ない。HIV対策としては予防や治療だけでなく社会的、文化的背景への対策も必要だ。

MARPs(もっとも危険にさらされている人々)にはMSM(men who have sex with men)やsex workers、drug usersなどがある。これらはhigh risk groupとは呼ばない。アフリカの新規感染者はこれらMARPsよりパートナーが一人の人が多い。HIVは特定の人々の問題ではなく、全ての人の問題であり、その中には常に自分自身も入っている。

> Most-at-risk populations (MARPs)
> (もっとも危険にさらされている人々)
>
> 「High risk group」とは言わない
>
> - MSM
>   - Men who have sex with men
>   (ゲイ、同性愛者、両性愛者、トランスジェンダー・・・)
> - Sex worker(セックスワーカー)とその顧客
> - Drug Users (injecting drug users: IDU)
>   (静脈注射薬物使用者)

●自分自身に偏見はないか?

例えば、「結婚しようとしていた彼が先日HIV検査に行った」ときかされたら、「そんな人と結婚しない方がいいのでは?」と応じるのは、HIVに対する偏見で、ポイントとしてはHIVとの距離感やsexについての意識の違いである。HIVは誰でも感染するが、誤った意識は日本の教育の問題かもしれない。

ここで、短いワークショップとして、「sex workerやinjecting drug userにどのようなHIV対策を考えるか?」近くの参加者同士で考え次のような意見が出された。

【Sex worker への対策】

- 事業主がコンドームを付けるように指導
- 定期的な健診を義務付ける
- コンドームの配布
- エイズの知識を普及させる
- 採用前に検査
- 陽性者に治療、転職紹介

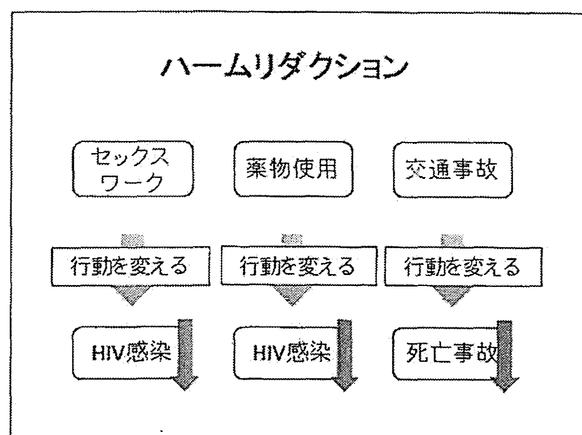
【Drug users への対策】

- 内服薬に変える
- やめるための支援
- 法律を変える
- 清潔な注射器を配る
- 薬物の流通を防ぐ
- HIVに対する教育、啓発

例えば、sex workerにこれらの対策をするためには、情報の把握や弱い立場の人への対応、法律などの整備が必要になる。ちなみに日本の売春防止法ではこれらの対策は管理売春として違法になるので、風俗嬢の健康が守られにくい環境にある。

●「ハーム・リダクション」

ある行動が原因となっている健康被害を行動変容などにより予防または軽減させることで、特にエイズ対策では、注射薬物使用者(IDU: Injection Drug User)が注射器や針を共有することによるHIV感染を、注射器交換や経口薬物への薬物代替を取り上げられることが多い。また、ハーム・リダクションは、この対策自体が薬物使用を抑制するのでも逆に奨励するものでもない。セックスワークに対しても取り締まるのではなく、コンドームを使ってもらうようにする。交通事故で言えばシートベルトが相当する。



●研究の紹介

カンボジアで妊婦を対象にHIV検査を導入した。検査を受けたい人は多いが実際に受ける人は少なかっ

た。原因として夫の許可が必要というのが多かった。実際に、パートナーが同行した妊婦はHIV検査受検率が高かった。

パートナー同行の有無による HIV検査受検率等の相違(02年7月～05年5月)

	一人で妊婦健診	パートナーが同行	p値
妊婦健診初診	17,340	3,417	
HIV検査を受けた	3,228	2,904	
受検率(%)	18.7%	85.1%	<0.001
検査結果の受取り	2,519	2,355	
受取った率(%)	78.0%	81.1%	<0.005

Kakimoto K. et al. AIDS Care. 2007

ジンバブエでなぜHIV検査を受けなかったのか？というアンケートを女性に行ったら、パートナーに告知がいるから、という理由が多かった。女性が直面している問題としては、家族計画やパートナーへの告知、HIV陽性に対する恐怖などがあった。また、カンボジアやアフリカ諸国では避妊についての知識を持っている人と実際に行っている人にはかい離がみられる。

いずれもHIVに対する偏見や差別、女性の立場の弱さが原因で、女性の保健行動は知識の有無のみでは無いことがわかった。

＊質疑応答

Q.カンボジアの避妊方法として男性のコンドームが低いがピルが多いのはなぜか？

A.男性コンドームは男性の協力が必要だが、男性の理解が少ないのが理由の一つ。支援側の宗教的な理由もあると個人的には感じる。

Q.コンドームを男性に使うために女性の交渉の方法としてどんなものがあるのか？

A.コミュニケーションスキルを上げることやコンドームで遊んでみる、性交渉時以外に事前に話し合いをしておくなど

Q.日本でのHIV感染対策について先生はどのようにお考えか？

A.文部科学省では現実とは異なった教育を考えている。もう少し現実にそくした教育を行うことが必要。

Q.日本では他の国に比べてどのようなリスク行動があるのか？

A.日本では新規感染者の6割近くがMSMである。大阪や東京などの大都会を中心に啓発活動をするが、大都会に出てこないMSMの人たちに対していかにアプローチするかが大切。

Q.HIV検査を受ける人を増やすためにバイク以外に行ったことは？

A.資料を配布して夫に見せてもらう、ポスターを貼ってもらう、テレビやラジオでの啓発活動などを行った。

Q.インドネシアで内服の麻薬に変えてHIVを減らすことに対して国の合意はあったか？

A.その通り。国の方針として合意を取った後に行った。

Q.HIV陽性者は実際どのような推移をしているのか？

A.スライドで出したのはあくまで推計なので幅がある。

Q.sex worker が陽性という告知を受けた後に、どういった行動をするのか？

A.自分でお金を稼ぐスキルを教えて自立の支援をする。そのまま続けてほしいと言うことはない。

Q.村での対策でのキーパーソンは協力してくれるのか？

A.初等教育、職場での教育活動が有用である。村での対策では村長さんなどのキーパーソンへのアプローチは必ず行っている。

講演の内容は下記のアドレスから動画でご覧いただけます

http://www.youtube.com/watch?v=aPJyAgo3vak

Cell Cycle 12:5, 773–782; March 1, 2013; © 2013 Landes Bioscience

Cyclin D1 overexpression perturbs DNA replication and induces replication-associated DNA double-strand breaks in acquired radioresistant cells

Tsutomu Shimura,[1,*] Yasushi Ochiai,[2] Naoto Noma,[2] Toshiyuki Oikawa,[2] Yui Sano[2] and Manabu Fukumoto[2]

[1]Department of Environmental Health; National Institute of Public Health; Saitama, Japan; [2]Department of Pathology; Institute of Development, Aging and Cancer; Tohoku University; Sendai, Japan

Keywords: cyclin D1, DSBs, Mus81, Perturbation of DNA replication, radioresistance

Abbreviations: BrdU, bromodeoxyuridine; CDK4-I, Cdk4 inhibitor; CldU, 5-chloro-2'-deoxyuridine; DDR, DNA damage response; DSBs, double-strand breaks; FR, fractionated irradiation; GSK3β, glycogen synthase kinase 3beta; IF, immunofluorescence; IdU, 5-Iodo-2'-deoxyuridine; RT, radiotherapy; SR, single radiation; Thr286, threonine286; CD1-WT, wild-type cyclin D1

Fractionated radiotherapy (RT) is widely used in cancer treatment, because it preserves normal tissues. However, repopulation of radioresistant tumors during fractionated RT limits the efficacy of RT. We recently demonstrated that a moderate level of long-term fractionated radiation confers acquired radioresistance to tumor cells, which is caused by DNA-PK/AKT/GSK3β-mediated cyclin D1 overexpression. The resulting cyclin D1 overexpression leads to forced progression of the cell cycle to S-phase, concomitant with induction of DNA double-strand breaks (DSBs). In this study, we investigated the molecular mechanisms underlying cyclin D1 overexpression-induced DSBs during DNA replication in acquired radioresistant cells. DNA fiber data demonstrated that replication forks progressed slowly in acquired radioresistant cells compared with corresponding parental cells in HepG2 and HeLa cell lines. Slowly progressing replication forks were also observed in HepG2 and HeLa cells that overexpressed a nondegradable cyclin D1 mutant. We also found that knockdown of Mus81 endonuclease, which is responsible for resolving aberrant replication forks, suppressed DSB formation in acquired radioresistant cells. Consequently, Mus81 created DSBs to remove aberrant replication forks in response to replication perturbation triggered by cyclin D1 overexpression. After treating cells with a specific inhibitor for DNA-PK or ATM, apoptosis rates increased in acquired radioresistant cells but not in parental cells by inhibiting the DNA damage response to cyclin D1-mediated DSBs. This suggested that these inhibitors might eradicate acquired radioresistant cells and improve fractionated RT outcomes.

