established a set of two-step protocols for inducing reversible assembly of chromosome structure in situ, namely within a whole cell. In this assay, mitotic chromosomes are first expanded in a hypotonic buffer containing a Mg^{2+}-chelating agent and then converted into different shapes in a NaCl concentration-dependent manner. Both chromatin and condensin-positive chromosome axes are converted into near-original shapes at 100 mM NaCl. This assay combined with small interfering RNA depletion demonstrates that the recovery of chromatin shapes and the reorganization of axes are highly sensitive to depletion of condensin II but less sensitive to depletion of condensin I or topoisomerase IIα. Furthermore, quantitative morphological analyses using the machine-learning algorithm wndchrm support the notion that chromosome shaping is tightly coupled to the reorganization of condensin II–based axes. We propose that condensin II makes a primary contribution to mitotic chromosome architecture and maintenance in human cells.**

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INTRODUCTION

When eukaryotic cells divide, chromatin residing within the interphase nucleus is converted into a discrete set of individual chromosomes, each composed of a pair of rod-shaped chromatids (sister chromatids). This process, known as mitotic chromosome assembly or condensation, is an essential prerequisite for faithful segregation of genetic information into two daughter cells. Despite enormous progress marked during the past two decades or so, its molecular mechanism remains not fully understood (Belmont, 2006; Marko, 2008; Kinoshita and Hirano, 2017).

It is generally thought that the protein composition of mitotic chromosomes is highly complex, especially because they represent one of the largest structures observed within the cell. In fact, a recent proteomics approach has identified ~4000 proteins in mitotic chromosomes isolated from chicken DT40 cells (Ohta *et al.*, 2010). It should be noted, however, that this number includes domain-specific components (e.g., centromere- and telomere-specific proteins) and contaminants that may artificially get associated with chromosomes during their isolation. It is therefore possible that the "core" components required for building the bulk part of mitotic chromosomes is much simpler, as had been shown in classical studies of metaphase chromosomes isolated from HeLa cells (Gasser and Laemmli, 1987) or mitotic chromatids assembled in *Xenopus* egg cell-free extracts (Hirano and Mitchison, 1994). In fact, only two factors, topoisomerase IIα (topo IIα) and condensin I, have been demonstrated so far to be essential for mitotic chromatid assembly in the cell-free extracts (Hirano and Mitchison, 1993; Hirano *et al.*, 1997). Equally important, a recent study has demonstrated that mitotic chromosome-like structures can be reconstituted in vitro by

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Abbreviations used: H3K9me3, histone H3 trimethylated at Lys-9; IMS, intrinsic metaphase structure; PFA, paraformaldehyde; PI, propidium iodide; SCC, sodium chloride-induced chromosome conversion; SMC, structural maintenance of chromosomes; topo IIα, topoisomerase IIα; wndchrm, weighted neighbor distances using a compound hierarchy of algorithms representing morphology.

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A novel inhibitor of farnesyltransferase with a zinc site recognition moiety and a farnesyl group

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ABSTRACT

Protein prenylation such as farnesylation and geranylgeranylation is associated with various diseases. Thus, many inhibitors of prenyltransferase have been developed. We report novel inhibitors of farnesyltransferase with a zinc-site recognition moiety and a farnesyl/dodecyl group. Molecular docking analysis showed that both parts of the inhibitor fit well into the catalytic domain of farnesyltransferase. The synthesized inhibitors showed activity against farnesyltransferase *in vitro* and inhibited proliferation of the pancreatic cell line AsPC-1. Among the compounds with farnesyl and dodecyl groups, the inhibitor with a farnesyl group was found to have stronger and more selective activity.

Modification of proteins by prenyl lipids, e.g., farnesyl (15-carbon) or geranylgeranyl (20-carbon) isoprenoid lipid, is often crucial for protein function in a cell.[1,2] The first step of prenylation is catalyzed by a zinc-containing farnesyltransferase or geranylgeranyltransferase, which conjugates a farnesyl/geranylgeranyl group of farnesyl/geranylgeranyl pyrophosphate to a cysteine residue of the C-terminal CAAX motif (X: aliphatic amino acid; A: any amino acid) of the substrate protein. Subsequently, the terminal AAX tripeptide is removed by endoprotease digestion and the resulting carboxyl of the prenylated cysteine is methylated by methyltransferase. The attached prenyl group, with its hydrophobic character, facilitates the translocation of the prenylated protein to the membrane.

Protein prenylation is associated with various diseases, such as cancer, progeria, infectious diseases, glaucoma and neurological diseases.[1,3] Thus, many inhibitors of farnesyltransferase/geranylgeranyltransferase have been developed, including some under clinical investigation.[1–4] Most of the inhibitors are CAAX analogues, farnesyl/geranylgeranyl pyrophosphate analogues and screened library compounds. However, the zinc site of these transferases has yet to be a major inhibitory target.

We developed previously a dithiol compound SN-1 (Fig. 1) and showed that SN-1 inhibits the function of zinc proteins[5–7] via a zinc-binding mechanism.[8] In particular, we successfully endowed SN-1 with farnesyltransferase specificity by introducing a protein-recognizing naphthyl group, i.e., naphthyl-SN-1 (Fig. 1).[9] This compound inhibited farnesyltransferase, which is known to farnesylate the Ras oncoprotein to cause cancer.[10–12] We herein report novel SN-1 derivatives with a farnesyl/dodecyl group that bind into the farnesyl pyrophosphate-binding pocket of farnesyltransferase.

Analogous to the case of naphthyl-SN-1, we placed a farnesyl group at the 4-position of pyridine, i.e., farnesyl-SN-1 (Fig. 1). The structure of farnesyl-SN-1 seemed rational, as indicated by the docking study using the Molecular Operating Environment (MOE) 2014.09. As a template for docking, we selected the X-ray crystal structure (PDB ID: 1SA4) of human farnesyltransferase complexed with farnesyl pyrophosphate and an inhibitor Tipifarnib, which binds to the catalytic domain with high affinity.[3,13]

Abbreviations: DTT, dithiothreitol.

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Correlation between histone acetylation and expression of Notch1 in human lung carcinoma and its possible role in combined small-cell lung carcinoma

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Combined small-cell lung carcinoma (cSCLC) is composed of small-cell lung carcinoma (SCLC) admixed with non-small-cell lung carcinoma (NSCLC). Evaluating the molecular differences between SCLC and NSCLC could lead to a better understanding of the pathogenesis of such neoplasms. Therefore, in this study, we investigated the correlation between histone acetylation and Notch1 expression in lung carcinoma. Using chromatin immunoprecipitation (ChIP) assay, we measured the level of acetylated histone H3 around the promoter region of *Notch1* in SCLC and NSCLC cells. We then treated SCLC cells with trichostatin A (TSA) and characterized the level of histone H3 acetylation at *Notch1*. In addition, TSA-treated cells were injected into immune-compromised mice, for analysis of the *ex vivo* tumor xenograft phenotype. The level of acetylated histone H3 surrounding the *Notch1* promoter was lower in lung cancer cells not expressing Notch1. Tumors originated from TSA-treated SCLC cells occasionally formed an epithelial-like glandular arrangement of cells; with Notch1 expression and decreased expression of neuroendocrine (NE) markers. Histone deacetylation around the promoter region of *Notch1* inhibits Notch1 protein expression in SCLC and the restoration of Notch1 expression in SCLC leads to the concurrent appearance of epithelial-like areas within the SCLC, which could provide a possible mechanism for histogenesis of cSCLC.

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Combined small-cell lung carcinoma (cSCLC) is defined by the World Health Organization (WHO) as small-cell carcinoma (SCLC) combined with any of the non-small-cell lung carcinoma (NSCLC) types including adenocarcinoma (ADC), squamous cell carcinoma (SCC), and large-cell carcinoma (LCC).[1] SCLC (including the cSCLC variant) comprises 15–20% of all lung cancers, with cSCLC accounting for 4–6%, with a high incidence of mortality.[2] Combined tumors in any organ raise biologic questions about its origin, pathogenesis, and treatment. In addition, cSCLC is considered as a type of SCLC, possibly due to the neuroendocrine (NE) differentiation of both tumor components,[3] yet the difference in their morphology needs further clarification. Due to the lack of cSCLC cell lines, studying such a unique type of lung carcinoma *in vitro* requires comparison of cellular models of both SCLC and NSCLC, to mimic the clinical scenario.

One of the most significant molecular differences between SCLC and NSCLC is the activation of Notch1 signaling in the latter.[4–6] Notch1 has different roles in SCLC and NSCLC, as we previously showed,[6,7] and its induction induced 'epithelial-like' features in SCLC tissue sections.[6] The exact mechanism of Notch1 silencing in SCLC remains unclear, although 25% of SCLC cases have mutations in Notch-related genes.[8] Moreover, histone acetylation has been reported to

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ORIGINAL ARTICLE

Analysis of estrogen receptor β gene methylation in autistic males in a Chinese Han population

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Abstract Autism spectrum disorder (ASD) is a neurodevelopment disorder with abnormalities of social interaction, communication and repetitive behaviors. The higher prevalence of ASD in men implies a potential relationship between sex hormones and ASD etiology. The *ESR2* gene encodes estrogen receptor beta (ESR2) and plays an important role during brain development. A relationship between *ESR2* and ASD has been suggested by studies on single nucleotide polymorphisms and mRNA and protein expression levels in ASD patients. Here, we explored the possible epigenetic regulation of the *ESR2* gene in autism. We collected genomic DNA from the peripheral blood of Chinese Han males with autism and age-matched normal males and measured DNA methylation of CpG islands in the *ESR2* gene, which consisted of 41 CpG sites among the proximal promoter region and an untranslated exon, by bisulfite sequencing. We also investigated a relationship between DNA methylation and phenotypic features of autism, as assessed by the Children Autism Rating Scale. We found little overall difference in the DNA methylation of the *ESR2* 5′-flanking region in individuals with autism compared with normal individuals. However, detailed analyses revealed that eight specific CpG sites were hypermethylated in autistic individuals and that four specific CpG sites were positively associated with the severity of autistic symptoms. Our study indicates that the epigenetic dysregulation of *ESR2* may govern the development of autism.

Keywords DNA methylation · Estrogen receptor beta · Autism · Promoter

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Introduction

Autism spectrum disorder (ASD) is characterized by difficulties in social communications and behaviors. ASD has long been known to occur predominantly in males, with a widely reported male:female ratio of 4–5:1 and a more recently reported male:female ratio of 2–3:1 from population-based studies (Kim et al. 2011; Lai et al. 2015; Werling 2016). Several mechanisms underlying the higher incidence in males have been suggested, including the "fetal testosterone (fT)" theory (Baron-Cohen et al. 2011). Additionally, ASD risk factors may have different impacts depending on sex, as females and males with autism have been found to exhibit different patterns of atypical brain structure (Nordahl et al. 2015). A female-specific effect that protects the developing female brain from autism may also exist (Werling and Geschwind 2015). Consequently, the male and female autism populations should be evaluated separately.

In the present study, we focused on male participants who were susceptible to sex steroid hormone exposure in early development. During fetal and perinatal development, the sexual differentiation of the brain is established under quite different hormonal environments. Males produce high levels of fetal

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Original Article

ORIGINAL ARTICLE

DNA methylation-independent removable insulator controls chromatin remodeling at the *HOXA* locus via retinoic acid signaling

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Abstract

Chromatin insulators partition the genome into functional units to control gene expression, particularly in complex chromosomal regions. The CCCTC-binding factor (CTCF) is an insulator-binding protein that functions in transcriptional regulation and higher-order chromatin formation. Variable CTCF-binding sites have been identified to be cell type-specific partly due to differential DNA methylation. Here, we show that DNA methylation-independent removable CTCF insulator is responsible for retinoic acid (RA)-mediated higher-order chromatin remodeling in the human *HOXA* gene locus. Detailed chromatin analysis characterized multiple CTCF-enriched sites and RA-responsive enhancers at this locus. These regulatory elements and transcriptionally silent *HOXA* genes are closely positioned under basal conditions. Notably, upon RA signaling, the RAR/RXR transcription factor induced loss of adjacent CTCF binding and changed the higher-order chromatin conformation of the overall locus. Targeted disruption of a CTCF site by genome editing with zinc finger nucleases and CRISPR/Cas9 system showed that the site is required for chromatin conformations that maintain the initial associations among insulators, enhancers and promoters. The results indicate that the initial chromatin conformation affects subsequent RA-induced *HOXA* gene activation. Our study uncovers that a removable insulator spatiotemporally switches higher-order chromatin and multiple gene activities via cooperation of CTCF and key transcription factors.

Introduction

Distal cis-regulatory elements such as enhancers and locus control regions (LCRs), are often involved in cell type- and developmental stage-specific gene expression (1,2). Previous studies have shown that higher-order chromatin dynamics facilitates interactions between distal cis-regulatory elements and their target gene promoters (3,4). In combination with DNA methylation and histone modification, this spatiotemporal epigenetic gene regulation is essential for various pathophysiological phenomena in mammals.

Chromatin insulators, also known as boundary elements, define functional domains that ensure precise enhancer-promoter interactions within domains (5). The CCCTC-binding

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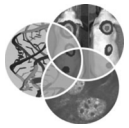
Advanced Review

Roles of long noncoding RNAs in chromosome domains



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The cell nucleus is highly organized and functionally compartmentalized. Double-stranded naked DNA is complexed with core histones and assembled into nucleosomes and chromatin, which are surrounded by nuclear domains composed of RNAs and proteins. Recently, three-dimensional views of chromosome organization beyond the level of the nucleosome have been established and are composed of several layers of chromosome domains. Only a small portion of the human genome encodes proteins; the majority is pervasively transcribed into noncoding RNAs whose functions are under intensive investigation. Importantly, the questions of how nuclear retained noncoding RNAs play roles in orchestrating the chromatin structure that have been addressed. We discuss the novel noncoding RNA clusters, *Eleanors*, which are derived from a large chromatin domain. They accumulate at the site of their own transcription to form RNA clouds in the nucleus, and they activate gene expression in the chromatin domain. Noncoding RNAs have emerging roles in genome regulation that are integrated into the spatial organization of chromatin and the nucleus.

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INTRODUCTION

Nuclear architecture is built with genomic DNA, proteins, and RNAs. Over recent years, high-throughput transcriptome analyses have identified thousands of mammalian long noncoding RNAs (lncRNAs), most of which are retained in the nucleus.[1] Generally, they show unique expression under specific conditions and function as key regulatory factors of various cellular processes, such as cell differentiation, X-chromosome inactivation (XCI), imprinting, and maintenance of pluripotency.[2–4] At a molecular level, they play roles in chromatin modulation, gene repression, enhancer/promoter function, and establishing the three-dimensional structure of chromosomes.[5–8]

In the nucleus, genomes do not exist just as one-dimensional structures that are defined by their linear nucleotide sequences. They are intricately folded into several layers of higher order structures, which are critical for maintaining genome stability and proper regulation of many nuclear functions.[9,10] Microscopy revealed that individual chromosomes occupy specific space in the nucleus, and are segmented into large domains.[11] In parallel, use of a wide range of techniques, including both molecular and biochemical approaches, elucidated that chromosomes are organized into domains. To date, various kinds of chromatin domains have been defined, ranging from hundreds of kilobases (kb) to megabases (Mb) in size. These domains have physiological characteristics, such as DNase sensitivity, histone modification patterns, and association with specific nuclear architectures. Of particular note, recently developed

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Review Article

Histone demethylase LSD1 controls the phenotypic plasticity of cancer cells

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Key words
Cancer metabolism, chromatin, histone demethylation, lysine-specific demethylase-1, stemness

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Epigenetic mechanisms underlie the phenotypic plasticity of cells, while aberrant epigenetic regulation through genetic mutations and/or misregulated expression of epigenetic factors leads to aberrant cell fate determination, which provides a foundation for oncogenic transformation. Lysine-specific demethylase-1 (LSD1, KDM1A) removes methyl groups from methylated proteins, including histone H3, and is frequently overexpressed in various types of solid tumors and hematopoietic neoplasms. While LSD1 is involved in a wide variety of normal physiological processes, including stem cell maintenance and differentiation, it is also a key player in oncogenic processes, including compromised differentiation, enhanced cell motility and metabolic reprogramming. Here, we present an overview of how LSD1 epigenetically regulates cellular plasticity through distinct molecular mechanisms in different biological contexts. Targeted inhibition of the context-dependent activities of LSD1 may provide a highly selective means to eliminate cancer cells.

Epigenetic gene regulation plays a central role not only in maintaining cell identity but also in reprogramming a cell's phenotype in response to environmental fluctuations.(1) Because cancer cells exhibit epigenomic signatures that are distinct from their normal counterparts, it is likely that their phenotypic plasticity is controlled in a unique way.(2)

Methylation of specific lysine residues in the N-terminal tails of histone proteins underlie diverse gene regulatory responses, including transcriptional activation and repression.(3) In general, methyl modifications at histone H3 lysine 4 (H3K4me) reflects transcriptional competency, while those at lysine 9 and 27 (H3K9me and H3K27me, respectively) are components of repressive chromatin structure.(4) These marks are dynamically regulated by specific methyltransferases and demethylases, both in steady-state cells and during cellular transitions. The proper regulation of these marks is essential for the maintenance of cell identity as well as for differentiation, and their misregulation is often linked to the development of cancer.(5)

Lysine-specific demethylase-1 (LSD1) was the first histone demethylase to be identified that demethylates histone H3K4 and H3K9. Extensive studies have established that LSD1 is essential for stem cell function and animal development. In addition, overexpression of LSD1 has been found in many types of cancer, and has been experimentally demonstrated to be a critical player in cancer development. Here, we provide an overview of how LSD1 contributes to phenotypic plasticity in cancer and normal stem cells through chromatin regulation. A number of proteins other than histones have also been identified as substrates of LSD1-mediated demethylation. A detailed review of LSD1 in non-histone protein demethylation can be found elsewhere.(6)

Molecular structure and function of lysine-specific demethylase-1. To date, according to the HUGO database (www.genenames.org), 21 lysine demethylases have been identified in the human genome, most of which target histones in a residue-selective manner.(5) Nineteen demethylases belong to the jumonji domain-containing dioxygenase family, while LSD1 and LSD2 (KDM1B) are the only members of the flavin-dependent amine oxidase family, which require flavin adenine dinucleotide for their enzymatic activity (Fig. 1a). Biochemical

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HMGA2 promotes adipogenesis by activating C/EBPβ-mediated expression of PPARγ

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ABSTRACT

Adipogenesis is orchestrated by a highly ordered network of transcription factors including peroxisome-proliferator activated receptor-gamma (PPARγ) and CCAAT-enhancer binding protein (C/EBP) family proteins. High mobility group protein AT-hook 2 (HMGA2), an architectural transcription factor, has been reported to play an essential role in preadipocyte proliferation, and its overexpression has been implicated in obesity in mice and humans. However, the direct role of HMGA2 in regulating the gene expression program during adipogenesis is not known. Here, we demonstrate that HMGA2 is required for C/EBPβ-mediated expression of PPARγ, and thus promotes adipogenic differentiation. We observed a transient but marked increase of *Hmga2* transcript at an early phase of differentiation of mouse 3T3-L1 preadipocytes. Importantly, *Hmga2* knockdown greatly impaired adipocyte formation, while its overexpression promoted the formation of mature adipocytes. We found that HMGA2 colocalized with C/EBPβ in the nucleus and was required for the recruitment of C/EBPβ to its binding element at the *Pparγ2* promoter. Accordingly, HMGA2 and C/EBPβ cooperatively enhanced the *Pparγ2* promoter activity. Our results indicate that HMGA2 is an essential constituent of the adipogenic transcription factor network, and thus its function may be affected during the course of obesity.

1. Introduction

Cellular differentiation is a highly ordered process, in which stepwise expression and/or activation of transcription factors (TFs) orchestrate the timely expression of a select set of genes [1–4]. During adipogenesis, preadipocytes undergo cell-cycle arrest followed by the reconstitution of a gene expression program, leading to the formation of lipid-accumulating mature adipocytes [5–7]. Upon adipogenic induction, key TFs including CCAAT-enhancer binding protein (C/EBP) family factors sequentially operate to activate the master adipogenic factor, peroxisome-proliferator activated receptor-gamma (PPARγ) [3]. Because the total adipocyte number has been intimately linked to obesity in humans, it is crucial to understand the precise mechanism of how preadipocytes cease proliferation and initiate the adipogenic program.

High mobility group protein AT-hook 2 (HMGA2) belongs to the non-histone chromosomal high mobility group (HMG) protein family and functions as an architectural transcriptional factor by recognizing AT-rich sequences in the minor groove of DNA and enhancing gene transcription [8–10]. *HMGA2* expression is absent or very low in adult tissues, while it is highly expressed in embryos and mesenchymal stem cells, implying that HMGA2 functions at an early developmental stage [9]. A previous study demonstrated that transgenic expression of *Hmga2* in mice caused a giant phenotype and hyperplasia of white adipose tissue (WAT) [11–13]. However, *Hmga2*-deficient mice exhibited a pygmy phenotype with drastically reduced fat tissue and resisted against diet-induced obesity [14,15]. Importantly, a recent study reported significantly elevated levels of *HMGA2* in human WAT of individuals with obesity and/or

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Glaucoma

Potential Neuroprotective Effects of an LSD1 Inhibitor in Retinal Ganglion Cells via p38 MAPK Activity

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Purpose. The epigenetic mechanisms associated with ocular neurodegenerative diseases remain unclear. The present study aimed to determine the role of lysine-specific demethylase 1 (LSD1), which represses transcription by removing the methyl group from methylated lysine 4 of histone H3, in retinal ganglion cell (RGC) survival, and to investigate the details of the neuroprotective mechanism of tranylcypromine, a major LSD1 inhibitor.

Methods. The authors evaluated whether tranylcypromine contributes to neuronal survival following stress-induced damage using primary cultured rat RGCs and in vivo N-methyl-D-aspartate (NMDA)-induced excitotoxicity. Additionally, the molecules associated with tranylcypromine treatment were assessed by microarray and immunoblot analysis.

Results. Tranylcypromine significantly suppressed neuronal cell death following glutamate neurotoxicity and oxidative stress. Microarray and immunoblot analyses revealed that p38 mitogen-activated protein kinase (MAPK)γ was a key molecule involved in the neuroprotective mechanisms induced by tranylcypromine because the significant suppression of p38 MAPKγ by glutamate was reversed by tranylcypromine. Moreover, although pharmacologic inhibition of the phosphorylation of the total p38 MAPKs interfered with neuroprotective effects of tranylcypromine, the specific inhibition of p38 MAPKα and p38 MAPKβ did not influence RGC survival. This suggests that the non-p38 MAPKα/β isoforms have important roles in neuronal survival by tranylcypromine. Additionally, the intravitreal administration of tranylcypromine significantly saved RGC numbers in an in vivo glaucoma model employing NMDA-induced excitotoxicity.

Conclusions. These findings indicate that tranylcypromine-induced transcriptional and epigenetic regulation modulated RGC survival via the promotion of p38 MAPKγ activity. Therefore, pharmacologic treatments that suppress LSD1 activity may be a novel therapeutic strategy that can be used to treat neurodegenerative diseases.

Keywords: tranylcypromine, neuroprotection, survival, glaucoma, epigenetic drug

Glaucoma is a major optic neuropathy characterized by the significant loss of retinal ganglion cells (RGCs)[1] and is a leading cause of blindness around the world.[2–4] Retinal ganglion cell death directly causes visual field deficits, and the primary risk factor associated with progressive damage to the visual field is elevated intraocular pressure (IOP).[5–7] However, RGCs may suffer damage even in normal-tension glaucoma patients in which IOP is within the normal range (10–21 mm Hg).[8]

Recent epidemiologic studies have demonstrated that normal-tension glaucoma is more common than high-tension glaucoma among primary open-angle glaucoma patients, particularly in Asian populations.[9–12] The causes of normal-tension glaucoma have yet to be completely elucidated; however, a variety of factors, including reduced blood flow in the optic nerve,[13] genetic variables,[14,15] and an enlarged gap between cerebrospinal fluid pressure and IOP,[16] may be involved in the pathophysiology of this disease. Evidence-based treatments for patients with glaucoma, including normal-tension patients, target reductions in IOP via ophthalmic solutions, laser therapy, or surgery.[17] However, the therapeutic efficacies of these treatments are often limited and may be clinically insufficient even though they lower IOP. Thus, novel treatment strategies for glaucoma, such as the neuroprotection of RGCs, are urgently required.

Except for alterations in the original DNA sequence via DNA methylation, the modulation of histone, or noncoding RNA, epigenetic changes influence gene expression and function.[18] It is well known that histone is specifically modified by a variety of mechanisms, including the acetylation of histone N-terminal tails, methylation, phosphorylation, ubiquitination, and adenosine diphosphate (ADP) ribosylation.[19] Histone modification involves switches that alter chromatin structure or form binding platforms that allow for downstream effector proteins to induce transcriptional activation or repression.[20]

Several recent reports have suggested that multiple epigenetic factors play important roles in the survival and pathogenesis of RGCs in models of glaucoma. For example,

Research Paper

UHRF1 regulates global DNA hypomethylation and is associated with poor prognosis in esophageal squamous cell carcinoma

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ABSTRACT

Background: Global DNA hypomethylation contributes to oncogenesis through various mechanisms. The level of long interspersed nucleotide element-1 (LINE-1) methylation is considered a surrogate marker of global DNA methylation, and is attracting interest as a good predictor of cancer prognosis. However, the mechanism how LINE-1 (global DNA) methylation is controlled in cancer cells remains to be fully elucidated. Ubiquitin-like with PHD and RING finger domain 1 (UHRF1) plays a crucial role in DNA methylation. UHRF1 is overexpressed in many cancers, and UHRF1 overexpression may be a mechanism underlying DNA hypomethylation in cancer cells. Nonetheless, the relationship between UHRF1, LINE-1 methylation level, and clinical outcome in esophageal squamous cell carcinoma (ESCC) remains unclear.

Results: In ESCC cell lines, vector-mediated UHRF1 overexpression caused global DNA (LINE-1) hypomethylation and, conversely, *UHRF1* knockdown using siRNA increased the global DNA methylation level. In ESCC tissues, UHRF1 expression was significantly associated with LINE-1 methylation levels. Furthermore, UHRF1 overexpression correlated with poor prognosis in our cohort of 160 ESCC patients.

Materials and Methods: The relationships between UHRF1 expression and LINE-1 methylation level (i.e., global DNA methylation level) were investigated using ESCC tissues and cell lines. In addition, we examined the correlation between UHRF1 expression, LINE-1 methylation, and clinical outcome in patients with ESCC.

Conclusions: Our results suggest that UHRF1 is a key epigenetic regulator of DNA methylation and might be a potential target for cancer treatment.

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is one of the most malignant tumors [1]. Despite remarkable progress in the advance of multidisciplinary treatments combining surgery, chemotherapy and/or radiotherapy, the outcome of ESCC patients remains unfavorable even after complete resection [2–4]. To develop novel strategies for treatment of ESCC, especially tumors that are molecularly targeted [5], it is extremely crucial to increase our understanding of the molecular basis of this disease. In particular, including alterations of DNA methylation, epigenetic changes are reversible and could be potential targets for cancer treatment and chemoprevention [6–8].

Alterations in DNA methylation correlated with human cancers include site-specific CpG island promoter hypermethylation and global DNA hypomethylation [9]. Global DNA hypomethylation contributes to oncogenesis through various mechanisms, including genomic instability [10–12]. Because long interspersed nucleotide element-1 (LINE-1) represents a considerable part of human genome (approximately 17%), LINE-1 methylation levels have been considered as a surrogate marker of global DNA methylation [13]. We have previously described

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Application of targeted enrichment to next-generation sequencing of retroviruses integrated into the host human genome

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The recent development and advancement of next-generation sequencing (NGS) technologies have enabled the characterization of the human genome at extremely high resolution. In the retrovirology field, NGS technologies have been applied to integration-site analysis and deep sequencing of viral genomes in combination with PCR amplification using virus-specific primers. However, virus-specific primers are not available for some epigenetic analyses, like chromatin immunoprecipitation sequencing (ChIP-seq) assays. Viral sequences are poorly detected without specific PCR amplification because proviral DNA is very scarce compared to human genomic DNA. Here, we have developed and evaluated the use of biotinylated DNA probes for the capture of viral genetic fragments from a library prepared for NGS. Our results demonstrated that viral sequence detection was hundreds or thousands of times more sensitive after enrichment, enabling us to reduce the economic burden that arises when attempting to analyze the epigenetic landscape of proviruses by NGS. In addition, the method is versatile enough to analyze proviruses that have mismatches compared to the DNA probes. Taken together, we propose that this approach is a powerful tool to clarify the mechanisms of transcriptional and epigenetic regulation of retroviral proviruses that have, until now, remained elusive.

Exogenous retroviruses, such as the human immunodeficiency virus type-1 (HIV-1) and the human T-cell leukemia virus type-1 (HTLV-1), integrate into host genomic DNA in the form of a provirus. The provirus works as a DNA template for viral mRNA transcription to produce viral proteins, later assembled to form viral particles for *de novo* infection. In addition, the provirus is thought to play an important role in persistent retroviral infections, because it is neither targeted by the host immune system, which is composed of virus-specific cytotoxic T-lymphocytes and antibodies, nor removed by the currently available anti-retroviral drugs[1].

HTLV-1 is the causative agent of adult T-cell leukemia (ATL), a leukemia of infected $CD4^+$ T cells, and is also associated with several inflammatory diseases[2–4]. A unique characteristic of HTLV-1 is that the virus increases its copy number not via viral particle production or *de novo* infection, but via the proliferation of infected cells in which it remains as a provirus integrated into host genomic DNA[5]. Therefore, understanding the regulation of proviral transcription is key to understanding the pathogenesis of HTLV-1 infection, including the mechanisms leading to transformation of infected cells or the establishment of chronic inflammatory diseases. In contrast, HIV-1 replicates via vigorous production of viral particles. However, once HIV-1-infected individuals are treated with combination anti-retroviral therapy (cART), the plasma viral RNA load is reduced to undetectable levels in most infected individuals[6]. Thanks to recent advances in the development of anti-retroviral drugs, reducing the onset of AIDS in patients infected with HIV-1 is currently possible[7]. However, a problem that remains is that HIV-1 cannot be completely eradicated from infected individuals by existing cART. Efficient and highly sensitive

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SHORT COMMUNICATION

Nuclear extrusion precedes discharge of genomic DNA fibers during tunicamycin-induced neutrophil extracellular trap-osis (NETosis)-like cell death in cultured human leukemia cells

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Abstract

We previously reported that the nucleoside antibiotic tunicamycin (TN), a protein glycosylation inhibitor triggering unfolded protein response (UPR), induced neutrophil extracellular trap-osis (NETosis)-like cellular suicide and, thus, discharged genomic DNA fibers to extracellular spaces in a range of human myeloid cell lines under serum-free conditions. In this study, we further evaluated the effect of TN on human promyelocytic leukemia HL-60 cells using time-lapse microscopy. Our assay revealed a previously unappreciated early event induced by TN-exposure, in which, at 30–60 min after TN addition, the cells extruded their nuclei into the extracellular space, followed by discharge of DNA fibers to form NET-like structures. Intriguingly, neither nuclear extrusion nor DNA discharge was observed when cells were exposed to inducers of UPR, such as brefeldin A, thapsigargin, or dithiothreitol. Our findings revealed novel nuclear dynamics during TN-induced NETosis-like cellular suicide in HL-60 cells and suggested that the toxicological effect of TN on nuclear extrusion and DNA discharge was not a simple UPR.