Introduction

The most severe form of DNA damage induced by ionizing radiation is DNA double-strand breaks (DSBs), which can trigger chromosomal aberrations such as deletions, insertions and translocations. A series of DNA damage responses (DDRs) are induced in eukaryotic cells after irradiation to maintain genomic stability. Cell cycle checkpoints are activated after irradiation resulting in blockage of cell cycle progression to achieve proper repair of DNA damage.[1] Cell death is induced in order to exclude abnormal cells in response to high doses of irradiation.[2] The molecular mechanisms involved in DDR have been well studied using single radiation (SR) exposure regimes; however, DDRs after multiple fractionated radiation (FR) exposure regime remain to be elucidated.

It is well known that cyclin D1 is degraded following SR exposure, which arrests cells at the G_1/S boundary as a G_1/S checkpoint.[3] Conversely, cyclin D1 is stabilized in human tumor cells after exposure to FR of X-ray at 0.5 Gy twice per day for 1 mo. This exposure regime confers acquired radioresistance to tumor cells.[4] By binding to Cdk4, cyclin D1 becomes an important regulator of cell cycle progression at the G_1/S transition. Cyclin D1-Cdk4 phosphorylates Rb, after which E2F is released to transactivate genes required for G_1- to S-phase progression.[5,6] Overexpression of cyclin D1/Cdk4 prevents FGF-mediated growth arrest by inhibiting downregulation of cyclin E/Cdk2 activity.[7,8] In addition to its role in activating Cdk4, cyclin D1 controls transcription of several genes in a Cdk-independent manner.[9,10]

The cyclin D1 level is tightly controlled for normal cell cycle progression, and its deregulation is linked to the development of

*Correspondence to: Tsutomu Shimura; Email: tsimura@niph.go.jp
Submitted: 11/05/12; Revised: 01/21/13; Accepted: 01/23/13
http://dx.doi.org/10.4161/cc.23719

©2013 Landes Bioscience. Do not distribute.

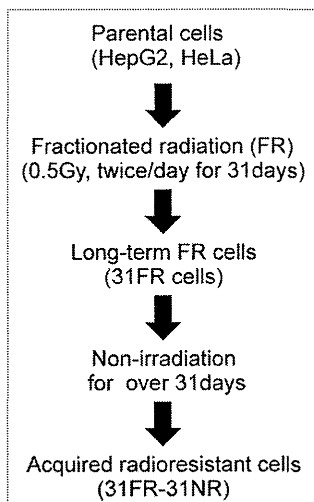


Figure 1. A flowchart describing the derivation of acquired radioresistant 31FR-31NR cells. Cells were exposed to FR consisting of a 0.5 Gy X-ray fraction dose at every 12 h, 6 d a week. The cells treated with this exposure scenario with 62 fractions for 31 d were referred to as 31FR cells. The 31FR cells were further cultured without irradiation for more than 31 d and the resulting cells were designated as 31FR-31NR cells.

cancer.[11-13] Cyclin D1 is implicated in induction of chromosomal instability in mammary gland tumors.[14] Abundance of cyclin D1 is also associated with cellular senescence in response to replicative stress.[15] Cyclin D1 accumulates during G_1-phase progression and is degraded during the S-phase.[16] During cell cycling, cyclin D1 expression is regulated both at the transcriptional and post-translational levels. Cyclin D1 expression is regulated by mitogenic signaling through small guanosine triphosphate-binding proteins such as Ras.[17]

Glycogen synthase kinase 3beta (GSK3β) is a protein kinase that phosphorylates cyclin D1 on threonine286 (Thr286) to facilitate its degradation. AKT-mediated phosphorylation of GSK3β on serine 9 decreases its kinase activity on cyclin D1 Thr286, which inhibits nuclear export and cytoplasmic proteasomal degradation of cyclin D1.[18,19] Thus, AKT positively regulates G_1/S cell cycle progression by inactivating GSK3β, which results in cyclin D1 accumulation in the nucleus. We previously reported that long-term FR-induced cyclin D1 overexpression was due to downregulation of cyclin D1 proteolysis via the activation of the DNA-PK/AKT/GSK3β pathway.[4,20]

Oncogene activation perturbs DNA replication and induces both DSBs and DDRs in nonmalignant cells during tumorigenesis.[21-23] Overexpression of cell cycle regulators such as cyclin D1, cyclin A and cyclin E induces DSBs and DNA damage checkpoints in human and mouse fibroblasts.[24-26] We recently reported that persistent cyclin D1 expression during S-phase induces DSBs in acquired radioresistant cells.[4] However, the molecular mechanisms underlying cyclin D1-mediated DSBs during DNA replication have not been completely characterized.

In this study, we investigated the effect of cyclin D1 overexpression on DNA replication in acquired radioresistant cells. We found that Mus81 created DSBs in response to aberrant replication forks that were induced by cyclin D1 overexpression. These DSBs were efficiently repaired, because acquired radioresistant cells continued to grow without any remarkable delay. We also demonstrated that either a DNA-PK inhibitor or an ATM inhibitor could induce cell death in acquired radioresistant cells. Thus, we provide evidence for a new strategy to suppress tumor radioresistance by targeting DDRs in response to cyclin D1-mediated DSBs in acquired radioresistant cells.

Results

Cyclin D1-mediated DSBs in acquired radioresistant 31FR-31NR cells. We previously established acquired radioresistant 31FR-31NR cells by 31-d FR exposure followed by 31-d non-FR (**Fig. 1**). This radioresistant phenotype was irreversible without FR exposure for > 1 mo; therefore, we termed it "acquired radioresistance."[4] In this study, we used the neutral comet assays to quantify the DSB levels in 31FR-31NR cells derived from HepG2 and HeLa cell lines (**Fig. 2A**). A higher tail moment value in 31FR-31NR cells compared with the corresponding parental (0FR) cells indicated that acquired radioresistant cells harbored large amounts of DSBs (**Fig. 2A**).

In a prior study, we found that cyclin D1 was overexpressed in 31FR-31NR cells derived from HepG2 and HeLa cell lines.[4] We examined whether the amount of DSBs decreased after repressing cyclin D1 gene expression using siRNA (**Fig. 2B**). Knock down of cyclin D1 by using cyclin D1 siRNA was confirmed by western blot analyses in HeLa cells (**Fig. 2C**). Distributions of tail moment values were the same in HeLa 0FR cells with both control siRNA and cyclin D1 siRNA. Thus, cyclin D1 siRNA did not affect the amount of DSBs in parental 0FR cells. In contrast, transfection with cyclin D1 siRNA clearly decreased the tail moment value in HeLa 31FR-31NR cells compared with HeLa cells transfected with control siRNA. These results demonstrated that cyclin D1 overexpression induced DSBs in 31FR-31NR cells.

Cdk4-independent DSBs formation in 31FR-31NR cells. Cyclin D1-mediated DSBs may affect cell cycle progression in 31FR-31NR cells. However, the percentage of BrdU-positive S-phase 31FR-31NR cells with control siRNA was the same as 0FR cells with control siRNA. Thus, 31FR-31NR cells continued to grow without any remarkable delay compared with 0FR cells (**Fig. 3A**).

Cyclin D1 is a regulator of Cdk4 and Cdk6 during the G_1/S transition of the cell cycle. We investigated whether Cdk4 was required for cyclin D1-mediated DSB formation in 31FR-31NR cells. In order to inactivate Cdk4, we used Cdk4 siRNA or a Cdk4 inhibitor (Cdk4-I). As we reported previously, 1.9 μM of Cdk4-I could suppress cyclin D1/Cdk4-dependent phosphorylation of Rb at Serine 795 in 0FR and 31FR-31NR cells of HeLa.[4] In HeLa cells, human papillomavirus E7 disrupts the formation of RB-E2F complexes, which results in increased expression levels of E2F-responsive genes. Therefore, Cdk4 inactivation did not affect the G_1/S transition in 0FR and 31FR-31NR cells derived from HeLa cells (**Fig. 3A**). The amounts of γ-H2AX did not decrease after treatment with either Cdk4 siRNA or a Cdk4-I in 31FR-31NR cells (**Fig. 3B and C**). These results demonstrated

©2013 Landes Bioscience. Do not distribute.