Keywords: cell death; extracellular chromatin; nucleus; tunicamycin

Introduction

Tunicamycin (TN) is a metabolite structurally analogous to uridin from *Streptomyces* spp. and is best known for its ability to inhibit N-glycan protein glycosylation, promoting an unfolded protein response (UPR) in a wide variety of cultured cell lines, including the predominantly neutrophilic precursor human HL-60 cell line (Prescher and Bertozzi, 2006; van Galen et al., 2014; Oakes and Papa, 2015). We previously reported that TN induced neutrophil extracellular trap-osis (NETosis)-like cellular suicide in HL-60 cells under serum-free conditions. However, the consecutive morphological alteration(s) during NETosis-like cellular suicide from the beginning of TN addition to the final event of extracellular DNA formation has not been described.

Here, we performed time-lapse observation of HL-60 cells exposed to TN under serum-free culture conditions and found that the cells exposed to TN extruded their nuclei to extracellular spaces, preceding the discharge of chromatin fibers from the nuclei. Intriguingly, neither nuclear extrusion nor extracellular chromatin release was observed when cells were exposed to other inducers of UPR, such as brefeldin A, thapsigargin, or dithiothreitol, suggesting that the TN-induced changes of the nuclear structure and function were not a simple UPR.

Materials and methods

Cell culture and drug treatments

Cells used in this study were obtained from the RIKEN cell bank (Tsukuba, Japan) or American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 supplemented with 5% (v/v) FBS, 50 U/mL penicillin, and 50 μg/mL streptomycin in a humidified atmosphere containing 5% (v/v) CO_2. TN, thapsigargin (TG), brefeldin A (BFA), and dithiothreitol (DTT) was obtained from Wako Pure

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Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; NETs, neutrophil extracellular traps; TN, tunicamycin; UPR, unfold protein response

The retrovirus HTLV-1 inserts an ectopic CTCF-binding site into the human genome

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Human T-lymphotropic virus type 1 (HTLV-1) is a retrovirus that causes malignant and inflammatory diseases in ∼10% of infected people. A typical host has between 10^4 and 10^5 clones of HTLV-1–infected T lymphocytes, each clone distinguished by the genomic integration site of the single-copy HTLV-1 provirus. The HTLV-1 bZIP (*HBZ*) factor gene is constitutively expressed from the minus strand of the provirus, whereas plus-strand expression, required for viral propagation to uninfected cells, is suppressed or intermittent in vivo, allowing escape from host immune surveillance. It remains unknown what regulates this pattern of proviral transcription and latency. Here, we show that CTCF, a key regulator of chromatin structure and function, binds to the provirus at a sharp border in epigenetic modifications in the pX region of the HTLV-1 provirus in T cells naturally infected with HTLV-1. CTCF is a zinc-finger protein that binds to an insulator region in genomic DNA and plays a fundamental role in controlling higher order chromatin structure and gene expression in vertebrate cells. We show that CTCF bound to HTLV-1 acts as an enhancer blocker, regulates HTLV-1 mRNA splicing, and forms long-distance interactions with flanking host chromatin. CTCF-binding sites (CTCF-BSs) have been propagated throughout the genome by transposons in certain primate lineages, but CTCF binding has not previously been described in present-day exogenous retroviruses. The presence of an ectopic CTCF-BS introduced by the retrovirus in tens of thousands of genomic locations has the potential to cause widespread abnormalities in host cell chromatin structure and gene expression.

retrovirus | latency | epigenetics | HTLV-1 | CTCF

Retroviruses integrate a dsDNA copy of their genome, the provirus, into the genome of the cell they infect. Human T-lymphotropic virus type 1 (HTLV-1) is an exogenous retrovirus, widespread in the tropics. Most infected people are asymptomatic carriers, but ∼10% develop a malignant or inflammatory disease. Adult T-cell leukemia (ATL) is a leukemia of HTLV-1–infected $CD4^+$ T cells. ATL cells frequently contain chromosomal abnormalities, and the disease is refractory to conventional chemotherapy. HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) is a chronic inflammatory disease of the spinal cord. HTLV-1 spreads within the individual both by cell-to-cell transmission and by clonal proliferation of infected cells: HTLV-1 gene products induce proliferation and enhance survival of infected cells (1, 2). In addition to the viral genes that encode enzymes and structural proteins, HTLV-1 encodes several regulatory and accessory genes in the pX region, between the *env* gene and the 3′ long terminal repeat (LTR). The *HBZ* gene is constitutively expressed from the minus strand of the integrated provirus (3), whereas plus-strand expression, required for viral propagation to uninfected cells, is suppressed or intermittently expressed in vivo, allowing escape from host immune surveillance (2, 4). It is unknown how HTLV-1 maintains this chromatin state and strand-selective transcription.

One mechanism used by HTLV-1 to suppress transcription of the plus strand is methylation of the 5′ LTR, whereas there is little DNA methylation in the 3′ LTR (5). The DNA methylation is sharply reduced at the middle of the provirus (6). This observation raised the question of whether there is a regulatory mechanism that divides the methylated 5′ part from the unmethylated 3′ part of the provirus, perhaps to allow the constitutive expression of the *HBZ* gene that appears to be required for clonal persistence of HTLV-1 (3, 7).

A chromatin insulator is a DNA region that separates transcriptionally active and inactive regions by binding to certain proteins. The best-characterized insulator-binding protein in higher eukaryotes is CTCF, an 11 zinc-finger protein highly conserved from flies to humans (8), which binds to tens of thousands of sites in the human genome and regulates chromatin structure, transcriptional activation, repression, silencing, imprinting, and alternative splicing (9). We therefore set out to test the hypothesis that CTCF binds to the HTLV-1 provirus at an epigenetic border and regulates proviral transcription.

Significance

The retrovirus human T-lymphotropic virus type 1 (HTLV-1) causes inflammatory and malignant diseases in humans. To maintain latency and avoid immune detection in vivo, HTLV-1 minimizes expression of genes on the plus-strand of the integrated provirus but allows constitutive expression of the minus-strand gene, which maintains clonal persistence. It is not understood how this gene expression is regulated. We show that CTCF, a master regulator of chromatin structure and gene expression, binds to HTLV-1, forms loops between the provirus and host genome, and alters expression of proviral and host genes. Because a typical HTLV-1–infected host carries $>10^4$ infected T-cell clones, each containing a provirus integrated in a different genomic site, CTCF binding gives HTLV-1 the potential to cause widespread abnormalities in the human genome.

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Data deposition: ChIP-sequencing data reported in this paper have been deposited in the DNA Data Bank of Japan (accession no. DRA004162).

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RESEARCH PAPER

Loss of the integral nuclear envelope protein SUN1 induces alteration of nucleoli

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ABSTRACT
A supervised machine learning algorithm, which is qualified for image classification and analyzing similarities, is based on multiple discriminative morphological features that are automatically assembled during the learning processes. The algorithm is suitable for population-based analysis of images of biological materials that are generally complex and heterogeneous. Here we used the algorithm wndchrm to quantify the effects on nucleolar morphology of the loss of the components of nuclear envelope in a human mammary epithelial cell line. The linker of nucleoskeleton and cytoskeleton (LINC) complex, an assembly of nuclear envelope proteins comprising mainly members of the SUN and nesprin families, connects the nuclear lamina and cytoskeletal filaments. The components of the LINC complex are markedly deficient in breast cancer tissues. We found that a reduction in the levels of SUN1, SUN2, and lamin A/C led to significant changes in morphologies that were computationally classified using wndchrm with approximately 100% accuracy. In particular, depletion of SUN1 caused nucleolar hypertrophy and reduced rRNA synthesis. Further, wndchrm revealed a consistent negative correlation between SUN1 expression and the size of nucleoli in human breast cancer tissues. Our unbiased morphological quantitation strategies using wndchrm revealed an unexpected link between the components of the LINC complex and the morphologies of nucleoli that serves as an indicator of the malignant phenotype of breast cancer cells.

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KEYWORDS
breast cancer; LINC complex; lamin A/C; nuclear morphology; nuclear envelope; SUN1; SUN2; wndchrm

Introduction

The cell nucleus is surrounded by the nuclear envelope (NE) that comprises the inner and outer nuclear membranes (INM and ONM). The multifunctional nuclear membrane protein assembly called the linker of nucleoskeleton and cytoskeleton (LINC) complex consists of the INM-spanning protein SUN and the ONM-spanning protein nesprin. SUN and nesprin interact in the lumen of the NE via their luminal domains.[1] The LINC complex directly connects the cytoskeleton (e.g. actin filaments or microtubule motors) and the nucleoskeleton (e.g., lamins or chromatin) and plays a major role in shaping and positioning the nucleus.[2] Further, the LINC complex connects specific chromosomal loci to the nucleoplasmic face to orchestrate chromosome organization and dynamics during meiosis[3] and acts in mechanotransduction by relaying extracellular signals to the nucleus.[4,5]

Morphological changes in the nucleus are hallmarks of cancer cells. In particular, an enlarged nucleus, abnormal nuclear shape, anisokaryosis, increased chromatin staining, and altered nucleolar size are often observed in cancer cells.[6,7] These phenotypes are used for histological and cytological diagnosis. Further, the diagnostic and prognostic pathology of the NE in human cancers was described.[8] For example, deregulation of lamin A/C expression occurs in many human tumors.[9-13] We reported recently that

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Lysine-specific demethylase-1 contributes to malignant behavior by regulation of invasive activity and metabolic shift in esophageal cancer

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Lysine-specific demethylase-1 (LSD1) removes the methyl groups from mono- and di-methylated lysine 4 of histone H3. Previous studies have linked LSD1 to malignancy in several human tumors, and LSD1 is considered to epigenetically regulate the energy metabolism genes in adipocytes and hepatocellular carcinoma. This study investigates the function of LSD1 in the invasive activity and the metabolism of esophageal cancer cells. We investigated whether LSD1 immunohistochemical expression levels are related to clinical and pathological features, including the maximum standard uptake value in fluorodeoxyglucose positron emission tomography assay. The influence of LSD1 on cell proliferation, invasion and glucose uptake was evaluated *in vitro* by using specific small interfering RNA for LSD1, and an LSD1 inhibitor. We also evaluated two major energy pathways (glycolytic pathway and mitochondrial respiration) by measuring the extracellular acidification rate (ECAR) and the oxygen consumption rate (OCR) with an extracellular flux analyzer. High LSD1 immunohistochemical expression was significantly associated with high tumor stage, lymphovascular invasion, poor prognosis, and high maximum standard uptake value in esophageal cancer patients. In the *in vitro* analysis, LSD1 knockdown significantly suppressed the invasive activity and glucose uptake of cancerous cells, reduced their ECAR and increased their OCR and OCR/ECAR. LSD1 may contribute to malignant behavior by regulating the invasive activity and metabolism, activating the glycolytic pathway and inhibiting the mitochondrial respiration of esophageal cancer cells. The results support LSD1 as a potential therapeutic target.

Molecular Cancer Biology

Key words: lysine-specific demethylase-1, Warburg effect, esophageal cancer, metabolic shift, invasive activity
Abbreviations: 2-DG: 2-deoxy-d-glucose;; ECAR: extracellular acidification rate; FDG-PET: fluorodeoxyglucose positron emission tomography; GLUT1: glucose transporter type 1; H3K4me1/me2: mono- and di-methylated lysine 4 of histone H3; HK2: hexokinase 2; HRs: hazard ratios; LDHA: lactate dehydrogenase A; LSD1: lysine-specific demethylase-1; OCR: oxygen consumption rate; qRT-PCR: quantitative reverse transcription PCR; siCtrl: control siRNA; siLSD1: small interfering RNA for LSD1; SUVmax: maximum standard uptake value
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Esophageal cancer is a worldwide concern. Although its geographic distribution varies, it is the eighth most common cancer and the sixth leading cause of cancer mortality.[1] In addition, despite remarkable advances in multimodal therapies combining surgery, chemotherapy, radiotherapy and chemoradiotherapy, the prognosis of esophageal cancer patients remains poor even after complete resection.[2–4] Recently, 18F-fluorodeoxyglucose positron emission tomography (FDG-PET) has become available for assessing the range of tumor progression and predicting prognoses.[5,6] As FDG-PET can also detect the glucose uptake of cancer cells, it may reveal a crucial link between cancer metabolism and malignancy in esophageal cancer.

The Warburg effect refers to the shift of glucose metabolism from oxygen-mediated respiration to glycolysis for lactate production.[7] Metabolic switching from oxidative phosphorylation to less energy-efficient glycolysis is characteristic of cancer cells, and the rate of glycolysis is correlated with tumor growth even when oxygen is sufficient.

Lysine-specific demethylase-1 (LSD1) is the first reported histone demethylase. LSD1 suppresses gene expression by removing the methyl groups from mono- and di-methylated lysine 4 of histone H3 (H3K4me1/me2). Flavin adenosine

Androgen Regulates *Mafb* Expression Through its 3′UTR During Mouse Urethral Masculinization

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External genitalia are prominent organs showing hormone-dependent sexual differentiation. Androgen is an essential regulator of masculinization of the genital tubercle, which is the anlage of external genitalia. We have previously shown that v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) is an androgen-inducible regulator of embryonic urethral masculinization in mice. However, it remains unclear how androgen regulates *Mafb* expression. The current study suggests that the *Mafb* 3′ untranslated region (UTR) is an essential region for its regulation by androgen. We identified 2 functional androgen response elements (AREs) in *Mafb* 3′UTR. Androgen receptor is bound to such AREs in 3′UTR during urethral masculinization. In addition to 3′UTR, *Mafb* 5′UTR also showed androgen responsiveness. Moreover, we also demonstrated that β-catenin, one of genital tubercle masculinization factors, may be an additional regulator of *Mafb* expression during urethral masculinization. This study provides insights to elucidate mechanisms of gene regulation through AREs present in *Mafb* 3′UTR for a better understanding of the processes of urethral masculinization. (*Endocrinology* 157: 844–857, 2016)

Sexual differentiation is an essential process to establish dimorphic male and female-type reproductive organs. External genitalia are prominent organs showing hormone-dependent organogenesis, and are involved in essential reproductive functions such as copulation (1). The structure of external genitalia is quite different between males and females. In mice, sexual differences in the genital tubercle (GT), which is the anlage of external genitalia, appear due to androgen signals starting from embryonic day 16.5 (E16.5) as canalization of the urethral plate to form the urethra (2). The process of male urethral formation is essential for sex differentiation of the GT. The urethra is incorporated into the glans in males by androgen action (3, 4). Androgen signaling in the mesenchyme adjacent to the urethra is essential for embryonic urethral masculinization (5, 6). Thus, urethral masculinization is a useful model for understanding of the mechanisms underlying hormone-dependent sexual differentiation. Hypospadias is one of the most common congenital anomalies associated with urethral defects (7–9). Many gene mutation sites have been identified in protein coding sequences (CDSs) in hypospadias patients (10–12). Moreover, mu-

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Abbreviations: AR, androgen receptor; ARE, androgen response element; CDS, coding sequence; ChIP, chromatin immunoprecipitation; DHT, 5α-dihydrotestosterone; DMSO, dimethylsulfoxide; E, embryonic day; Enz, enzalutamide; Flu, flutamide; GT, genital tubercle; KO, knockout; MAFB, v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog B; TK, thymidine kinase; UTR, untranslated region.

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A cluster of noncoding RNAs activates the *ESR1* locus during breast cancer adaptation

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Estrogen receptor-α (ER)-positive breast cancer cells undergo hormone-independent proliferation after deprivation of oestrogen, leading to endocrine therapy resistance. Up-regulation of the ER gene (*ESR1*) is critical for this process, but the underlying mechanisms remain unclear. Here we show that the combination of transcriptome and fluorescence *in situ* hybridization analyses revealed that oestrogen deprivation induced a cluster of noncoding RNAs that defined a large chromatin domain containing the *ESR1* locus. We termed these RNAs as *Eleanors* (ESR1 locus enhancing and activating noncoding RNAs). *Eleanors* were present in ER-positive breast cancer tissues and localized at the transcriptionally active *ESR1* locus to form RNA foci. Depletion of one *Eleanor, upstream (u)-Eleanor*, impaired cell growth and transcription of intragenic *Eleanors* and *ESR1* mRNA, indicating that *Eleanors* *cis*-activate the *ESR1* gene. *Eleanor*-mediated gene activation represents a new type of locus control mechanism and plays an essential role in the adaptation of breast cancer cells.

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Retinoblastoma protein promotes oxidative phosphorylation through upregulation of glycolytic genes in oncogene-induced senescent cells

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Summary

Metabolism is closely linked with cellular state and biological processes, but the mechanisms controlling metabolic properties in different contexts remain unclear. Cellular senescence is an irreversible growth arrest induced by various stresses, which exhibits active secretory and metabolic phenotypes. Here, we show that retinoblastoma protein (RB) plays a critical role in promoting the metabolic flow by activating both glycolysis and mitochondrial oxidative phosphorylation (OXPHOS) in cells that have undergone oncogene-induced senescence (OIS). A combination of real-time metabolic monitoring, and metabolome and gene expression analyses showed that OIS-induced fibroblasts developed an accelerated metabolic flow. The loss of RB down-regulated a series of glycolytic genes and simultaneously reduced metabolites produced from the glycolytic pathway, indicating that RB upregulates glycolytic genes in OIS cells. Importantly, both mitochondrial OXPHOS and glycolytic activities were abolished in RB-depleted or downstream glycolytic enzyme-depleted OIS cells, suggesting that RB-mediated glycolytic activation induces a metabolic flux into the OXPHOS pathway. Collectively, our findings reveal that RB essentially functions in metabolic remodeling and the maintenance of the active energy production in OIS cells.

Key words: gene regulation; glycolysis; metabolic remodeling; oncogene-induced senescence; oxidative phosphorylation; retinoblastoma protein.

Introduction

Cellular senescence is a state of essentially irreversible growth arrest that is induced by various stresses, such as shortened telomeres, DNA damage, and oncogene activation. It is thought to function as an exclusion of unnecessary cells or as an anticancer mechanism. Senescent cells usually exhibit morphological features including an enlarged size, flattened shape, increased β-galactosidase activity, a senescence-associated secretory phenotype, and the formation of senescence-associated heterochromatin foci (SAHF) (Campisi & d'Adda di Fagagna, 2007; Kuilman *et al.*, 2010; Rodier & Campisi, 2011; Lopez-Otin *et al.*, 2013; Munoz-Espin & Serrano, 2014; Perez-Mancera *et al.*, 2014; Salama *et al.*, 2014). Senescent cells also possess some dynamic functions that together make up the metabolically active phenotype.

Several signaling pathways that activate the senescence program have been identified *in vitro*, and a deficiency of these pathways is often found in tumors *in vivo* (Collado & Serrano, 2010), suggesting that bypassing cellular senescence could lead to tumorigenesis. Among such pathways, the retinoblastoma protein (RB) has been shown to play a pivotal role in the transcriptional repression of cell cycle genes in both oncogene-induced senescence (OIS) and replicative senescence (RS) (Ben-Porath & Weinberg, 2005). In senescent cells, RB represses E2F target genes including cell cycle regulators (Narita *et al.*, 2003), and this repression is partly caused by the RB-mediated recruitment of transcriptional repressors such as histone deacetylases to the regulatory sites of its target genes (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Interestingly, RB is required for SAHF formation through cooperation with histone chaperone proteins HIRA and antisilencing function 1A (Narita *et al.*, 2003; Ye *et al.*, 2007). RB is also known to act as a transcriptional activator in some contexts (Talluri & Dick, 2012), although it remains unclear whether RB-mediated transcriptional activation contributes to senescent phenotypes. Thus, RB is likely to orchestrate several senescence-associated phenomena and to contribute to the stable maintenance of the senescence program.

Cellular metabolism produces energy and the materials required for cell structure and function, and its regulation is necessary to meet the energy demands of the cellular state. To generate ATP as a cellular fuel, normal cells mainly use OXPHOS in the presence of oxygen and anaerobic glycolysis in its absence. However, proliferative and cancer cells typically show a bias toward aerobic glycolysis even in the presence of oxygen because of their high energy demand for rapid growth. Recently, the metabolic profiling of mouse embryonic stem cells (ESCs), mouse epiblast stem cells (EpiSC), and human embryonic stem cells (hESCs) found that EpiSCs/hESCs possess higher glycolytic and lower OXPHOS activities compared with ESCs (Zhou *et al.*, 2012). Regulation of a set of mitochondrial IV *COX* genes was thought to be involved in this phenomenon. Human-induced pluripotent stem cells also displayed higher glycolytic rates relative to the original somatic cells, similar to hESCs (Varum *et al.*, 2011). Although it is still uncertain whether metabolic remodeling is a cause or consequence of cellular reprogramming, these studies clearly indicate that metabolic pathways can change depending on cellular states.

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Tumor and Stem Cell Biology

Cancer Research

Lysine Demethylase LSD1 Coordinates Glycolytic and Mitochondrial Metabolism in Hepatocellular Carcinoma Cells

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Abstract

The hallmark of most cancer cells is the metabolic shift from mitochondrial to glycolytic metabolism for adapting to the surrounding environment. Although epigenetic modification is intimately linked to cancer, the molecular mechanism, by which epigenetic factors regulate cancer metabolism, is poorly understood. Here, we show that lysine-specific demethylase-1 (LSD1, KDM1A) has an essential role in maintaining the metabolic shift in human hepatocellular carcinoma cells. Inhibition of LSD1 reduced glucose uptake and glycolytic activity, with a concurrent activation of mitochondrial respiration. These metabolic changes coexisted with the inactivation of the hypoxia-inducible factor HIF1α, resulting in a decreased expression of *GLUT1* and glycolytic enzymes. In contrast, during LSD1 inhibition, a set of mitochondrial metabolism genes was activated with the concomitant increase of methylated histone H3 at lysine 4 in the promoter regions. Consistently, both LSD1 and GLUT1 were significantly overexpressed in carcinoma tissues. These findings demonstrate the epigenetic plasticity of cancer cell metabolism, which involves an LSD1-mediated mechanism. *Cancer Res; 75(7); 1445–56. ©2015 AACR.*

Introduction

Patterns of gene expression are maintained and often reprogrammed by the combination of transcription factors and epigenetic factors involved in modifications of DNAs and histones, leading to conversion of cellular phenotypes. In particular, alterations of these epigenetic marks are hallmarks of many types of cancer cells (1–3). As another notable feature, proliferative cancer cells are thought to rely on energy production from the glycolytic pathway, even under high oxygen conditions: the so-called aerobic glycolysis (4–6). This metabolic remodeling may be interpreted as an adaptation to the hypoxic microenvironment where the cancer cells originally reside (7). Recent reports have highlighted that metabolic enzymes involved in such process are manipulated in cancer cells (8). Mitochondrial function is also modified such that it supports the production of biomacromolecules and reactive oxygen species rather than ATP (9). These lines of evidence support the notion that metabolic alteration in cancer cells is not merely a consequence of impaired cellular functions by transformation, but rather an ordered reprogramming of energy flow that fuels the accelerated cell growth. Epigenetic plasticity has been discussed as an underlying mechanism for metabolic reprogramming (10). In addition, recent studies on isocitrate dehydrogenase mutations in various cancers defined that misguided metabolic flow leads to the impaired activities of epigenetic factors (11). Thus metabolism–epigenome crosstalk may profoundly contribute to the dysregulated gene expression in cancer (12, 13). However, little is known about how specific epigenetic factors control cancer cell metabolism.

Lysine-specific demethylase-1 (LSD1, also known as KDM1A) is a flavin-dependent amine oxidase, which, in general, suppresses gene expression by removing the methyl group from mono- and dimethylated histone H3 at lysine 4 (H3K4; ref. 14). LSD1 knockout mice die early in development (15), and LSD1-null embryonic stem cells showed impaired viability (16), suggesting that LSD1 plays a crucial role in cell functions. Several studies showed that overexpression of LSD1 drives cell proliferation in various cancers (17–20). We have recently found that LSD1 suppresses mitochondrial respiration and maintains energy storage in murine adipocytes under the obese condition (21). Therefore, it is fascinating to test whether LSD1 facilitates the metabolic reprogramming in cancer cells.

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Materials and Methods

Cell culture

HepG2 and Huh-7 cells from human hepatocellular carcinomas (HCC) were grown in DMEM (Sigma), supplemented with 10% (v/v) heat-inactivated FBS. Human telomerase-immortalized hepatic NeHepLxHT cells were cultured in DMEM/Nutrient

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Lysine-Specific Demethylase 2 Suppresses Lipid Influx and Metabolism in Hepatic Cells

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Cells link environmental fluctuations, such as nutrition, to metabolic remodeling. Epigenetic factors are thought to be involved in such cellular processes, but the molecular basis remains unclear. Here we report that the lysine-specific demethylase 2 (LSD2) suppresses the flux and metabolism of lipids to maintain the energy balance in hepatic cells. Using transcriptome and chromatin immunoprecipitation-sequencing analyses, we revealed that LSD2 represses the genes involved in lipid influx and metabolism through demethylation of histone H3K4. Selective recruitment of LSD2 at lipid metabolism gene loci was mediated in part by a stress-responsive transcription factor, c-Jun. Intriguingly, LSD2 depletion increased the intracellular levels of many lipid metabolites, which was accompanied by an increased susceptibility to toxic cell damage in response to fatty acid exposure. Our data demonstrate that LSD2 maintains metabolic plasticity under fluctuating environment in hepatocytes by mediating the cross talk between the epigenome and metabolism.

Organisms and cells must adjust their energy strategy to fluctuating nutrient availability and other environmental conditions. Epigenetic mechanisms have been implicated in the phenotypic plasticity in response to environmental changes, as well as in consistent execution of the developmental program (1). It has been shown that nutrients and dietary composition potently influence epigenetic marks, including DNA methylation and histone methylation and acetylation, in both humans and animal models (2).

Because chromatin-modifying enzymes utilize nutrient-derived metabolites as substrates and coenzymes, epigenome formation is, by nature, influenced by nutritional and metabolic conditions (3–6). Lysine-specific demethylases 1 and 2 (LSD1 and LSD2), also known as KDM1A and KDM1B, respectively, comprise the flavin-dependent amine oxidase family of histone demethylases (7). These enzymes require flavin adenine dinucleotide (FAD) as a coenzyme for the removal of methyl groups from the lysine residue of histone H3 and other proteins (8, 9). FAD is a vitamin B_2-derived metabolite that serves as a redox cofactor in key metabolic processes such as fatty acid oxidation and succinate dehydrogenation in the tricarboxylic acid (TCA) cycle (10). Thus, the cellular metabolic state may influence the demethylase activity of these proteins. Indeed, we and others have previously demonstrated that LSD1 controls energy metabolism genes in response to extracellular conditions (11, 12), suggesting that FAD-dependent epigenetic factors may link environmental information to metabolic programming. LSD2 was identified as a second flavin-dependent histone demethylase that targets methylated lysines 4 and 9 of histone H3 (H3K4 and H3K9, respectively) (8, 13–15). Although LSD2 has been implicated in the establishment of maternal genomic imprinting in oocytes (16), little is known about its biological functions, particularly in relation to metabolic control.

In the liver, hepatocytes play a crucial role in the homeostatic control of lipid metabolism. Hepatocytes incorporate adipose- and diet-derived fatty acids, which are either stored by themselves as neutral lipids or redistributed to other tissues in the form of very-low-density lipoproteins (17). When hepatocytes are exposed to an intolerably large amount of fatty acids, for example, due to overfeeding, excessive fatty acids and their toxic metabolites accumulate in the cells, often leading to the lipotoxic liver injury known as nonalcoholic fatty liver disease (NAFLD) (18, 19). Epigenetic alterations in the liver have been linked to insulin resistance and NAFLD in humans (20) and diet-induced steatosis in mice (21). A recent study by Ahrens et al. examined the DNA methylation profiles of liver biopsy specimens from patients with NAFLD and nonalcoholic steatohepatitis (NASH), an advanced form of NAFLD (22). Of particular note, some disease state-dependent methylation patterns could be reversed after improvement of the disease condition by bariatric surgery (22), suggesting that hepatic lipid homeostasis is associated with epigenetic plasticity. However, we still lack knowledge of whether a specific epigenetic factor could be involved in the homeostatic control of hepatic lipid metabolism.

Here, we provide direct evidence that LSD2 plays an essential role in the homeostatic control of lipid metabolism in hepatocytes. Our integrative investigations using transcrip-

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H3K4/H3K9me3 Bivalent Chromatin Domains Targeted by Lineage-Specific DNA Methylation Pauses Adipocyte Differentiation

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SUMMARY

Bivalent H3K4me3 and H3K27me3 chromatin domains in embryonic stem cells keep active developmental regulatory genes expressed at very low levels and poised for activation. Here, we show an alternative and previously unknown bivalent modified histone signature in lineage-committed mesenchymal stem cells and preadipocytes that pairs H3K4me3 with H3K9me3 to maintain adipogenic master regulatory genes (*Cebpa* and *Pparg*) expressed at low levels yet poised for activation when differentiation is required. We show lineage-specific gene-body DNA methylation recruits H3K9 methyltransferase SETDB1, which methylates H3K9 immediately downstream of transcription start sites marked with H3K4me3 to establish the bivalent domain. At the *Cebpa* locus, this prevents transcription factor C/EBPβ binding, histone acetylation, and further H3K4me3 deposition and is associated with pausing of RNA polymerase II, which limits *Cebpa* gene expression and adipogenesis.

INTRODUCTION

During development, pluripotent stem cells undergo commitment to lineage-specific multipotent stem cells and progenitor cells that eventually develop into unique types of terminally differentiated mature cells. For self-renewal and repair upon injury, adult tissues need to maintain lineage-committed progenitor cells. Although mechanisms by which the pluripotency of embryonic stem cells (ESCs) are maintained have been extensively studied (reviewed in Tee and Reinberg, 2014), mechanisms that keep lineage committed progenitor cells paused for differentiation or maintained in an undifferentiated state are not clearly understood and are of significant current interest.

DNA methylation and histone modifications regulate gene expression and are altered during cell lineage decisions (Margueron et al., 2005). The four core histones (H2A, H2B, H3, and H4) are subjected to several types of covalent posttranslational modifications that in turn modulate nucleosome structure and influence chromatin dynamics (Kouzarides, 2007). Histone modifications regulate gene expression either positively or negatively depending on the selective type of modification that is referred to as the histone code (Lee and Mahadevan, 2009). Relevant here, methylation of H3K27 and H3K9 are considered as hallmark signatures of condensed heterochromatin and low expression of neighboring genes (Margueron et al., 2005).