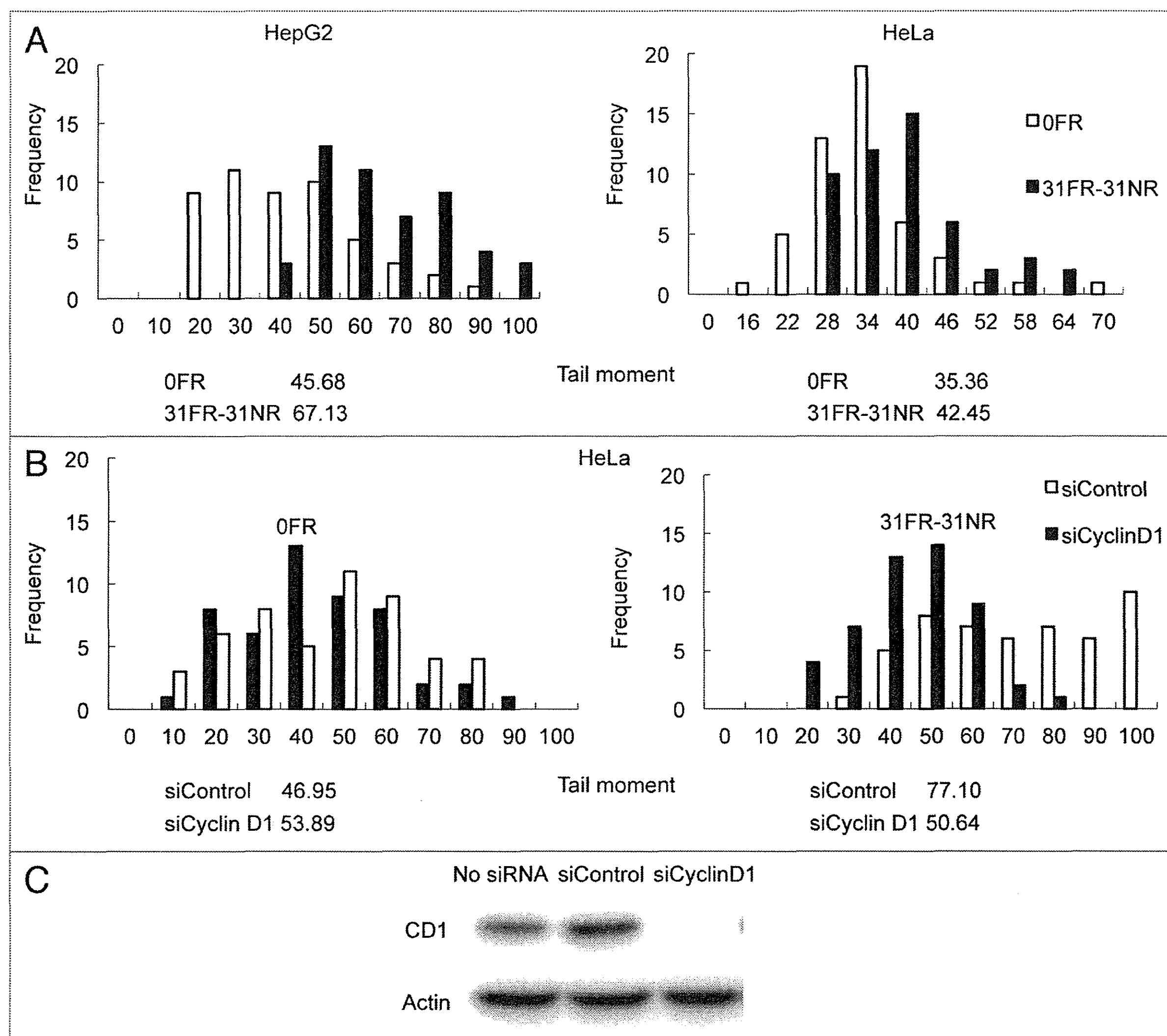


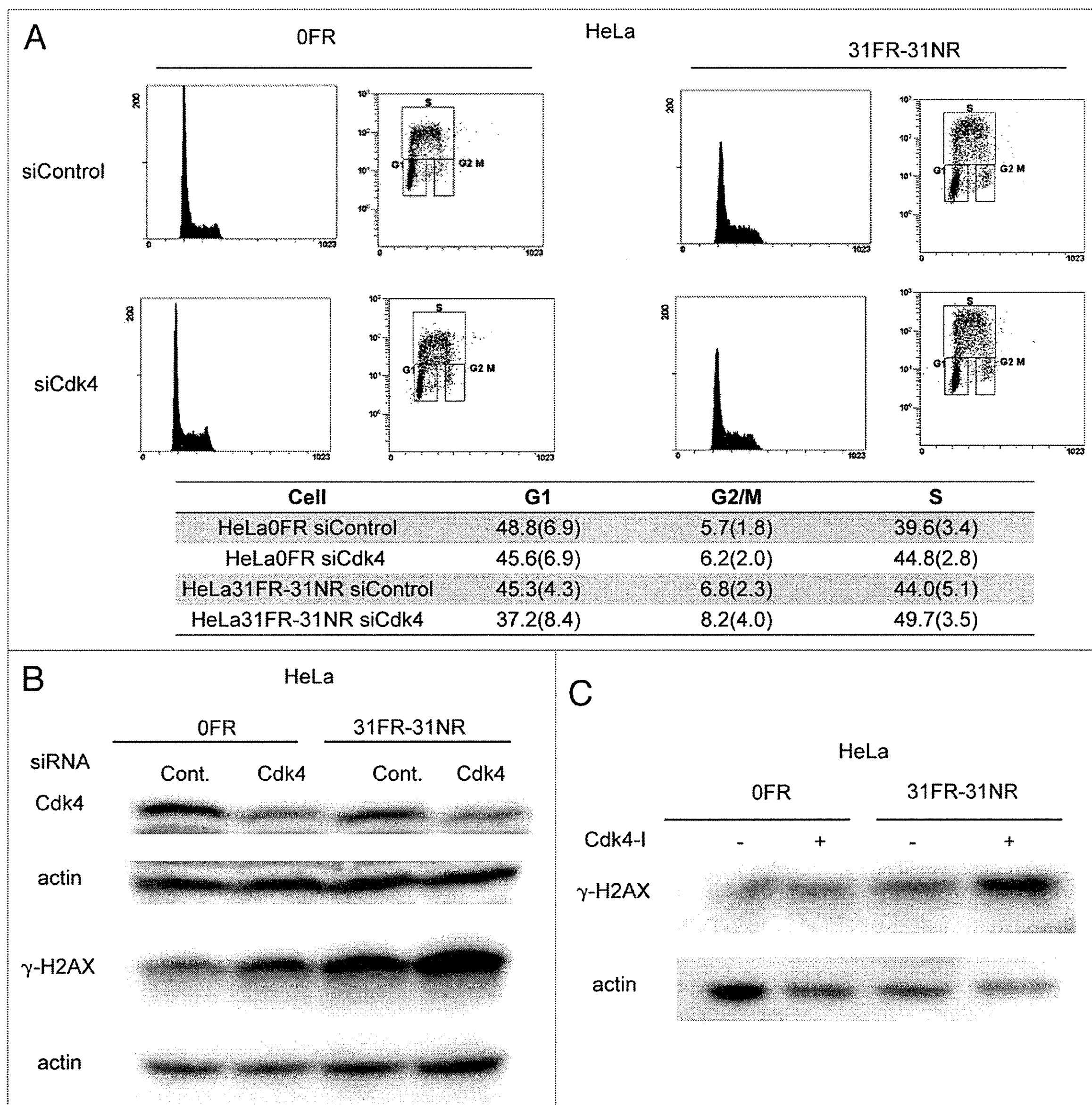
Figure 2. Results for neutral comet assays. (**A**) Distributions of tail moment values for 0FR and 31FR-31NR cells. Results for HepG2 and HeLa cells are shown in the left and right panels, respectively. The median tail moment values are indicated at the bottom of the graph. (**B**) Distributions of tail moment values for 0FR and 31FR-31NR cells derived from the HeLa cell line. Samples were prepared at 48 h after transfection with control siRNA (siControl) or cyclin D1 siRNA (siCyclin D1). Median tail moment values are indicated at the bottom of the graph.

©2013 Landes Bioscience. Do not distribute.

that cyclin D1/Cdk4 activity was unnecessary for DSB formation in 31FR-31NR cells.

Slowing down of replication fork progression due to cyclin D1 overexpression in 31FR-31NR cells. Persistent cyclin D1 expression during S-phase may perturb DNA replication in 31FR-31NR cells. We used the DNA fiber assay to determine if cyclin D1 affected the elongation stages of DNA replication. The cells were first pulse-labeled with 5-iodo-2'-deoxyuridine (IdU; detected by Cy3, red signal) and subsequently labeled with 5-chloro-2'-deoxyuridine (CldU; detected by Alexa 488, green signal). Replication fork elongation was detected as unidirectional red-green tracks (R-G, **Fig. 4A**). Short-length R-G tracks were evident in 31FR-31NR cells but not in 0FR cells (indicated by the arrowhead in **Fig. 4A**). Lengths of the green-labeled tracks in the R-G tracks were measured in 50 DNA tracks. The average replicating DNA tracks with the standard deviations was shown in **Figure 4B and C**. The lengths of replicating DNA in the 31FR-31NR cells were shorter than those in the 0FR cells derived from the HepG2 and HeLa cell lines (**Fig. 4B**). These results indicated that replication forks in 31FR-31NR cells progressed more slowly than those in 0FR cells.

We have made cells overexpressing wild-type cyclin D1 (CD1-WT) and a nondegradable cyclin D1 mutant (CD1-T286A), mutated at the phosphorylation on Thr286, and confirmed their expression by western blotting in a prior study.[4] Enforced expression of a CD1-T286A but not CD1-WT resulted in its overexpression. Short replicating DNA tracks increased



Cell	G1	G2/M	S
HeLa0FR siControl	48.8(6.9)	5.7(1.8)	39.6(3.4)
HeLa0FR siCdk4	45.6(6.9)	6.2(2.0)	44.8(2.8)
HeLa31FR-31NR siControl	45.3(4.3)	6.8(2.3)	44.0(5.1)
HeLa31FR-31NR siCdk4	37.2(8.4)	8.2(4.0)	49.7(3.5)

Figure 3. Cdk4-independent DSBs formation in 31FR-31NR cells. **(A)** Cell cycle distributions for HeLa cells with either control siRNA (siControl) or Cdk4 siRNA (siCdk4). Percentages of G_1-, G_2/M- and S-phase cells are shown with the standard deviations in parentheses in the lower panel. **(B)** Western blotting results for Cdk4, γ-H2AX and actin in 0FR and 31FR-31NR cells derived from HeLa cell line. Cell extracts were prepared at 48 h after transfection with either control siRNA or Cdk4 siRNA. **(C)** Western blotting results for γ-H2AX and actin in the 0FR and 31FR-31NR cells derived from HeLa cell line. The cells were treated with 1.9 μM Cdk4-I for 24 h.