Direct DNA methylation of cytosine residues at CpG dinucleotides is crucially involved in embryonic development and transcription. DNA methylation patterns are thought to be erased during early embryogenesis and then re-established during development (Illingworth et al., 2008; Kafri et al., 1992). CpG methylation of DNA within promoter regions represses transcription by inhibiting the specific binding of transcription factors or recruiting chromatin-modifying repression complexes (Jones, 2012). DNA methylation is more prevalent in gene bodies than in promoters (Lister et al., 2009), where it is positively correlated with gene expression (Ball et al., 2009). However, the functional consequences of DNA methylation in the gene body remains elusive, especially in relation to the cross-talk with histone methylation (Cedar and Bergman, 2009; Jones, 2012).

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Endoplasmic Reticulum (ER) Stress Induces Sirtuin 1 (SIRT1) Expression via the PI3K-Akt-GSK3β Signaling Pathway and Promotes Hepatocellular Injury*

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Sirtuin 1 (SIRT1), an NAD^+-dependent histone deacetylase, plays crucial roles in various biological processes including longevity, stress response, and cell survival. Endoplasmic reticulum (ER) stress is caused by dysfunction of ER homeostasis and exacerbates various diseases including diabetes, fatty liver, and chronic obstructive pulmonary disease. Although several reports have shown that SIRT1 negatively regulates ER stress and ER stress-induced responses *in vitro* and *in vivo*, the effect of ER stress on SIRT1 is less explored. In this study, we showed that ER stress induced SIRT1 expression *in vitro* and *in vivo*. We further determined the molecular mechanisms of how ER stress induces SIRT1 expression. Surprisingly, the conventional ER stress-activated transcription factors XBP1, ATF4, and ATF6 seem to be dispensable for SIRT1 induction. Based on inhibitor screening experiments with SIRT1 promoter, we found that the PI3K-Akt-GSK3β signaling pathway is required for SIRT1 induction by ER stress. Moreover, we showed that pharmacological inhibition of SIRT1 by EX527 inhibited the ER stress-induced cellular death *in vitro* and severe hepatocellular injury *in vivo*, indicating a detrimental role of SIRT1 in ER stress-induced damage responses. Collectively, these data suggest that SIRT1 expression is up-regulated by ER stress and contributes to ER stress-induced cellular damage.

The endoplasmic reticulum (ER)[2] is an important organelle functioning in protein folding, transport, processing, and storage of calcium ion. Homeostasis of ER is crucial for cellular activity and survival (1–3). Intracellular and extracellular conditions that interfere with ER function lead to the accumulation of unfolded proteins in the ER, resulting in ER stress. Cells exposed to ER stress activate the unfolded protein response (UPR), which consists of three major branches: the activating transcription factor (ATF) 6, inositol-requiring enzyme (IRE) 1-X-box binding protein (XBP) 1, and PERK-eIF2α-ATF4 pathways (4). These signaling pathways include both translational and transcriptional control mechanisms that reduce protein synthesis, increase the protein folding capacity by up-regulating the transcription of molecular chaperones, and activate the ER-associated protein degradation. By using these systems, living organisms maintain homeostasis against various stress conditions (4). Thus, malfunction of ER stress responses is associated with various diseases including diabetes, fatty liver, neurodegeneration, and chronic obstructive pulmonary disease (5–7).

Sirtuins are the mammalian orthologues of the yeast Sir2 protein that is involved in chromatin silencing and lifespan regulation. The mammalian Sirtuin 1 (SIRT1) gene encodes a nicotinamide adenosine dinucleotide (NAD^+)-dependent histone deacetylase that is the closest structural ortholog of the yeast Sir2 protein (8, 9). Although the yeast Sir2 protein has been extensively characterized as a histone deacetylase, the mammalian SIRT1 protein deacetylates not only histone but also non-histone substrates including transcription factors. For example, SIRT1 expression is induced in response to acute nutritional stress leading to SIRT1 deacetylation of FOXO1, a cell metabolism regulator, thereby contributing to the activation of gluconeogenesis (10). The deacetylation of E2F1 by SIRT1 inhibits its transcriptional activity that contributes to the suppression of apoptosis induced by DNA damage (11).

Recent reports have shown that SIRT1 deacetylates XBP1 and inhibits its transcriptional activity to promote ER stress-induced apoptosis, and SIRT1 suppresses the PERK-eIF2α-dependent translational inhibition in mammals (12–14). Thus, SIRT1 has been known to be a negative regulator for ER stress response. However, compared with the effect of SIRT1 on ER stress response, the effect of ER stress on SIRT1 is less studied.

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[2] The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; ATF, activating transcription factor; IRE, inositol-requiring enzyme; XBP, X-box-binding protein; XBP1s, spliced XBP1; SIRT1, Sirtuin 1; QPCR, quantitative RT-PCR; CHOP, CCAAT/enhancer-binding protein (C/EBP) homologous protein; GRP78, glucose-related protein 78 kDa; ERdj4, ER DnaJ homologue 4; m, mouse; mCTGF, mouse connective tissue growth factor; TG, thapsigargin; TM, tunicamycin; PERK, protein *kinase RNA-like endoplasmic reticulum kinase;* PTEN, phosphatase and tensin homologue.

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Novel metal chelating molecules with anticancer activity. Striking effect of the imidazole substitution of the histidine–pyridine–histidine system

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ABSTRACT

Previously we have reported a metal chelating histidine–pyridine–histidine system possessing a trityl group on the histidine imidazole, namely HPH-2Trt, which induces apoptosis in human pancreatic adenocarcinoma AsPC-1 cells. Herein the influence of the imidazole substitution of HPH-2Trt was examined. Five related compounds, HPH-1Trt, HPH-2Bzl, HPH-1Bzl, HPH-2Me, and HPH-1Me were newly synthesized and screened for their activity against AsPC-1 and brain tumor cells U87 and U251. HPH-1Trt and HPH-2Trt were highly active among the tested HPH compounds. In vitro DNA cleavage assay showed both HPH-1Trt and HPH-2Trt completely disintegrate pUC19 DNA. The introduction of trityl group decisively potentiated the activity.

1. Introduction

Bleomycin is an antitumor antibiotic consisting of a metal-binding/oxygen-activating site and a DNA-binding site, inducing oxidative cleavage of DNA strand.[1] Bleomycin forms an iron complex by the β-aminoalanine-pyrimidine-β-hydroxyhistidine region (Fig. 1A). Previously we have designed β-aminoalanine-pyridine-β-hydroxyhistidine ligands, namely PYML compounds, based on the direct analogy to the bleomycin metal core (Fig. 1B).[1] In particular, the Fe(II) complex of PYML-6 activates molecular oxygen as efficiently as bleomycin does,[2] and PYML-8 was found to be superior to bleomycin in oxygen activation.[3] However, although PYML-6-Fe(II) complex showed efficient oxygen activation, it was virtually inactive in the DNA cleaving reaction.[4] This must be due to the lack of the DNA-binding moiety in PYML-6, and, in fact, introduction of the DNA binding site into the PYML-6 skeleton resulted in the acquisition of the DNA cleaving capability.[1]

For the cellular DNA cleavage, bleomycin must be delivered inside the nucleus to contact DNA. Bleomycin is capable of this presumably owing to the complicated total structure including the L-gulose-3-*O*-carbamoyl-D-mannose disaccharide for the nuclear transport and the bithiazole moiety for the DNA binding.

For the further investigation of such DNA-binding/oxygen-activation mode of action, we designed a novel ligands of symmetrized coordination environment histidine–pyridine–histidine ligand, namely HPH compounds (Fig. 2).[5] Iron complex of HPH showed efficient oxygen activation that was plausibly optimized by introducing an electron donating dimethylamino substituent into the pyridine ring, as demonstrated by the ESR spin trapping experiment.[5] Iron complex of unsymmetrical HPH-M showed oxygen-activating property as demonstrated by ESR spin trapping

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The actin family protein ARP6 contributes to the structure and the function of the nucleolus

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Abstract

The actin family members, consisting of actin and actin-related proteins (ARPs), are essential components of chromatin remodeling complexes. ARP6, one of the nuclear ARPs, is part of the Snf-2-related CREB-binding protein activator protein (SRCAP) chromatin remodeling complex, which promotes the deposition of the histone variant H2A.Z into the chromatin. In this study, we showed that ARP6 influences the structure and the function of the nucleolus. ARP6 is localized in the central region of the nucleolus, and its knockdown induced a morphological change in the nucleolus. We also found that in the presence of high concentrations of glucose ARP6 contributed to the maintenance of active ribosomal DNA (rDNA) transcription by placing H2A.Z into the chromatin. In contrast, under starvation, ARP6 was required for cell survival through the repression of rDNA transcription independently of H2A.Z. These findings reveal novel pleiotropic roles for the actin family in nuclear organization and metabolic homeostasis.

Keywords

Actin-related protein; ARP6; Histone H2A.Z; Nucleolus; Wndchrm

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Appendix A. Supplementary data

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STAT5 Orchestrates Local Epigenetic Changes for Chromatin Accessibility and Rearrangements by Direct Binding to the TCRγ Locus

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The transcription factor STAT5, which is activated by IL-7R, controls chromatin accessibility and rearrangements of the TCRγ locus. Although STAT-binding motifs are conserved in Jγ promoters and Eγ enhancers, little is known about their precise roles in rearrangements of the TCRγ locus in vivo. To address this question, we established two lines of Jγ1 promoter mutant mice: one harboring a deletion in the Jγ1 promoter, including three STAT motifs ($J\gamma1P^{\Delta/\Delta}$), and the other carrying point mutations in the three STAT motifs in that promoter ($J\gamma1P^{mS/mS}$). Both $J\gamma1P^{\Delta/\Delta}$ and $J\gamma1P^{mS/mS}$ mice showed impaired recruitment of STAT5 and chromatin remodeling factor BRG1 at the Jγ1 gene segment. This resulted in severe and specific reduction in germline transcription, histone H3 acetylation, and histone H4 lysine 4 methylation of the Jγ1 gene segment in adult thymus. Rearrangement and DNA cleavage of the segment were severely diminished, and Jγ1 promoter mutant mice showed profoundly decreased numbers of γδ T cells of γ1 cluster origin. Finally, compared with controls, both mutant mice showed a severe reduction in rearrangements of the Jγ1 gene segment, perturbed development of γδ T cells of γ1 cluster origin in fetal thymus, and fewer $V\gamma3^+$ dendritic epidermal T cells. Furthermore, interaction with the Jγ1 promoter and Eγ1, a TCRγ enhancer, was dependent on STAT motifs in the Jγ1 promoter. Overall, this study strongly suggests that direct binding of STAT5 to STAT motifs in the Jγ promoter is essential for local chromatin accessibility and Jγ/Eγ chromatin interaction, triggering rearrangements of the TCRγ locus. ***The Journal of Immunology*****, 2015, 195: 1804–1814.**

V(D)J recombination of lymphocyte AgR genes is carried out by conserved recombinatorial signals and RAG recombinases. The accessibility model postulates that, in developing lymphocytes, specific molecular mechanisms exist to make the appropriate AgR loci accessible to the common recombination machinery in a lineage- and stage-specific manner (1). At least two kinds of *cis*-regulatory elements control chromatin accessibility. First, enhancer elements govern locus-wide accessibility; deletion of their respective enhancers greatly reduces rearrangements at the IgH, Igκ, TCRβ, and TCRα loci (2–7). Second, germline promoters control local chromatin accessibility: deletion of Dβ and Jα germline promoters severely impairs rearrangements of their respective segments (8, 9). In addition to chromatin accessibility, higher-order chromatin architecture, such as repositioning of AgR loci within the nucleoplasm and formation of chromatin loops, plays a significant role in V(D)J recombination (10).

Several transcription factors function in accessibility control through *cis*-regulatory elements. For example, Oct-1 recruits STAT5 to distal V_H gene segments and controls their accessibility and recombination (11). IRF4 plays a role in accessibility of the IgL loci and counteracts association of an Igκ allele with het-

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The online version of this article contains supplemental material.

Abbreviations used in this article: BAC, bacterial artificial chromosome; 3C, chromosome conformation capture; ChIP, chromatin immunoprecipitation; DETC, dendritic epidermal T cell; Eγ, TCRγ 3′ enhancer; ES, embryonic stem; H3ac, histone H3 acetylation; H3K4, histone H3 lysine 4; H3K4me1, H3K4 monomethylation; H3K4me2, H3K4 dimethylation; H3K4me3, H3K4 trimethylation; HsA, DNase I hypersensitivity site; IEL, intraepithelial lymphocyte; LM, ligation mediated; TN, $CD3^-CD4^-CD8^-$ triple negative.

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TET family proteins and 5-hydroxymethylcytosine in esophageal squamous cell carcinoma

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ABSTRACT

Mammalian DNA is epigenetically marked by 5′-cytosine methylation (5-methylcytosine [5-mC]). The Ten-eleven translocation (TET) enzymes (TET1, TET2, and TET3) are implicated in DNA demethylation, through dioxygenase activity that converts 5-mC to 5-hydroxymethylcytosine (5-hmC). Although decreased TET is reportedly associated with decreased 5-hmC levels in various cancers, functions of 5-hmC and TET expression in esophageal squamous cell carcinoma (ESCC) are unclear. We used ELISA and immunohistochemistry tests to analyze 5-hmC status in ESCC tissues, RT-qPCR to analyze *TET* family mRNA expression in normal and tumor tissues, and pyrosequencing to quantify LINE-1 (i.e., global DNA methylation) levels. ELISA and immunohistochemical testing showed 5-hmC levels were significantly lower in ESCC than in paired normal tissues ($P < 0.0001$). *TET2* expression was significantly lower in ESCCs than paired normal tissues ($P < 0.0001$), and significantly associated with 5-hmC levels in ESCCs ($P = 0.003$, $r = 0.33$). 5-hmC levels were also significantly associated with LINE-1 methylation level ($P = 0.0002$, $r = 0.39$). Patients with low 5-hmC levels had shorter overall survival than those with higher levels, although not significantly so ($P = 0.084$). In conclusion, 5-hmC expression was decreased in ESCC tissues, and was associated with *TET2* expression level. *TET2* reduction and subsequent 5-hmC loss might affect ESCC development.

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC), the most common esophageal cancer in East Asian countries, is a very aggressive malignancy that requires combined modality therapies [1]. However, the limited improvement provided by conventional therapies has prompted us to seek innovative strategies for treating ESCC, especially molecularly targeted treatments. Importantly, epigenetic changes, including alterations in DNA methylation, are reversible, and can thus be targets for therapy or chemoprevention [2–6].

DNA methylation—conversion of cytosine to 5-methylcytosine (5-mC)—is a primary epigenetic mechanism involved in imprinting, X-chromosome inactivation, and repression of endogenous retroviruses. In human cancers, DNA methylation alterations include global DNA hypomethylation and site-specific CpG island

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Computational image analysis of colony and nuclear morphology to evaluate human induced pluripotent stem cells

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Non-invasive evaluation of cell reprogramming by advanced image analysis is required to maintain the quality of cells intended for regenerative medicine. Here, we constructed living and unlabelled colony image libraries of various human induced pluripotent stem cell (iPSC) lines for supervised machine learning pattern recognition to accurately distinguish bona fide iPSCs from improperly reprogrammed cells. Furthermore, we found that image features for efficient discrimination reside in cellular components. In fact, extensive analysis of nuclear morphologies revealed dynamic and characteristic signatures, including the linear form of the promyelocytic leukaemia (PML)-defined structure in iPSCs, which was reversed to a regular sphere upon differentiation. Our data revealed that iPSCs have a markedly different overall nuclear architecture that may contribute to highly accurate discrimination based on the cell reprogramming status.

The generation of human induced pluripotent stem cells (iPSCs) is simple and highly reproducible[1]. However, only a small proportion of cells become pluripotent after introduction of the reprogramming factors, possibly resulting in a mixture of bona fide iPSCs and partially reprogrammed cells[2,3]. It is essential to develop reliable methods to select completely reprogrammed iPSCs by eliminating the contamination of non-iPSCs[4]. Previous studies have shown changes in gene expression, DNA methylation, and histone modifications during iPSC reprogramming[1,5]. Furthermore, reporter genes have been integrated into the genomic loci of pluripotency genes to visualize bona fide iPSCs[4]. However, there are no non-invasive methods that reliably identify live human iPSCs in large and heterogeneous populations of reprogramming cells.

Recent advances in automated biological image analyses enable objective measurements of cellular morphologies[6]. A supervised machine learning algorithm, wndchrm (weighted neighbour distances using a compound hierarchy of algorithms representing morphology), has been developed for automated image classification and mining of image similarities or differences[7]. It is a flexible, multi-purpose image classifier that can be applied to a wide range of bio-image problems. Unlike conventional image analysis, where users are required to specify target morphologies, choose specific algorithms, and try different parameters depending on the imaging problem, wndchrm users define classes by providing example images for each class; completely reprogrammed cells or partially reprogrammed cells, for example. Once classes are defined, classifications and similarity measurements are performed automatically. As the first step of the classification, wndchrm computes a large set of image features for each image in the defined classes and then selects image features that are informative for discrimination of the groups and constructs a classifier in an automated fashion[6,7]. Next, the dataset is tested by multiple rounds of cross validation to measure classification accuracy (CA) as well as class similarity, which can be visualized with phylogenetic tree. The wndchrm algorithm has been successfully used for early detection of osteoarthritis[8], measurement of muscle decline with aging, sarcopenia[9], classification of malignant lymphoma[10], and many other applications[10].

Nuclear structure and function are closely linked to cellular reprogramming and epigenomic regulation[5]. During cell differentiation, nuclear structures are reconfigured dynamically. Previous studies have identified numerous distinct nuclear bodies[11–13]. For example, promyelocytic leukaemia (PML) nuclear bodies typically exist as small spheres of 0.3–1.0 μm in diameter, and are implicated in various cellular pathways including

Sall1 Maintains Nephron Progenitors and Nascent Nephrons by Acting as Both an Activator and a Repressor

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ABSTRACT

The balanced self-renewal and differentiation of nephron progenitors are critical for kidney development and controlled, in part, by the transcription factor Six2, which antagonizes canonical Wnt signaling-mediated differentiation. A nuclear factor, Sall1, is expressed in Six2-positive progenitors as well as differentiating nascent nephrons, and it is essential for kidney formation. However, the molecular functions and targets of Sall1, especially the functions and targets in the nephron progenitors, remain unknown. Here, we report that *Sall1* deletion in Six2-positive nephron progenitors results in severe progenitor depletion and apoptosis of the differentiating nephrons in mice. Analysis of mice with an inducible *Sall1* deletion revealed that Sall1 activates genes expressed in progenitors while repressing genes expressed in differentiating nephrons. Sall1 and Six2 co-occupied many progenitor-related gene loci, and Sall1 bound to Six2 biochemically. In contrast, Sall1 did not bind to the *Wnt4* locus suppressed by Six2. Sall1-mediated repression was also independent of its binding to DNA. Thus, Sall1 maintains nephron progenitors and their derivatives by a unique mechanism, which partly overlaps but is distinct from that of Six2: Sall1 activates progenitor-related genes in Six2-positive nephron progenitors and represses gene expression in Six2-negative differentiating nascent nephrons.

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The nephron is a basic functional unit of the kidney, which includes the glomerulus, proximal and distal renal tubules, and the loop of Henle. The mammalian kidney, the metanephros, is formed by reciprocally inductive interactions between two precursor tissues, namely the metanephric mesenchyme and the ureteric bud. The mesenchyme contains nephron progenitors that express a transcription factor, Six2. When Six2-positive cells are labeled using *Six2GFPCre*, a mouse strain expressing Cre recombinase fused to green fluorescent protein (GFP) under the control of the *Six2* promoter, they give rise to nephron epithelia *in vivo*.[1] *Six2* opposes the canonical Wnt-mediated differentiation evoked by ureteric bud-derived *Wnt9b*, thereby maintaining the self-renewal of nephron progenitors.[2–4] However, the progenitors gradually lose *Six2* expression

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Lys-63-linked Ubiquitination by E3 Ubiquitin Ligase Nedd4-1 Facilitates Endosomal Sequestration of Internalized α-Synuclein*

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Background: Nedd4-1 catalyzes the Lys-63-linked ubiquitination of aS.
Results: The Lys-63-linked ubiquitination of aS by Nedd4-1 facilitates endosomal targeting of extracellular aS.
Conclusion: Compared with C-terminal deficient mutants, wild type-aS is preferentially internalized and translocates to endosomes. The overexpression of Nedd4-1 leads to the accumulation of aS in endosomes.
Significance: Nedd4-1-mediated Lys-63 ubiquitination specifies the fate of internalized aS.

α-Synuclein (aS) is a major constituent of Lewy bodies, which are not only a pathological marker for Parkinson disease but also a trigger for neurodegeneration. Cumulative evidence suggests that aS spreads from cell to cell and thereby propagates neurodegeneration to neighboring cells. Recently, Nedd4-1 (neural precursor cell expressed developmentally down-regulated protein 4-1), an E3 ubiquitin ligase, was shown to catalyze the Lys-63-linked polyubiquitination of intracellular aS and thereby facilitate aS degradation by the endolysosomal pathway. Because Nedd4-1 exerts its activity in close proximity to the inner leaflet of the plasma membrane, we speculate that after the internalization of aS the membrane resident aS is preferentially ubiquitinated by Nedd4-1. To clarify the role of Nedd4-1 in aS internalization and endolysosomal sequestration, we generated aS mutants, including ΔPR1(1–119 and 129–140), ΔC(1–119), and ΔPR2(1–119 and 134–140), that lack the proline-rich sequence, a putative Nedd4-1 recognition site. We show that wild type aS, but not ΔPR1, ΔPR2, or ΔC aS, is modified by Nedd4-1 *in vitro*, acquiring a Lys-63-linked ubiquitin chain. Compared with the mutants lacking the proline-rich sequence, wild type-aS is preferentially internalized and translocated to endosomes. The overexpression of Nedd4-1 increased aS in endosomes, whereas RNAi-mediated silencing of Nedd4-1 decreased endosomal aS. Although aS freely passes through plasma membranes within minutes, a pulse-chase experiment revealed that the overexpression of Nedd4-1 markedly decreased the re-secretion of internalized aS. Together, these findings demonstrate that Nedd4-1-linked Lys-63 ubiquitination specifies the fate of extrinsic and *de novo* synthesized aS by facilitating their targeting to endosomes.

The intraneuronal aggregation of misfolded α-synuclein (aS),[3] known as a component of Lewy bodies (LB), is a pathological hallmark of Parkinson disease (PD). After the discovery of LB-like inclusions in the grafted neurons of PD patients who had previously received transplants of fetal mesencephalic neurons (1), increasing evidence has suggested that both monomeric and oligomeric aS can be secreted into the extracellular milieu (2), thereby affecting the physiological state of neighboring cells. Previous studies have revealed that the cellular uptake of fibrillar aS requires physiological temperatures and dynamin-1 (3, 4), a master regulator of endocytic vesicle formation, suggesting the

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[3] The abbreviations used are: aS, α-synuclein; CQ, chloroquine; HECT, homologous to the E6-AP C terminus; HMW, high molecular weight; LB, Lewy body; LMW, low molecular weight; Nedd4, neural precursor cell expressed developmentally down-regulated protein 4; pAb, polyclonal antibody; PD, Parkinson disease; PR, proline-rich; aS, α-synuclein; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; ESCRT, endosomal sorting complex required for transport; BN, blue native.

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ARTICLE

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Glycolytic genes are targets of the nuclear receptor Ad4BP/SF-1

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Genetic deficiencies in transcription factors can lead to the loss of certain types of cells and tissue. The steroidogenic tissue-specific nuclear receptor Ad4BP/SF-1 (NR5A1) is one such gene, because mice in which this gene is disrupted fail to develop the adrenal gland and gonads. However, the specific role of Ad4BP/SF-1 in these biological events remains unclear. Here we use chromatin immunoprecipitation sequencing to show that nearly all genes in the glycolytic pathway are regulated by Ad4BP/SF-1. Suppression of Ad4BP/SF-1 by small interfering RNA reduces production of the energy carriers ATP and nicotinamide adenine dinucleotide phosphate, as well as lowers expression of genes involved in glucose metabolism. Together, these observations may explain tissue dysgenesis as a result of Ad4BP/SF-1 gene disruption *in vivo*. Considering the function of estrogen-related receptor α, the present study raises the possibility that certain types of nuclear receptors regulate sets of genes involved in metabolic pathways to generate energy carriers.

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ORIGINAL ARTICLE – THORACIC ONCOLOGY

IGF2 DMR0 Methylation, Loss of Imprinting, and Patient Prognosis in Esophageal Squamous Cell Carcinoma

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ABSTRACT

Background. Insulin like growth factor 2 gene (*IGF2*) is normally imprinted. Loss of imprinting (LOI) of *IGF2* in humans is associated with an increased risk of cancer and is controlled by CpG-rich regions known as differentially methylated regions (DMRs). Specifically, the methylation level at *IGF2* DMR0 is correlated with *IGF2* LOI and is a suggested surrogate marker for *IGF2* LOI. A relationship between *IGF2* DMR0 hypomethylation and poor prognosis has been shown in colorectal cancer. However, to our knowledge, no study has examined the relationships among the *IGF2* DMR0 methylation level, LOI, and clinical outcome in esophageal squamous cell carcinoma (ESCC).

Methods. The *IGF2* imprinting status was screened using *Apa*I polymorphism, and IGF2 protein expression was evaluated by immunohistochemistry with 30 ESCC tissue specimens. For survival analysis, *IGF2* DMR0 methylation was measured using a bisulfite pyrosequencing assay with 216 ESCC tissue specimens.

Results. Twelve (40 %) of 30 cases were informative (i.e., heterozygous for *Apa*I), and 5 (42 %) of 12 informative cases displayed *IGF2* LOI. *IGF2* LOI cases exhibited lower DMR0 methylation levels (mean 23 %) than *IGF2* non-LOI cases (37 %). The *IGF2* DMR0 methylation level was significantly associated with IGF2 protein expression. Among 202 patients eligible for survival analysis, *IGF2* DMR0 hypomethylation was significantly associated with higher cancer-specific mortality.

Conclusions. The *IGF2* DMR0 methylation level in ESCC was associated with *IGF2* LOI and IGF2 protein expression. In addition, *IGF2* DMR0 hypomethylation was associated with a shorter survival time, suggesting its potential role as a prognostic biomarker.

Esophageal squamous cell carcinoma (ESCC), the major histological type of esophageal cancer in East Asian countries, is one of the most aggressive malignant tumors.[1] The limited improvement in past treatment outcomes provided by conventional therapies has prompted us to seek innovative strategies for treating ESCC, especially molecularly targeted treatments.[2–6]

Insulinlike growth factor 2 (IGF2) is an important autocrine growth factor in human tumors because of its mitogenic and antiapoptotic functions mediated by the IGF-I receptor.[7–10] Aberrant expression of IGF2 is frequently found in a wide variety of human cancers, and accumulating evidence implicates IGF2 as a major factor contributing to oncogenesis.[8,11,12] The *IGF2* gene is located within a cluster of imprinted genes on chromosome 11p15 and is expressed predominantly from the paternal allele.[13] Loss of imprinting (LOI) and biallelic expression of IGF2 are common epigenetic alterations in various human cancers. These phenomena increase mitogenic gene expression and facilitate progression

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Clinical Cancer Research

Human Cancer Biology

LINE-1 Hypomethylation, DNA Copy Number Alterations, and *CDK6* Amplification in Esophageal Squamous Cell Carcinoma

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Abstract

Purpose: Global DNA hypomethylation plays a crucial role in genomic instability and carcinogenesis. DNA methylation of the long interspersed nucleotide element-1, L1 (LINE-1) repetitive element is a good indicator of the global DNA methylation level, and is attracting interest as a useful marker for predicting cancer prognosis. Our previous study using more than 200 esophageal squamous cell carcinoma (ESCC) specimens demonstrated the significant relationship between LINE-1 hypomethylation and poor prognosis. However, the mechanism by which LINE-1 hypomethylation affects aggressive tumor behavior has yet to be revealed.

Experimental Design: To examine the relationship between LINE-1 hypomethylation and DNA copy number variations, we investigated LINE-1–hypomethylated and LINE-1–hypermethylated ESCC tumors by comparative genomic hybridization array.

Results: LINE-1–hypomethylated tumors showed highly frequent genomic gains at various loci containing candidate oncogenes such as *CDK6*. LINE-1 methylation levels were significantly associated with *CDK6* mRNA and CDK6 protein expression levels in ESCC specimens. In our cohort of 129 patients with ESCC, cases with CDK6-positive expression experienced worse clinical outcome compared with those with CDK6-negative expression, supporting the oncogenic role of CDK6 in ESCC. In addition, we found that the prognostic impact of LINE-1 hypomethylation might be attenuated by CDK6 expression.

Conclusion: LINE-1 hypomethylation (i.e., global DNA hypomethylation) in ESCC might contribute to the acquisition of aggressive tumor behavior through genomic gains of oncogenes such as *CDK6*. *Clin Cancer Res; 20(5); 1114–24. ©2014 AACR.*

Introduction

Esophageal squamous cell carcinoma (ESCC), the major histologic type of esophageal cancer in East Asian countries, is one of the most aggressive malignant tumors (1). Despite remarkable advances in multimodal therapies, including surgery, chemotherapy, radiotherapy, and chemoradiotherapy, the prognosis of patients remains poor, even for those whose carcinomas have been completely resected (2–4). To develop innovative strategies for treating ESCC, especially those that are molecularly targeted (5), it is of particular importance to increase our understanding of the cellular and molecular basis of this disease. Importantly, epigenetic changes, including alterations in DNA methylation, are reversible, and can thus be targets for cancer therapy or chemoprevention (6–8).

DNA methylation alterations occurring in human cancers include global DNA hypomethylation and site-specific CpG island promoter hypermethylation (9). Global DNA hypomethylation seems to play a crucial role in genomic instability, leading to cancer development (10–12). Because long interspersed nucleotide element-1 (LINE-1; L1 retrotransposon) constitutes a substantial portion (approximately 17%) of the human genome, the level of LINE-1 methylation is regarded to be a surrogate marker of global DNA methylation (13). In many types of human neoplasms, LINE-1 methylation has been shown to be highly variable (14–16), and LINE-1 hypomethylation is strongly associated with a poor prognosis (17–22). In a previous study of 217 curatively resected ESCC specimens (23), we

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The Transcriptional Cofactor MCAF1/ATF7IP Is Involved in Histone Gene Expression and Cellular Senescence

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Abstract

Cellular senescence is post-mitotic or oncogene-induced events combined with nuclear remodeling. MCAF1 (also known as hAM or ATF7IP), a transcriptional cofactor that is overexpressed in various cancers, functions in gene activation or repression, depending on interacting partners. In this study, we found that MCAF1 localizes to PML nuclear bodies in human fibroblasts and non-cancerous cells. Interestingly, depletion of MCAF1 in fibroblasts induced premature senescence that was characterized by cell cycle arrest, SA-β-gal activity, and senescence-associated heterochromatic foci (SAHF) formation. Under this condition, core histones and the linker histone H1 significantly decreased at both mRNA and protein levels, resulting in reduced nucleosome formation. Consistently, in activated Ras-induced senescent fibroblasts, the accumulation of MCAF1 in PML bodies was enhanced via the binding of this protein to SUMO molecules, suggesting that sequestration of MCAF1 to PML bodies promotes cellular senescence. Collectively, these results reveal that MCAF1 is an essential regulator of cellular senescence.