©2013 Landes Bioscience. Do not distribute.

by CD1-T286A overexpression in HepG2 and HeLa cells compared with parental cells (**Fig. 4C**). Thus, cyclin D1 overexpression disrupted DNA replication by suppressing replication fork progression.

Mus81-mediated DSBs in 31FR-31NR cells. Mus81 cleaves an aberrant fork structure to generate DSB, which removes a stalled fork from replication sites.[27-29] Therefore, DSBs may be generated by Mus81 in response to replication perturbations caused by cyclin D1 overexpression. Mus81 expression was suppressed by its siRNA but not by random control siRNA in 0FR and 31FR-31NR cells (**Fig. 5A**). Mus81 knockdown decreased γ-H2AX signals in 31FR-31NR cells compared with the control siRNA cells. We further performed immunostaining of cyclin D1 and γ-H2AX in 0FR and 31FR-31NR cells derived from HeLa cells. γ-H2AX was observed in cyclin D1-positive 31FR-31NR cells as indicated by arrow on the lower panel in **Figure 5B**. Upon Mus81 depletion by using Mus81 siRNA, double-positive cells with γ-H2AX and cyclin D1 were disappeared in 31FR-31NR cells (**Fig. 5B**). These results demonstrated that cyclin D1-dependent DSBs were created by Mus81 endonuclease

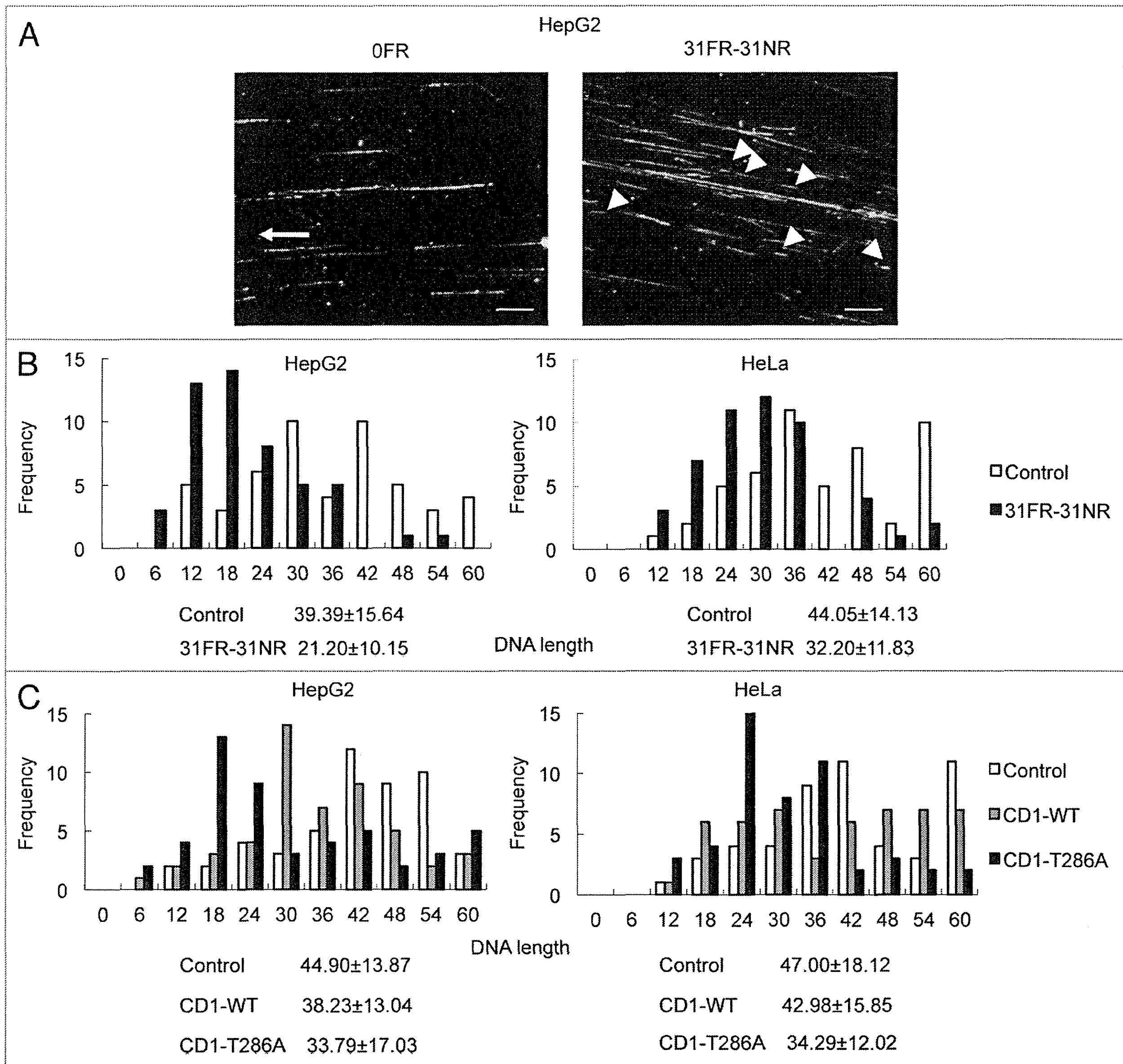


Figure 4. Cyclin D1-mediated slowing down of replication forks. **(A)** Image of red- and green-labeled DNA tracks. Replication of DNA in 0FR cells is shown by the arrow. Short-labeled DNA tracks in 31FR-31NR cells are shown by arrowheads. **(B)** Histograms of labeled DNA lengths in 0FR and 31FR-31NR cells. Results for HepG2 and HeLa cells are shown in the left and right panels, respectively. The average lengths are indicated at the bottom of the graph. **(C)** Histograms of labeled DNA lengths in the HepG2 and HeLa cells that expressed wild-type cyclin D1(CD1-WT) or cyclin D1 mutants (CD1-T286A).

©2013 Landes Bioscience. Do not distribute.

in response to aberrant replication forks triggered by cyclin D1 overexpression in 31FR-31NR cells.

Eradication of 31FR-31NR cells by inhibition of DNA-PK and ATM. We next investigated DDR in 31FR-31NR cells in response to cyclin D1-mediated DSBs. Activation of the DNA damage-activated serine/threonine protein kinases DNA-PK and ATM was examined in 31FR-31NR cells using an anti-phospho-DNA-PKcs-Thr2609 and anti-phospho-ATM-Ser1981 antibody, respectively. In order to identify cells in S phase, cells were stained with PCNA, which is a replication fork processivity protein at the ongoing replication fork during S phase. DNA-PK and ATM phosphorylation was not observed in the control 0FR cells but was observed in the PCNA-positive 31FR-31NR cells (**Fig. 6A**). These results indicated that cyclin D1-mediated DSBs activated DNA-PK and ATM during S-phase in 31FR-31NR cells.

We expected DNA-PK and ATM inactivation to prevent DNA repair of cyclin D1-mediated DSBs and induce 31FR-31NR cell death. Treatment with either a DNA-PK inhibitor NU7026

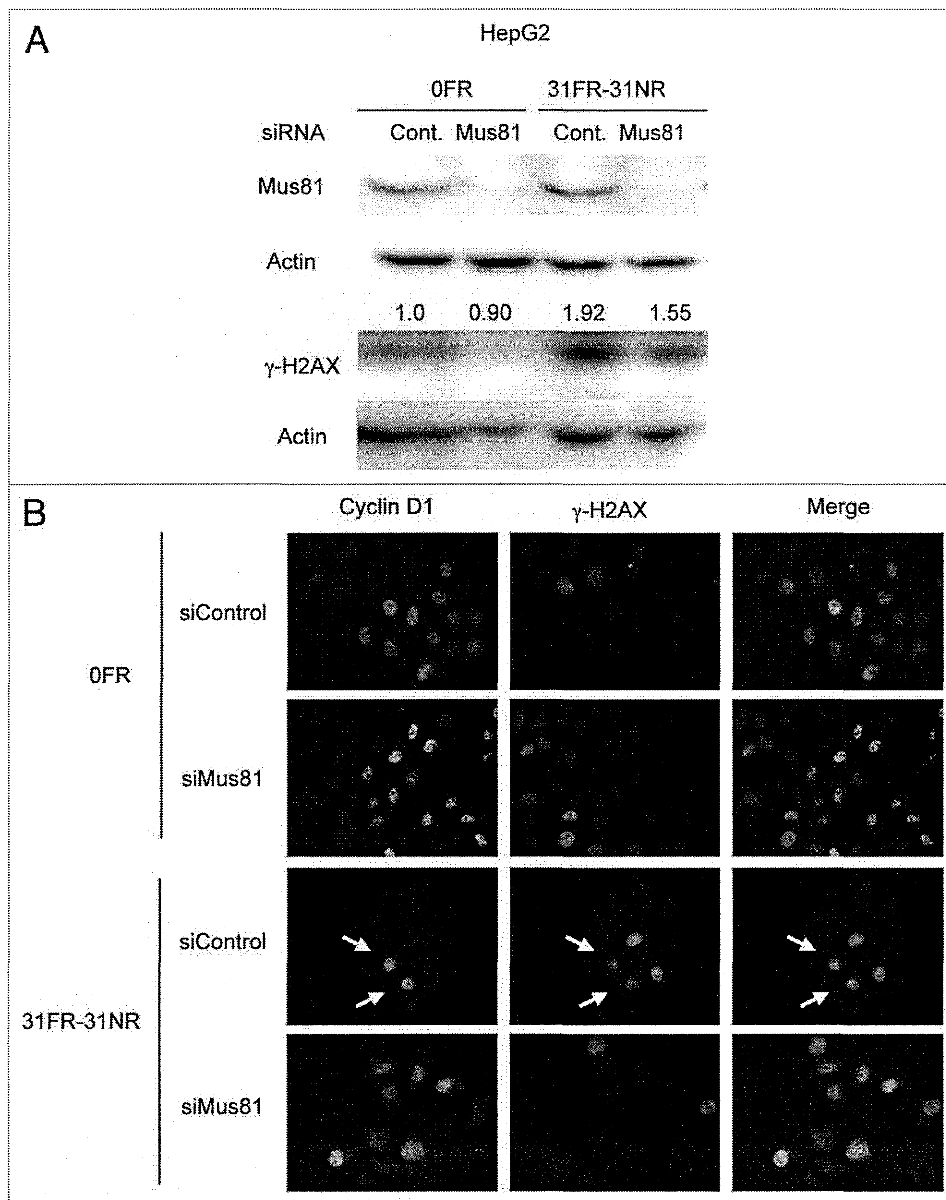


Figure 5. Mus81-mediated DSBs in 31FR-31NR cells. **(A)** Western blotting results for Mus81, γ-H2AX and actin in 0FR and 31FR-31NR cells derived from HepG2 cell line. Cell extracts were prepared at 24 h after transfection with either control siRNA (Cont.) or Mus81 siRNA. The amounts of γ-H2AX were normalized by corresponding actin level. The values are expressed relative to the control value of 0FR cells with control siRNA. **(B)** Cyclin D1 (green) and γ-H2AX (red) in 0FR cells and 31FR-31NR cells of HeLa are shown. Immunofluoresence were performed 48 h after transfection with control siRNA or Mus81 siRNA. Double-staining cells with cyclin D1 and γ-H2AX are indicated by arrows.

or an ATM inhibitor KU55236 did not affect 0FR cell survival; however, these inhibitors reduced the overall survival of the 31FR-31NR cells with cyclin D1-mediated DSBs (**Fig. 5B**). We also examined HeLa cell apoptosis using annexin V staining. After treatment with either the DNA-PK inhibitor or the ATM inhibitor, the proportions of the apoptotic 31FR-31NR cells increased, but that of the parental 0FR cells did not change. Thus, both these inhibitors could induce cell death only in 31FR-31NR cells with cyclin D1-mediated DSBs.

Discussion

Cyclin D1-mediated DSBs following long-term FR. We have been investigating the biological effects of long-term FR-induced cyclin D1 overexpression in human tumor cell lines. In response to long-term FR with a moderate dose, cyclin D1 behavior is completely different from that observed after SR with high doses. Cyclin D1 is degraded following SR and causes G_1/S arrest through inactivating Cdk4, while cyclin D1 is stabilized

©2013 Landes Bioscience. Do not distribute.

by downregulating its proteolysis via the DNA-PK/AKT/GSK3β pathway in response to long-term FR.

Long-term FR-induced cyclin D1 overexpression is mediated by its protein expression level and not by its mRNA expression level.[4] This epigenetic change in DNA-PK/AKT/GSK3β-mediated cyclin D1 overexpression is long-lasting, even after discontinuing FR for over 1 mo. In this study, we investigated the level of cyclin D1-mediated DSBs in 31FR-31NR cells using the neutral comet assay. Some of 31FR-31NR cells contained large amounts of DSBs, as shown by their high tail moment values compared with the values of the parental 0FR cells. Thus, DSBs are generated only at a specific stage of the cell cycle.

We previously reported that γ-H2AX-positive 31FR-31NR cells were also positive for IdU.[4] In this study, we showed that activation of the DNA damage sensor kinases DNA-PK and ATM was observed in PCNA-positive 31FR-31NR cells. Thus, DSBs were generated during DNA replication and activated DNA damage signaling pathways in 31FR-31NR cells. Cyclin D1 knockdown by siRNA decreased the amounts of DSBs in 31FR-31NR cells, whereas inactivation of Cdk4 by either siRNA or Cdk4-I had no effect. Therefore, DSB formation was mediated by cyclin D1 itself but not by cyclin D1/Cdk4 in 31FR-31NR cells.

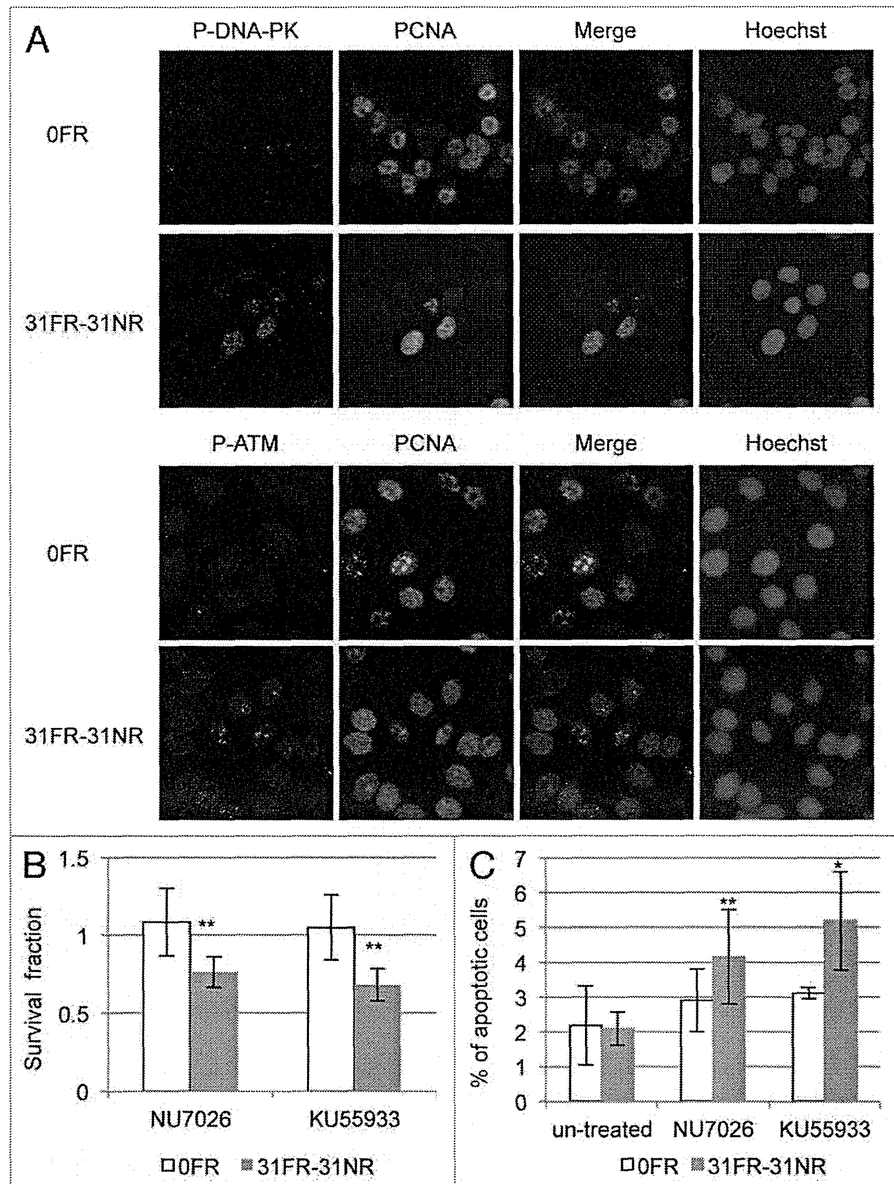


Figure 6. DDR and cell death in acquired radioresistant cells. **(A)** Double immunostaining with p-DNA-PK and PCNA in HepG2 cells is shown in the upper panel. Double immunostaining with p-ATM and PCNA in HeLa cells is shown in the lower panel. **(B)** Colony survival of HeLa cells treated with 10 μM NU7026 or 1 μM KU55933. Asterisk indicates significant sensitivity to drugs by 31FR-31NR cells compared with 0FR cells. **(C)** Percentage of annexin V-positive HeLa cells. The cells were treated with either NU7026 or KU55933. Asterisk indicates a significant difference in the frequency of apoptotic FR cells compared with 0FR cells.