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Introduction

Cellular senescence is a permanent cell cycle arrest that is induced by various stresses such as activated oncogenes, short telomeres, oxidative stress, and inadequate growth conditions [1]. In vivo evidence revealed that cellular senescence occurs in benign or premalignant lesions and acts as an important anti-tumor mechanism [2,3]. Senescent cells are characterized by several features including permanent cell cycle arrest, senescence-associated β-galactosidase (SA-β-gal) activity, morphological changes, activation of DNA damage signaling, and expression of cytokines or secreted factors [1]. Dynamic chromatin changes, including the formation of senescence-associated heterochromatin foci (SAHF), are also observed in senescent cells. The condensed chromatin in senescent cells contributes to the stable repression of proliferation-promoting genes [4]. Increasing number of proteins have been reported to be involved in the chromatin changes during the senescence process [5]. However, little is known about how the epigenetic factors are involved in and contribute to the senescence pathway.

MCAF1 (also known as hAM or ATF7IP) is a transcriptional cofactor that was originally identified as a binding protein of the transcription factor ATF7 [6]. In addition, MCAF1 associates with general transcription factors [6], RNA polymerase II [6,7], and a transcriptional activator SP1 [8]. While MCAF1 associates with the transcriptional apparatus, it also interacts with a methyl-CpG binding protein MBD1 and a H3K9 methyltransferase SETDB1 to form heterochromatin [9,10], suggesting that MCAF1 may function as both a transcriptional activator and a repressor depending on the situation. Biochemical analysis revealed that MCAF1 is an enzymatic cofactor of SETDB1. SETDB1 itself has ability to mono- and di-methylates H3K9, but in the presence of MCAF1 it can also tri-methylate H3K9 [9]. In the cancer cell line C33a, MCAF1, MBD1, and SETDB1 co-localize at the H3K9me3-containing heterochromatin region [8,11]. MCAF1 contains the SUMO-interacting motif (SIM) which preferentially binds to SUMO2/3 [12]. Modification of MBD1 with SUMO2/3 is considered to be required for the recruitment of the MCAF1/SETDB1 complex to DNA-methylated loci to form heterochromatin [11]. Although MCAF1 is overexpressed in various types of cancers [7], the biological significance of MCAF1 remains largely unknown.

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REVIEW

Metabolism–epigenome crosstalk in physiology and diseases

Shinjiro Hino[1], Katsuya Nagaoka[1] and Mitsuyoshi Nakao[1,2]

The way in which energy is used in cells is determined under the influence of environmental factors such as nutritional availability. Metabolic adaptation is mainly achieved through the modulation of metabolic gene expression, and may also involve epigenetic mechanisms that enable long-term regulation. Recent studies have identified that nutrients and their metabolites exert an important influence on the epigenome, as they serve as substrates and/or coenzymes for epigenetic-modifying enzymes. Some epigenetic factors have been shown to regulate metabolic genes leading to a shift in energy flow. These findings suggest the concept of metabolism–epigenome crosstalk that may contribute to the formation of a long-term metabolic phenotype. This is particularly relevant to the pathogenesis of obesity and associated metabolic disorders, in which pre- and post-natal nutritional conditions affect disease risks in adulthood. Moreover, most cancer cells exploit metabolic pathways for their hyperproliferative activity, while metabolic misregulation leads to aberrant epigenetic regulation in some cancers. This review explores the possible mechanisms of metabolism–epigenome crosstalk that may facilitate our understanding of physiology and diseases.
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Keywords: DNA methylation; energy metabolism; epigenome; histone modification; obesity; warburg effect

INTRODUCTION

To survive nutritionally threatening conditions, organisms have evolved strategies to exploit limited energy sources for their biological activities. Such strategies are determined at a cellular level, as each cell type harbors differences in energy demands and access to energy sources and oxygen.[1] Thus, cells can sense their own energetic status and environment, enabling them to remodel their methods of energy utilization. It is now well accepted that differences in metabolic gene expression largely account for adaptive metabolic changes.[2] Recent progress in epigenetics research has suggested the concept that nutrients and their metabolites influence the activities of epigenetic factors, as they often function as coenzymes or substrates for chromatin modifications.[3] A plausible biological meaning of this is that nutritional availability affects the epigenomic landscape, which assists the cells in adapting to their environment. Such metabolism–epigenome crosstalk may support the formation of a long-term energy strategy.

The failure to maintain appropriate energy strategies leads to the development of diseases, such as obesity-associated metabolic disorders and cancer.[4,5] Nutritional availability in prenatal and early life affects the metabolic disease risks, indicating that long-term epigenetic alterations may be involved in the development of metabolic diseases.[6] Indeed, the impact of caloric excess, deficiencies and imbalances on the epigenome of metabolic organs including skeletal muscle, liver and adipose tissue has been demonstrated in many animal models and some human subjects.[7] Although the underlying mechanisms are yet to be revealed, metabolism–epigenome crosstalk is likely to have a central role in connecting dietary habit to disease susceptibility. Cancer cells exhibit extraordinary metabolic flow as they vigorously consume glucose to support rapid cell growth.[5] Such metabolism is referred to as the Warburg effect or aerobic glycolysis, meaning a glycolytic-biased metabolism not based on oxygen availability. As epigenetic dysregulation is one of the prominent hallmarks of cancer,[8] it is plausible that cancer metabolism could be regulated through epigenetic mechanisms. Conversely, altered metabolic flow has been shown to drive epigenetic misregulation in cancer cells.[9,10]

In this review, we will discuss recent progress in understanding how diet and cellular metabolism affects the epigenome, and how epigenetic factors regulate energy metabolism. By focusing on observations in mammalian systems, we will consider the nutritional aspects of lifestyle-associated diseases.

NUTRIENT SENSING BY EPIGENETIC FACTORS

Genomic DNA in eukaryotes is incorporated into a structure called chromatin that enables the efficient packaging of a large DNA in a

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ARTICLE

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FAD-dependent lysine-specific demethylase-1 regulates cellular energy expenditure

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Environmental factors such as nutritional state may act on the epigenome that consequently contributes to the metabolic adaptation of cells and the organisms. The lysine-specific demethylase-1 (LSD1) is a unique nuclear protein that utilizes flavin adenosine dinucleotide (FAD) as a cofactor. Here we show that LSD1 epigenetically regulates energy-expenditure genes in adipocytes depending on the cellular FAD availability. We find that the loss of LSD1 function, either by short interfering RNA or by selective inhibitors in adipocytes, induces a number of regulators of energy expenditure and mitochondrial metabolism such as PPARγ coactivator-1α resulting in the activation of mitochondrial respiration. In the adipose tissues from mice on a high-fat diet, expression of LSD1-target genes is reduced, compared with that in tissues from mice on a normal diet, which can be reverted by suppressing LSD1 function. Our data suggest a novel mechanism where LSD1 regulates cellular energy balance through coupling with cellular FAD biosynthesis.

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Higher-Order Chromatin Regulation and Differential Gene Expression in the Human Tumor Necrosis Factor/Lymphotoxin Locus in Hepatocellular Carcinoma Cells

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The three-dimensional context of endogenous chromosomal regions may contribute to the regulation of gene clusters by influencing interactions between transcriptional regulatory elements. In this study, we investigated the effects of tumor necrosis factor (TNF) signaling on spatiotemporal enhancer-promoter interactions in the human *tumor necrosis factor* (*TNF*)/*lymphotoxin* (*LT*) gene locus, mediated by CCCTC-binding factor (CTCF)-dependent chromatin insulators. The cytokine genes *LTα*, *TNF*, and *LTβ* are differentially regulated by NF-κB signaling in inflammatory and oncogenic responses. We identified at least four CTCF-enriched sites with enhancer-blocking activities and a TNF-responsive TE2 enhancer in the *TNF*/*LT* locus. One of the CTCF-enriched sites is located between the early-inducible *LTα*/*TNF* promoters and the late-inducible *LTβ* promoter. Depletion of CTCF reduced *TNF* expression and accelerated *LTβ* induction. After TNF stimulation, via intrachromosomal dynamics, these insulators mediated interactions between the enhancer and the *LTα*/*TNF* promoters, followed by interaction with the *LTβ* promoter. These results suggest that insulators mediate the spatiotemporal control of enhancer-promoter associations in the *TNF*/*LT* gene cluster.

Chromosomal regions harboring different tissue-specific or cellular-state-specific gene clusters may be influenced by long-range regulatory elements and higher-order chromatin organization (45, 53, 60). Recent studies suggest that transcriptional regulatory elements, such as enhancers, promoters, and chromatin insulators, contribute to gene activation and inactivation via genome accessibility and chromosomal interactions (8, 18). Among these, chromatin insulators are boundary elements that partition the genome into chromosomal subregions, probably through their ability to block interactions between enhancers and promoters when positioned between them (enhancer-blocking effect) (7, 17, 41). However, the precise mechanisms responsible for the enhancer-blocking effect and the relationship with long-range chromatin interactions remain unclear (47, 49). The CCCTC-binding factor CTCF is a highly conserved 11-zinc-finger protein that plays crucial roles at insulator sites (44). CTCF is also reported to function in transcriptional activation (62, 73) and repression (16, 36). In the *IGF2*/*H19* locus, CTCF binds to the differentially methylated region (DMR) of the *H19* gene to form a predicted chromatin loop structure (6, 22, 42). Genome-wide analyses identified the distribution of the putative CTCF-binding sites and their consensus sequences (4, 27, 28, 69). We and other groups recently determined that CTCF is enriched with cohesin in at least 14,000 sites on the human genome (46, 54, 65). CTCF and cohesin cooperatively form compact chromatin loops, leading to the colocalization of gene promoters and their common enhancer in the human *apolipoprotein* gene locus (40). CTCF has been reported to interact with nuclear substructures (71, 72), chromatin remodeling factors (26, 33), RNA polymerase II (10), and CTCF itself (34, 72), as well as undergoing several posttranslational modifications of the protein (12, 29, 37, 70).

Inflammation involves the activation of a highly coordinated gene expression program (43). The tumor necrosis factor (TNF) superfamily members, TNF (initially termed TNF-α), lymphotoxin α (LTα, also termed TNF-β), and lymphotoxin β (LTβ), are major proinflammatory cytokines that mediate inflammatory responses in autocrine/paracrine manners (63). TNF and LTα form homotrimers and act as soluble ligands for the TNF receptor. In contrast, LTβ forms a heterotrimer with LTα and functions as a membrane-bound ligand for the LTβ receptor. In addition to their physiological roles, the aberrant or unbalanced expression of these cytokines is linked to pathological conditions, such as tissue damage/remodeling (38), metabolic diseases (14, 20), and cancer development (19, 23). Hepatic TNF expression is closely related to steatohepatitis (64), and LTβ expression is significantly involved in liver regeneration (3) and hepatocellular carcinomas (HCCs) (23, 67). The *TNF*/*LT* genes are clustered within the major histocompatibility complex (MHC) class III region on human chromosome 6p21.3, which is the most gene-dense region of the human genome (68). Interestingly, it is reported that NF-κB does not directly interact with the proximal human *TNF* promoter (9, 15, 59) and that NF-κB activation induced by TNF treatment in-

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SHORT TAKE

Quantitative assessment of higher-order chromatin structure of the *INK4/ARF* locus in human senescent cells

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Summary

Somatic cells can be reset to oncogene-induced senescent (OIS) cells or induced pluripotent stem (iPS) cells by expressing specified factors. The *INK4/ARF* locus encodes $p15^{INK4b}$, *ARF*, and $p16^{INK4a}$ genes in human chromosome 9p21, the products of which are known as common key reprogramming regulators. Compared with growing fibroblasts, the CCCTC-binding factor CTCF is remarkably up-regulated in iPS cells with silencing of the three genes in the locus and is reversely down-regulated in OIS cells with high expression of $p15^{INK4b}$ and $p16^{INK4a}$ genes. There are at least three CTCF-enriched sites in the *INK4/ARF* locus, which possess chromatin loop-forming activities. These CTCF-enriched sites and the $p16^{INK4a}$ promoter associate to form compact chromatin loops in growing fibroblasts, while CTCF depletion disrupts the loop structure. Interestingly, the loose chromatin structure is found in OIS cells. In addition, the *INK4/ARF* locus has an intermediate type of chromatin compaction in iPS cells. These results suggest that senescent cells have distinct higher-order chromatin signature in the *INK4/ARF* locus.

Key words: chromatin organization; CTCF; *INK4/ARF*; senescent cells; stem cells.

Somatic fibroblasts can be reprogrammed to senescent cells by oncogenic activation (Serrano *et al.*, 1997) or to induced pluripotent stem (iPS) cells by the expression of transcription factors associated with pluripotency (Takahashi *et al.*, 2007). The *INK4/ARF* locus, which encodes $p15^{INK4b}$, $p16^{INK4a}$, and *ARF* genes (Gil & Peters, 2006; Kim & Sharpless, 2006), is an inducer of senescence (Collado *et al.*, 2007) and a barrier for reprogramming into iPS cells (Banito *et al.*, 2009; Li *et al.*, 2009). In fact, the conversion to iPS cells is strongly impaired by senescent program. The CCCTC-binding factor CTCF is known to possess essential functions including transcriptional control, chromatin insulation, and chromosomal interactions within the three-dimensional context of the nucleus (Phillips & Corces, 2009; Ohlsson *et al.*, 2010).

Here, we analyzed the role of CTCF in well-established human cell lines: 201B7 cells, induced from fibroblasts and recognized as the iPS cell standard, human diploid fibroblasts (IMR90), and the same cells undergoing oncogene-induced senescence after Ras activation (oncogene-induced senescent, OIS) (Fig. S1, Supporting information). To avoid the variability between the cells, we used early passage IMR90 cells (Narita *et al.*, 2003) and repeated experiments in other cell lines such as 253G1 cells for iPS cells. Interestingly, *CTCF* mRNA was remarkably up-regulated in iPS cells and reversely down-regulated in OIS cells, compared with that of IMR90 cells (Fig. 1A). We also found increased CTCF protein in iPS cells, but decreased levels of expression in senescent cells (Fig. S2, Supporting information). Using the same samples, $p15^{INK4b}$, *ARF*, and $p16^{INK4a}$ were significantly silenced in iPS cells and IMR90 cells (Fig. 1A and Fig. S2, Supporting information). In contrast, as previously shown, $p15^{INK4b}$ and $p16^{INK4a}$ were up-regulated in OIS cells (day 6), with no change in the expression of *ARF*.

Using genome-wide CTCF-binding profiles available at the websites and our published data (Wendt *et al.*, 2008; Mishiro *et al.*, 2009), there were at least three CTCF-enriched sites in this locus, named IC1, IC2, and IC3 (Fig. S3, Supporting information). IC1 and IC2 were downstream of the $p15^{INK4b}$ and *ARF* transcription start sites, respectively, while IC3 was downstream of $p16^{INK4a}$ exon 3. Chromatin immunoprecipitation showed that CTCF bound to IC1, IC2, and IC3 sites in all three cell lines (Fig. 1B), but did not bind to $p16^{INK4a}$ exon 1 as a negative control (data not shown). Compared with IMR90 cells, the amount of CTCF decreased at IC1 and IC3 sites in OIS cells. In contrast, CTCF binding was significantly high in iPS cells.