Cyclin D1 overexpression suppresses replication fork progression. It has been reported that the accumulation of cyclin D1 in the nucleus loads replicative MCM helicase onto the chromatin and triggers DNA re-replication, which is required for Cdk4 activity.[24] Our results revealed that Cdk4 inactivation did not affect the amount of DSBs in the 31FR-31NR cells. Thus, cyclin D1-mediated DSBs are not induced by DNA re-replication in 31FR-31NR cells.

We also found that cyclin D1 overexpression perturbed DNA replication by downregulating replication fork progression. Cyclin D1 is associated with the replication factor PCNA, a clamp loader for DNA polymerase.[30-32] Thus, PCNA may recruit cyclin D1 to replication forks and the interaction between cyclin D1 and PCNA may prevent replication fork movement in 31FR-31NR cells. However, further studies will be needed to determine the molecular mechanisms underlying slowing down on replication fork progression triggered by cyclin D1 overexpression.

Replication-associated DSBs are created by Mus81 endonuclease to remove stalled replication forks. Oncogene-induced replication stress is associated with induction of genomic

©2013 Landes Bioscience. Do not distribute.

instability and acceleration of tumor progression.[21,22] The structure-specific Mus81/Eme1 complex is produced in response to this type of replication perturbation to control genomic stability during DNA replication.[23] Mus81 creates DSBs by cleaving DNA to resolve stalled replication forks that are induced by treatments with DNA synthesis inhibitors such as aphidicolin and hydroxyurea.[27-29] Our present results showed that Mus81 created DSBs in response to cyclin D1-mediated slowing down of replication forks in 31FR-31NR cells.

DSBs induced by radiation spread randomly over an entire genome and subsequently activate DNA damage signals involving DNA damage sensor kinases such as ATM and DNA-PK. In contrast, Mus81 cleaves aberrant fork structures to generate one-sided DSBs only at the sites of stalled replication forks.[21-23] Mus81-induced DSBs are thought to be repaired more easily than radiation-induced DNA damage, because these DSBs are created during homologous recombination repair (HRR).[27,29] Thus, cyclin D1-mediated DSBs are efficiently repaired in 31FR-31NR cells to promote the growth of these cells.

Eradication of acquired radioresistant cells by ATM and DNA-PK inhibition. Tumor radioresistance is one of the major obstacles in accomplishing complete cure of cancer with fractionated radiotherapy (RT).[33,34] Thus, it is important to identify molecular targets to suppress tumor radioresistance during cancer treatment. Our findings indicated that treatment with either a DNA-PK inhibitor or an ATM inhibitor efficiently induces apoptosis of acquired radioresistant cells harboring cyclin D1-mediated DSBs but does not affect apoptosis of parental cells. Thus, both of these inhibitors may affect acquired radioresistant cells without harmful side effects to normal cells.

In conclusion, this is the first study to demonstrate that cyclin D1 overexpression perturbs DNA replication by suppressing replication fork progression. DSBs could be induced by Mus81 for the recovery of cyclin D1-mediated slowing down of replication forks. The combination of fractionated RT with a DNA-PK inhibitor or an ATM inhibitor can suppress tumor radioresistance by eradicating acquired radioresistant cells and may improve outcomes with fractionated RT.

Materials and Methods

Cell culture condition and drugs. The human liver cancer cell line HepG2 and the human cervical cancer cell line HeLa were obtained from the Cell Resource Center for Biomedical Research (IDAC, Tohoku University). Cells were grown in RPMI 1640 medium (NacalaiTesque) supplemented with 5% heat-inactivated fetal calf serum. A Cdk4 inhibitor,[35] a DNA-PK inhibitor (NU7026) and an ATM inhibitor (KU55933) were purchased from Calbiochem. pFlex-cyclin D1 vectors[36] were introduced into HepG2 and HeLa cells, as described previously.[4]

Neutral comet assay. The neutral comet assays were performed using CometAssay kits (Trevigen) following the manufacturer's protocol, as described previously.[37] Images were captured with a CCD camera attached to a fluorescence microscope. The tail moment was determined by multiplying the fraction of DNA in the tail by the length of the tail.

RNA interference. Cells were transfected with siRNA using Lipofectamine RNAiMAX reagent (Invitrogen). Cyclin D1 and control siRNAs were purchased from Santa Cruz Biotechnology. Mus81 siRNA was purchased from Invitrogen. Cells were incubated with 40 nM of these siRNAs for 24 h. The medium was then removed and replaced with fresh medium for another 24 h. Neutral comet assay, cell cycle analysis, western blotting and immunofluoresence staining were performed 48 h after siRNA transfection.

Cell cycle analysis. Cell cycle analysis was performed as described previously.[37] The cells were pulse-labeled with 20 μM bromodeoxyuridine (BrdU) for 1 h, washed with PBS and then fixed in 70% ethanol overnight. BrdU-positive cells were quantified by a FACScan (Cytomics FC500, Becton Dickinson).

Western blot analyses. Western blotting was performed as described previously.[38] Histone extracts were prepared as described by Tung and Winn.[39] Proteins were separated by sodium-lauryl-sulfate-PAGE and transferred electrophoretically to PVDF membranes (Bio-Rad). The membranes were blocked with 5% (w/v) phospho-blocker (Cell Biolabs, Inc.) for 1 h and incubated with each primary antibody, including anti-β-actin (Sigma, A2066), anti-Cdk4 (Santa Cruz Biotechnology, SC-260), anti-cyclin D1 (Nichirei Bioscience) and anti-γ-H2AX (Upstate), either for 1 h at room temperature or overnight at 4°C. The membranes were then incubated for 1 h at room temperature with either HRP-conjugated goat anti-rabbit IgG (Nichirei Bioscience) or HRP-conjugated goat anti-mouse IgG (R&D Systems). Protein bands were visualized with Chemi-Lumi One L western blotting substrate (NacalaiTesquea). Band intensity was measured by densitometry using Image Lab Software (Bio-Rad).

Immunofluorescence staining. Immunofluorescence staining was performed as described previously.[28] Cells were seeded onto coverslips placed in 10 mm tissue culture dishes. The coverslips were fixed with ice-cold acetone (5 min), ice-cold methanol (5 min) and then washed twice with PBS. The cells were permeabilized and blocked for 30 min at room temperature in 5% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS. Anti-PCNA antibody (Mouse IgG, PC10; Oncogen) (Rabbit IgG, SC-7007; Santa Cruz), anti-ATM-phosphoserine 1981 (Rockland), anti-DNA-PKcs-phosphothreonine 2609 (Thermo Scientific), anti-cyclin D1 (Nichirei) and anti-γ-H2AX (Trevigen) were diluted in PBS with 0.5% BSA and incubated with the coverslips for 1 h. The coverslips were then washed three times with 0.1% Triton X-100 in PBS, incubated for 1 h with secondary antibodies conjugated with Alex 488 (Molecular Probes for mouse IgG) or Cy-3 (Jackson Immuno Research Laboratories, Inc. for mouse IgG). The coverslips were washed three times with 0.1% Triton X-100 in PBS, counterstained for DNA with 4, 6-diamidino-2-phenylindole (DAPI) (4 μg/ml prepared in Vectashield mouting medium; Vector Laboratories). Images were captured with a CCD camera attached to a fluorescence microscope.

DNA fiber analysis. DNA fiber analysis was performed as described previously.[38] Cells were labeled with 20 μM IdU for 10 min and then with 20 μM CldU for 20 min. The cells were trypsinized and resuspended in PBS at 1×10^6 cells/ml. The cells (2.5 μl) were then mixed with 7.5 μl lysis buffer (0.5% SDS in

©2013 Landes Bioscience. Do not distribute.

200 mM TRIS-HCl, pH 7.4, 50 mM ethylene diaminetetraacetic acid) on a glass slide. After 8 min, DNA spreads were fixed in 3:1 methanol/acetic acid and then stored in 70% ethanol at 4°C. CldU and IdU staining used a previously described protocol.[40] The length of fork extension was studied during second (green CldU, 20 min) labeling period. Signals were measured by using Photoshop software (Adobe Systems). The results of the analyses on 50 tracks are shown.

Clonogenic assay. Cells were treated with NU7026 or KU55933 for 24 h. They were then seeded in 60-mm dishes coated with 0.1% gelatin (Wako) at 1×10^3 cells per dish and incubated for 10 d until colonies were visible. Colonies were fixed with ethanol for 30 min and stained with Giemsa solution (Merck and Co., Inc.). Colonies with > 50 cells were counted under a light microscope (Olympus, SZX10).

Annexin V staining. Apoptotic cells were identified and quantified using the annexin V-FITC apoptosis detection kit (Bio Vision) following the manufacturer's protocol. Cells were stained with annexin V-FITC and propidium iodide at 72 h after treatment with NU7026 or KU55933. Annexin V-positive apoptotic cells were analyzed by FACScan (Becton Dickinson).

Statistical analysis. All experiments were repeated at least three times with independent samples. Results are given as means + standard deviations. Group comparisons were made by Student's t-test. A single asterisk and double asterisks indicate significance at $p < 0.01$ and $p < 0.05$, respectively.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This study was supported in part by research grants from the Japanese Ministry of Education and Science Kiban C (24510063), the Mishima Kaiun Memorial Foundation of Japan and grant for Cancer Research from National Cancer Institute (Japan).

References

1. Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. Science 1989; 246:629-34; PMID:2683079; http://dx.doi.org/10.1126/science.2683079
2. Szumiel I. Ionizing radiation-induced cell death. Int J Radiat Biol 1994; 66:329-41; PMID:7930835; http://dx.doi.org/10.1080/09553009414551271
3. Agami R, Bernards R. Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell 2000; 102:55-66; PMID:10929713; http://dx.doi.org/10.1016/S0092-8674(00)00010-6
4. Shimura T, Kakuda S, Ochiai Y, Nakagawa H, Kuwahara Y, Takai Y, et al. Acquired radioresistance of human tumor cells by DNA-PK/AKT/GSK3beta-mediated cyclin D1 overexpression. Oncogene 2010; 29:4826-37; PMID:20562919; http://dx.doi.org/10.1038/onc.2010.238
5. Sherr CJ, McCormick F. The RB and p53 pathways in cancer. Cancer Cell 2002; 2:103-12; PMID:12204530; http://dx.doi.org/10.1016/S1535-6108(02)00102-2
6. Giacinti C, Giordano A. RB and cell cycle progression. Oncogene 2006; 25:5220-7; PMID:16936740; http://dx.doi.org/10.1038/sj.onc.1209615
7. Kolupaeva V, Basilico C. Overexpression of cyclin E/CDK2 complexes overcomes FGF-induced cell cycle arrest in the presence of hypophosphorylated Rb proteins. Cell Cycle 2012; 11:2557-66; PMID:22713240; http://dx.doi.org/10.4161/cc.20944
8. Kolupaeva V, Laplantine E, Basilico C. PP2A-mediated dephosphorylation of p107 plays a critical role in chondrocyte cell cycle arrest by FGF. PLoS ONE 2008; 3:e3447; PMID:18927618; http://dx.doi.org/10.1371/journal.pone.0003447
9. Hanse EA, Mashek DG, Becker JR, Solmonson AD, Mullany LK, Mashek MT, et al. Cyclin D1 inhibits hepatic lipogenesis via repression of carbohydrate response element binding protein and hepatocyte nuclear factor 4α. Cell Cycle 2012; 11:2681-90; PMID:22751438; http://dx.doi.org/10.4161/cc.21019
10. Sladek FM. The yin and yang of proliferation and differentiation: cyclin D1 inhibits differentiation factors ChREBP and HNF4α. Cell Cycle 2012; 11:3156-7; PMID:22895168; http://dx.doi.org/10.4161/cc.21721
11. Bartkova J, Lukas J, Strauss M, Bartek J. The PRAD-1/cyclin D1 oncogene product accumulates aberrantly in a subset of colorectal carcinomas. Int J Cancer 1994; 58:568-73; PMID:8056453; http://dx.doi.org/10.1002/ijc.2910580420
12. Bartkova J, Lukas J, Müller H, Lützhøft D, Strauss M, Bartek J. Cyclin D1 protein expression and function in human breast cancer. Int J Cancer 1994; 57:353-61; PMID:8168995; http://dx.doi.org/10.1002/ijc.2910570311
13. Bani-Hani K, Martin IG, Hardie LJ, Mapstone N, Briggs JA, Forman D, et al. Prospective study of cyclin D1 overexpression in Barrett's esophagus: association with increased risk of adenocarcinoma. J Natl Cancer Inst 2000; 92:1316-21; PMID:10944553; http://dx.doi.org/10.1093/jnci/92.16.1316
14. Casimiro MC, Pestell RG. Cyclin d1 induces chromosomal instability. Oncotarget 2012; 3:224-5; PMID:22538871
15. Leontieva OV, Lenzo F, Demidenko ZN, Blagosklonny MV. Hyper-mitogenic drive coexists with mitotic incompetence in senescent cells. Cell Cycle 2012; 11:4642-9; PMID:23187803; http://dx.doi.org/10.4161/cc.22937
16. Hitomi M, Stacey DW. Cyclin D1 production in cycling cells depends on ras in a cell-cycle-specific manner. Curr Biol 1999; 9:1075-84; PMID:10531005; http://dx.doi.org/10.1016/S0960-9822(99)80476-X
17. Filmus J, Robles AI, Shi W, Wong MJ, Colombo LL, Conti CJ. Induction of cyclin D1 overexpression by activated ras. Oncogene 1994; 9:3627-33; PMID:7970723
18. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002; 2:489-501; PMID:12094235; http://dx.doi.org/10.1038/nrc839
19. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007; 129:1261-74; PMID:17604717; http://dx.doi.org/10.1016/j.cell.2007.06.009
20. Shimura T. Acquired radioresistance of cancer and the AKT/GSK3β/cyclin D1 overexpression cycle. J Radiat Res 2011; 52:539-44; PMID:21881296; http://dx.doi.org/10.1269/jrr.11098
21. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 2006; 444:633-7; PMID:17136093; http://dx.doi.org/10.1038/nature05268
22. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 2006; 444:638-42; PMID:17136094; http://dx.doi.org/10.1038/nature05327
23. Murfuni I, Nicolai S, Baldari S, Crescenzi M, Bignami M, Franchitto A, et al. The WRN and MUS81 proteins limit cell death and genome instability following oncogene activation. Oncogene 2012; PMID:22410776
24. Aggarwal P, Lessie MD, Lin DI, Pontano L, Gladden AB, Nuskey B, et al. Nuclear accumulation of cyclin D1 during S phase inhibits Cul4-dependent Cdt1 proteolysis and triggers p53-dependent DNA rereplication. Genes Dev 2007; 21:2908-22; PMID:18006686; http://dx.doi.org/10.1101/gad.1586007
25. Tane S, Chibazakura T. Cyclin A overexpression induces chromosomal double-strand breaks in mammalian cells. Cell Cycle 2009; 8:3900-3; PMID:19901524; http://dx.doi.org/10.4161/cc.8.23.10071
26. Li Z, Jiao X, Wang C, Shirley LA, Elsaleh H, Dahl O, et al. Alternative cyclin D1 splice forms differentially regulate the DNA damage response. Cancer Res 2010; 70:8802-11; PMID:20940395; http://dx.doi.org/10.1158/0008-5472.CAN-10-0312
27. Hanada K, Budzowska M, Davies SL, van Drunen E, Onizawa H, Beverloo HB, et al. The structure-specific endonuclease Mus81 contributes to replication restart by generating double-strand DNA breaks. Nat Struct Mol Biol 2007; 14:1096-104; PMID:17934473; http://dx.doi.org/10.1038/nsmb1313
28. Shimura T, Torres MJ, Martin MM, Rao VA, Pommier Y, Katsura M, et al. Bloom's syndrome helicase and Mus81 are required to induce transient double-strand DNA breaks in response to DNA replication stress. J Mol Biol 2008; 375:1152-64; PMID:18054789; http://dx.doi.org/10.1016/j.jmb.2007.11.006
29. Regairaz M, Zhang YW, Fu H, Agama KK, Tata N, Agrawal S, et al. Mus81-mediated DNA cleavage resolves replication forks stalled by topoisomerase I-DNA complexes. J Cell Biol 2011; 195:739-49; PMID:22123861; http://dx.doi.org/10.1083/jcb.201104003
30. Fukami-Kobayashi J, Mitsui Y. Cyclin D1 inhibits cell proliferation through binding to PCNA and cdk2. Exp Cell Res 1999; 246:338-47; PMID:9925749; http://dx.doi.org/10.1006/excr.1998.4306
31. Prosperi E, Scovassi AI, Stivala LA, Bianchi L. Proliferating cell nuclear antigen bound to DNA synthesis sites: phosphorylation and association with cyclin D1 and cyclin A. Exp Cell Res 1994; 215:257-62; PMID:7982468; http://dx.doi.org/10.1006/excr.1994.1341
32. Xiong Y, Zhang H, Beach D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 1992; 71:505-14; PMID:1358458; http://dx.doi.org/10.1016/0092-8674(92)90518-H

©2013 Landes Bioscience. Do not distribute.