Using a chromosome conformation capture (3C) assay, we measured the interaction frequencies of the reference IC1/$p15^{INK4b}$ with nine distinct *Eco*RI fragments in the locus in IMR90 cells (Fig. 1C). The IC1 site was colocalized with IC2/*ARF*, the $p16^{INK4a}$ promoter, and IC3 (red line). The IC2/*ARF* reference strongly interacted with IC1/$p15^{INK4b}$ (Fig. 1D). Further, interaction frequencies of the IC3 reference increased at IC1/$p15^{INK4b}$ (Fig. 1E). These data indicate that IC1/$p15^{INK4b}$, IC2/*ARF*, the $p16^{INK4}$ promoter, and IC3 are closely localized in nuclei, leading to possible formation of chromatin loops in the *INK4/ARF* locus (as modeled in Fig. 2D). Importantly, CTCF knockdown decreased their colocalization (purple line), resulting in increased expression of $p15^{INK4b}$ and $p16^{INK4a}$ (Fig. S4A–D, Supporting information). The depletion of the cofactor cohesin RAD21 (Wendt *et al.*, 2008), which coexisted with CTCF at the IC sites, also induced the *INK4/ARF* genes (Fig. S4E–G, Supporting information). These data suggest that CTCF complex is involved in the compact chromatin formation at the *INK4/ARF* locus.

We then performed a 3C assay in IMR90, OIS, and iPS cells (Fig. 2). Using three reference sites (yellow bars), the IC sites and the $p16^{INK4a}$ pro-

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The distribution of phosphorylated SR proteins and alternative splicing are regulated by RANBP2

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ABSTRACT **The mammalian cell nucleus is functionally compartmentalized into various substructures. Nuclear speckles, also known as interchromatin granule clusters, are enriched with SR splicing factors and are implicated in gene expression. Here we report that nuclear speckle formation is developmentally regulated; in certain cases phosphorylated SR proteins are absent from the nucleus and are instead localized at granular structures in the cytoplasm. To investigate how the nuclear architecture is formed, we performed a phenotypic screen of HeLa cells treated with a series of small interfering RNAs. Depletion of Ran-binding protein 2 induced cytoplasmic intermediates of nuclear speckles in G1 phase. Detailed analyses of these structures suggested that a late step in the sequential nuclear entry of mitotic interchromatin granule components was disrupted and that phosphorylated SR proteins were sequestered in an SR protein kinase–dependent manner. As a result, the cells had an imbalanced subcellular distribution of phosphorylated and hypophosphorylated SR proteins, which affected alternative splicing patterns. This study demonstrates that the speckled distribution of phosphorylated pre-mRNA processing factors is regulated by the nucleocytoplasmic transport system in mammalian cells and that it is important for alternative splicing.**

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INTRODUCTION

Mammalian nuclei are highly organized and compartmentalized into a large number of membrane-free structures that increase the local concentrations of essential molecules and facilitate nuclear events, including transcription, pre-mRNA processing, DNA replication, and DNA repair/recombination (Lamond and Earnshaw, 1998; Lanctot *et al.*, 2007; Sexton *et al.*, 2007; Takizawa *et al.*, 2008; Zhao *et al.*, 2009). Spatial and temporal coordination of chromosomes and the nuclear microenvironment significantly influence gene expression. Although the general principles underlying nuclear body formation are not well determined, several mechanisms have been proposed. One uses scaffold proteins to which additional components are added by ordered assembly. The other is by self-organization in which the components simply associate with each other in a stochastic manner (Misteli, 2001; Kaiser *et al.*, 2008). In addition, RNAs or proteins may serve as seeds for nucleation of a nuclear body, followed by either the stochastic or ordered assembly (Mao *et al.*, 2011). Chromosomes with high gene densities, active gene loci, or coordinately regulated gene loci are clustered at, or adjacent to, nuclear speckles, also called interchromatin granule clusters (IGCs; Shopland *et al.*, 2003; Brown *et al.*, 2008; Hu *et al.*, 2008, 2010; Zhao *et al.*, 2009; Spector and Lamond, 2011). Previous proteomics analyses showed that nuclear speckles contain a variety of proteins involved in gene expression, including pre-mRNA splicing and processing, transcription, subunits of RNA polymerase II (RNAPII), mRNA export, nonsense-mediated mRNA decay, and translation,

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Abbreviations used: FISH, fluorescence in situ hybridization; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IGC, interchromatin granule cluster; MIG, mitotic interchromatin granule; NPC, nuclear pore complex; RANBP2, Ran-binding protein 2; RCC1, regulator of chromosome condensation; RNP, ribonucleoprotein; siRNA, small interfering RNA; SUMO, small ubiquitin-like modifiers; TNPO3, transportin 3.

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Hmga1 is differentially expressed and mediates silencing of the *CD4/CD8* loci in T cell lineages and leukemic cells

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High-mobility group A1 (Hmga1) protein is an architectural chromatin factor, and aberrant Hmga1 expression in mice causes hematopoietic malignancies with defects in cellular differentiation. However, the functional involvement of Hmga1 in hematopoietic development and leukemic cells remains to be elucidated. Using *Hmga1-green fluorescent protein* (*GFP*) knock-in mice that endogenously express an Hmga1-GFP fusion protein, we examined Hmga1 expression in undifferentiated and differentiated populations of hematopoietic cells. During early T cell development in the thymus, Hmga1 is highly expressed in CD4/CD8-double negative (DN) cells and is transiently downregulated in CD4/CD8-double positive (DP) cells. Consistently, Hmga1 directly binds to *cis*-regulatory elements in the *CD4/CD8* loci and the heterochromatin foci in DN-stage cells, but not in DP cells. Interestingly, CD4/CD8 expression in DN-stage leukemic cells is induced by inhibition of Hmga1 binding to nuclear DNA or RNA interference-mediated Hmga1 knockdown. In addition, Hmga1-depleted leukemic T cells markedly diminish proliferation, with transcriptional activation of cyclin-dependent kinase inhibitor genes as a direct target of Hmga1. The data in the present study reveal a role of Hmga1 in transcriptional silencing in T cell lineages and leukemic cells. (*Cancer Sci* 2012; 103: 439–447)

Hematopoietic development has been investigated extensively through identification of stage-specific cell surface markers, as well as multiple transcription factors, which play an important role in establishing lineage diversity.[1–3] Recent studies have emphasized that the cell-fate decision is ultimately determined by the chromatin and nuclear machineries that affect protein-DNA structures and histone modifications.[1,2,4,5] Therefore, it is crucial to investigate the involvement of DNA/chromatin-binding factors to understand key events during hematopoietic differentiation and malignant transformation.

High-mobility group A1 protein (Hmga1) is a non-histone architectural chromatin protein and is characterized by the presence of three AT-hook DNA-binding motifs that preferentially bind AT-rich DNA.[6–10] Hmga1 binding to DNA acts as the architectural structure, which alters the chromatin conformation of target DNA and facilitates the assembly of a high-order multiprotein transcription complex called enhanceosome on transcriptionally active promoter/enhancer regions in specific genes.[11–15] However, Hmga1 is present in the nucleus and predominantly localized to condensed chromatin, suggesting that the major sites of Hmga1 accumulation are not transcriptionally active in somatic cells.[16,17] Thus, Hmga1 possesses distinct and independent biological functions within cells.

The *Hmga1* gene is highly expressed in early embryos, whereas its expression is commonly downregulated in differentiated cells, suggesting its biological role during mammalian development.[18] *Hmga1* haploinsufficiencies in mice cause myeloid leukemia and B cell lymphoma in the peripheral spleen.[19] Conversely, mice with *Hmga1* overexpression develop T and natural killer cell lymphomas.[20,21] In addition, altered hematopoietic differentiation that leads to reduced T cell precursors and preferential B cell differentiation is observed in *Hmga1* null mouse embryonic stem cells.[22] Thus, Hmga1 possesses a crucial role in hematopoietic development and malignant transformation. However, the molecular mechanism of Hmga1 that regulates hematopoietic cells remains to be elucidated.

Hmga1 overexpression is a common feature in the transformation and progression of human cancers, including leukemia and lymphoma.[20,23–26] In our previous study, we showed that Hmga1 overexpression can abolish the retinoblastoma protein (RB) function through direct binding in glioblastoma cells.[27] Furthermore, an anti-apoptotic function has been suggested through a direct Hmga1-p53 interaction with both proteins overexpressed *in vitro*.[28] Because the loss of RB protein and/or the p53 mutations are frequently found in leukemia and lymphoma cells,[29] Hmga1 overexpression may have an uncovered role in hematopoietic tumors.[20,24]

Using *Hmga1-green fluorescent protein* (*GFP*) knock-in mice that express an Hmga1-GFP fusion protein,[25] here we found that Hmga1 has a distinct expression level and mediates silencing of the *CD4/CD8* loci during early T cell development in the thymus. We also demonstrate that Hmga1-depleted T leukemic cells decrease proliferation, together with transcriptional activation of the cyclin-dependent kinase (Cdk) inhibitor genes *p21* and *p27*. Collectively, our results suggest that Hmga1 is actively involved in the transcriptional silencing of these genes in T cell lineages and leukemic cells.

Materials and Methods

Animals. *Hmga1-EGFP* knock-in mice were prepared as previously described.[25] All procedures and protocols were approved by the Committee on Animal Research at Kumamoto University, Japan.

Cell culture and treatments. Human T cell acute lymphoblastic leukemia (T-ALL) Jurkat cells and mouse thymoma EL4 cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated FBS and 50 μM β-mercaptoethanol. To study inhibition of Hmga1 binding to DNA, EL4 cells (2×10^5 cells/mL) were treated with Hoechst 33342 (0.1, 0.2 and 0.5 μg/mL) (Dojindo, Kumamoto, Japan) or Hoechst 33258 (1.0, 2.5 and 5.0 μg/mL) (Dojindo) for 24 h.

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Rb/E2F1 Regulates the Innate Immune Receptor Toll-Like Receptor 3 in Epithelial Cells

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Tumor suppressor genes regulate the antiviral host defense through molecular mechanisms that are not yet well explored. Here, we show that the tumor suppressor retinoblastoma (Rb) protein positively regulates Toll-like receptor 3 (TLR3) expression, the sensing receptor for viral double-stranded RNA and poly(I·C). TLR3 expression was lower in Rb knockout ($Rb^{-/-}$) mouse embryonic fibroblasts (MEF) and in mammalian epithelial cells transfected with Rb small-interfering RNA (siRNA) than in control cells. Consequently, induction of cytokines interleukin-8 and beta interferon after poly(I·C) stimulation was impaired in $Rb^{-/-}$ MEF and Rb siRNA-transfected cells compared to controls. TLR3 promoter analysis showed that Rb modulates the transcription factor E2F1, which directly binds to the proximal promoter of TLR3. Exogenous addition of E2F1 decreased TLR3 promoter activity, while Rb dose dependently curbed the effect of E2F1. Interestingly, poly(I·C) increased the Rb expression, and the poly(I·C)-induced TLR3 expression was impaired in Rb-depleted cells, suggesting the importance of Rb in TLR3 induction by poly(I·C). Together, these data indicated that E2F1 suppresses TLR3 transcription, but during immune stimulation, Rb is upregulated to block the inhibitory effect of E2F1 on TLR3, highlighting a role of Rb-E2F1 axis in the innate immune response in epithelial cells.

Oncogenic virus infections, such as hepatitis C virus, human papillomavirus, Kaposi's sarcoma herpesvirus, and human T-cell leukemia virus 1 infections, are well-known causative factors of hepatocellular carcinoma, cervical carcinoma, lymphoma, and leukemia, respectively (30). These tumorigenic viruses attenuate tumor suppressor genes, activate proto-oncogenes, and consequently induce host cell abnormal growth. The tumor suppressor proteins p53 and Rb are frequently downregulated by oncogenic viral proteins during viral infection (11, 34, 51). Conversely, reactivation of p53 and Rb induces cell cycle arrest and apoptosis in virus-induced cancer (43, 61). Therefore, p53 and Rb are considered as critical proteins that prevent tumorigenesis caused by oncogenic viral infection (21, 30, 47). p53 activates interferon (IFN) signaling and induces apoptosis in infected cells by activating its target gene, the interferon regulatory factor 9 (IRF9) gene (36). p53 also increases viral sensing molecule Toll-like receptor 3 (TLR3) expression and function in human epithelial cells and mouse tissues (48), which correlates with the antiviral effect of p53 (35). Thus, p53 multiply regulates antiviral host defense, from sensing viral infection to signal transduction and viral removal by apoptosis, at the level of transcriptional regulation of p53 target genes (42).

The tumor suppressor Rb was identified as the protein responsible for the congenital tumor retinoblastoma, and this established the tumor suppressor paradigm for Rb in cancer research (8, 17, 37). Rb regulates cellular proliferation by directly binding to E2F transcription factors (9, 16, 45), a family of transcription factors that play a pivotal role in the regulation of cellular proliferation, growth, and differentiation (39, 58). The Rb-E2F binding negatively affects E2F-dependent transcription by at least three independent mechanisms: (i) masking the E2F's transactivation domain, (ii) sequestering the E2F from target promoters, and (iii) recruiting chromatin-modifying repressive complexes (18, 19, 23). The functional loss of Rb promotes the deregulated E2F activity that is observed in vast majority of human tumors (22, 25). Although the Rb-E2F pathway is well known to have a generally crucial role in oncogenesis, its functions during viral infection and antiviral host defense are not well studied. The founding member of the E2F family, E2F1, is mostly known for its transcriptional activating functions but, intriguingly, recent studies have revealed that E2F1 may act as a repressor as well. E2F1 was found to suppress, directly and independently of Rb, the promoters of vascular endothelial factor A (31), human telomerase reverse transcriptase (12), the antiapoptotic protein Mcl-1 (13), endoplasmic reticulum chaperone GRP78/Bip (41), and IRF3 (55). These surprisingly diverse gene targets of E2F1 revealed a wide influence of E2F1 on cellular events not only by activating but also by suppressing gene expression.

Double-stranded RNA (dsRNA) is an intermediate component during viral replication in host cells that is recognized by host innate immune molecules, including TLR3, protein kinase recep-

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The role of methyl-binding proteins in chromatin organization and epigenome maintenance

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Abstract

Methylated DNA can be specifically recognized by a set of proteins called methyl-CpG-binding proteins (MBPs), which belong to three different structural families in mammals: the MBD family, the Kaiso and Kaiso-like proteins and the SRA domain proteins. A current view is that, once bound to methylated DNA, MBPs translate the DNA methylation signal into appropriate functional states, through interactions with diverse partners. However, if some of the biological functions of MBPs have been widely described—notably transcriptional repression—others are poorly understood, and more generally the extent of MBP activities remains unclear. Here we propose to discuss the role of MBPs in two crucial nuclear events: chromatin organization and epigenome maintenance. Finally, important challenges for future research as well as for biomedical applications in pathologies such as cancers—in which DNA methylation patterns are widely altered—will be mentioned.

Keywords: *DNA methylation recognition; transcriptional repression; heterochromatin formation and maintenance; epigenetic cooperation; cancer*

INTRODUCTION: WHERE THE METHYLATED DNA IS, AND HOW WE KNOW DNA METHYLATION IS IMPORTANT

In eukaryotes, only cytosine can be methylated—this differs from bacteria that can also display adenine methylation. In mammals, DNA methylation takes place mostly in the context of CpG dinucleotides, and 60–80% of all CpG dinucleotides are methylated, in a heterogeneous fashion. Most CpG islands are unmethylated, whereas the rest of the genome is globally methylated. In other words, the regions of the genome that are methylated include repeated elements, intergenic regions, gene bodies and certain CpG islands [1]. The regions that are unmethylated are mainly CpG islands. There are excellent recent reviews on CpG islands, to which the reader can refer for further information [2, 3], including a discussion of the possible mechanisms by which some, but not all, CpG islands may escape DNA methylation.

Three DNA methyltransferases exist in mammals: Dnmt1, Dnmt3a and Dnmt3b [4]. Their invalidation in mouse is lethal at different stages, showing that

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