33. Kim JJ, Tannock IF. Repopulation of cancer cells during therapy: an important cause of treatment failure. Nat Rev Cancer 2005; 5:516-25; PMID:15965493; http://dx.doi.org/10.1038/nrc1650
34. Nguyen GH, Murph MM, Chang JY. Cancer Stem Cell Radioresistance and Enrichment: Where Frontline Radiation Therapy May Fail in Lung and Esophageal Cancers. Cancers (Basel) 2011; 3:1232-52; PMID:21603589; http://dx.doi.org/10.3390/cancers3011232
35. Bommi-Reddy A, Almeciga I, Sawyer J, Geisen C, Li W, Harlow E, et al. Kinase requirements in human cells: III. Altered kinase requirements in VHL-/- cancer cells detected in a pilot synthetic lethal screen. Proc Natl Acad Sci USA 2008; 105:16484-9; PMID:18948595; http://dx.doi.org/10.1073/pnas.0806574105
36. Diehl JA, Zindy F, Sherr CJ. Inhibition of cyclin D1 phosphorylation on threonine-286 prevents its rapid degradation via the ubiquitin-proteasome pathway. Genes Dev 1997; 11:957-72; PMID:9136925; http://dx.doi.org/10.1101/gad.11.8.957
37. Shimura T, Martin MM, Torres MJ, Gu C, Pluth JM, DeBernardi MA, et al. DNA-PK is involved in repairing a transient surge of DNA breaks induced by deceleration of DNA replication. J Mol Biol 2007; 367:665-80; PMID:17280685; http://dx.doi.org/10.1016/j.jmb.2007.01.018
38. Shimura T, Toyoshima M, Adiga SK, Kunoh T, Nagai H, Shimizu N, et al. Suppression of replication fork progression in low-dose-specific p53-dependent S-phase DNA damage checkpoint. Oncogene 2006; 25:5921-32; PMID:16682953; http://dx.doi.org/10.1038/sj.onc.1209624
39. Tung EW, Winn LM. Valproic acid-induced DNA damage increases embryonic p27(KIP1) and caspase-3 expression: a mechanism for valproic-acid induced neural tube defects. Reprod Toxicol 2011; 32:255-60; PMID:21708246; http://dx.doi.org/10.1016/j.reprotox.2011.05.020
40. Dimitrova DS, Gilbert DM. The spatial position and replication timing of chromosomal domains are both established in early G1 phase. Mol Cell 1999; 4:983-93; PMID:10635323; http://dx.doi.org/10.1016/S1097-2765(00)80227-0

©2013 Landes Bioscience. Do not distribute.

Cell Cycle 12:17, 2738–2743; September 1, 2013; © 2013 Landes Bioscience

The role of cyclin D1 in response to long-term exposure to ionizing radiation

Tsutomu Shimura,[1,*] Manabu Fukumoto[2], and Naoki Kunugita[1]

[1]Department of Environmental Health; National Institute of Public Health 2-3-6 Minami; Wako, Saitama, Japan; [2]Department of Pathology; Institute of Development, Aging, and Cancer; Tohoku University; Sendai, Japan

Keywords: Cyclin D1, long-term fractionated radiation, AKT, double-strand breaks, radioprotection

The health-related hazards resulting from long-term exposure to radiation remain unknown. Thus, an appropriate molecular marker is needed to clarify these effects. Cyclin D1 regulates the cell cycle transition from the G1 phase to the S phase. Cyclin D1 is degraded as a G1/S checkpoint after 10 Gy of single acute radiation exposure, whereas conversely, cyclin D1 is stabilized when human tumor cells are exposed to fractionated radiation (FR) with 0.5 Gy of x-rays for 31 d. In this article, we review new findings regarding cyclin D1 overexpression in response to long-term exposure to FR. Cyclin D1 overexpression is associated with induction of genomic instability in irradiated cells. Therefore, repression of cyclin D1 expression is likely to cancel the harmful effects of long-term exposure to FR. Thus cyclin D1 may be a marker of long-term exposure to radiation and is a putative molecular radioprotection target for radiation safety.

Introduction

Ionizing radiation (IR) can induce various types of DNA damage, such as DNA base damage, DNA single-strand breaks (SSBs), and DNA double-strand breaks (DSBs). Radiation transfers energy to DNA and disrupts DNA chemical bonds, which results in direct induction of DNA damage. Radiation also affects water and causes the generation of reactive oxygen species (ROS) that react with DNA. In this case, radiation indirectly induces DNA damage via ROS production. Gamma irradiation or X-rays (1 Gy) can damage 500 DNA bases and create 1000 SSBs and 40 DDBs per cell.[1,2] The most important biological consequence of IR is believed to be DSBs, which can trigger genomic instability in cells. For example, chromosomal aberrations, such as deletions, insertions, or translocations, can occur even after DSB repair.

Mammalian cells harbor a series of DNA damage responses (DDRs) that are induced after radiation in order to maintain genomic stability. The molecular mechanisms involved with DDRs have been thoroughly investigated using single radiation (SR) exposure regimes. DNA damage sensor kinases, ataxia telangiectasia mutated protein (ATM), and DNA-dependent protein kinase (DNA-PK) recognize DNA lesions and transfer these DNA damage signals to transducers, such as p53, checkpoint kinase1 (Chk1), and checkpoint kinase2 (Chk2). The G_1/S checkpoint, which regulates the entry into the S phase in the presence of DSBs, functions by inactivating cyclin-dependent kinases (Cdks) via the degradation of positive regulators of Cdks, such as cyclin D1 and Cdc25A, and by activating a negative regulator such as p21.[3-6]

Cyclin D1 degradation occurs rapidly after irradiation and results in the release of p21 in a p53-independent manner.[3] Thus, cell cycle progression ceases at the G_1/S boundary by suppressing Cdks activity. Cell cycle checkpoints block cell cycle progression in the presence of DSBs to achieve adequate DNA damage repair after irradiation. After repairing DNA damage, the cells resume cell cycling by activating Cdks. On the other hand, cell death is induced in order to exclude abnormal cells that have been exposed to high doses of radiation. Induction of apoptosis after exposure to high doses of radiation is associated with mitochondrial transmembrane potential collapse, caspase activation, and DNA degradation.[7,8]

In order to analyze the biological effect of long-term exposure to radiation, human cells were exposed to fractionated radiation (FR) with 0.5-Gy x-ray fraction at dose rate of 1 Gy per minutes twice per day, 6 d a week for 31 d (**Fig. 1**). ATM and DNA-PK were constitutive activated by FR for 31 d.[9] The cells treated with these exposure regimes were referred to as 31FR cells. A total dose of 27 Gy was delivered to cells for 31 d (**Fig. 1**). We further established 31FR-31NR cells by 31-d FR exposure followed by 31-d non-FR. We recently identified a unique DDR involving cyclin D1 in 31FR and 31FR-31NR cells.[9,10] Cells acquired radioresistance because of cyclin D1 overexpression by downregulating the cyclin D1 degradation pathway after exposure to 31 d FR. Glycogen synthase kinase 3β (GSK3β) is a protein kinase that phosphorylates cyclin D1 on threonine 286 (Thr286) to facilitate its degradation.[11] AKT-mediated phosphorylation of GSK3β on serine 9 decreases its kinase activity on cyclin D1 Thr286, which inhibits the nuclear export and cytoplasmic proteasomal degradation of cyclin D1.[12,13] Constitutive activation of the AKT pro-survival pathway in 31FR cells resulted in downregulation of cyclin D1 proteolysis. Consequently, 31-d FR conferred radioresistance to tumor cells by cyclin D1 overexpression, which

*Correspondence to: Tsutomu Shimura; Email: tsimura@niph.go.jp
Submitted: 06/13/2013; Revised: 07/11/2013; Accepted: 07/13/2013
http://dx.doi.org/10.4161/cc.25746

©2013 Landes Bioscience. Do not distribute.

was mediated by the AKT/GSK3β pathway.[9,10] Thus, cyclin D1 could be used as a marker of long-term exposure to radiation but not of SR exposure.

This paper reviews a novel attractive DDR involving cyclin D1 in 31FR cells. Deregulation of cyclin D1 expression generates DSBs during DNA replication and is associated with induction of genomic instability in cells. Therefore, cyclin D1 may serve not only as a marker of long-term exposure to radiation, but may also be a molecular target to reduce the effects of radiation exposure for radioprotection.

Cyclin D1 Expression during Cell Cycling

Cyclin D1 is a positive cell cycle regulator during the G_1/S transition. Cyclin D1 expression is regulated both at the transcriptional and post-translational levels. Cyclin D1 expression is upregulated by mitogenic signaling through the Ras-signaling pathway involving Ras/Raf/mitogen-activated protein (extracellular signal-regulated kinase [ERK] kinase) (MEK)/ERK.[14] Cyclin D1 levels are also post-translationally regulated by its degradation through the following ubiquitin-proteasome pathway.

Phosphorylation of the cyclin D1 Thr286 residue facilitates the nuclear exclusion of cyclin D1 by the nuclear exporter chromosome maintenance region 1 (CRM1), and then it is degraded. When stimulated by insulin or a growth factor, phosphatidylinositol-3-OH kinase (PI3K) generates phosphatidylinositol-3,4,5-triphosphate, a lipid messenger that is essential for the translocation of AKT (protein kinase B) to the plasma membrane, where it is phosphorylated and activated by 3-phosphoinositide-dependent kinase 1.[15] Activated AKT phosphorylates the GSK3β serine 9 residue to inactivate its kinase activity on the cyclin D1 Thr286 residue. This blocks cyclin D1 nuclear export, and cytoplasmic cyclin D1 subsequently undergoes proteasomal degradation.[16] Thus, cyclin D1 accumulates in the nucleus as a result of PI3K- and AKT-mediated GSK3β inactivation.

Cyclin D1 mediates the G_1/S transition by binding to Cdk4. Cyclin D1/Cdk4 phosphorylates Rb, and E2F is then released to transactivate genes required for the G_1/S transition.[17] In addition, cyclin D1 plays a role in the G_1/S transition by sequestering a Cdk inhibitor of p21 and p27.[18,19]

Cyclin D1 levels vary throughout the cell cycle; cyclin D1 levels increase in the nucleus during the G_1 phase and decline because of cyclin D1 nuclear exclusion when cells enter the S phase concomitant with an increase in GSK3β levels.[20] Because cyclin D1 degradation during the S phase is essential for cell cycling, deregulating cyclin D1 expression during the S phase perturbs DNA replication.[21,22]

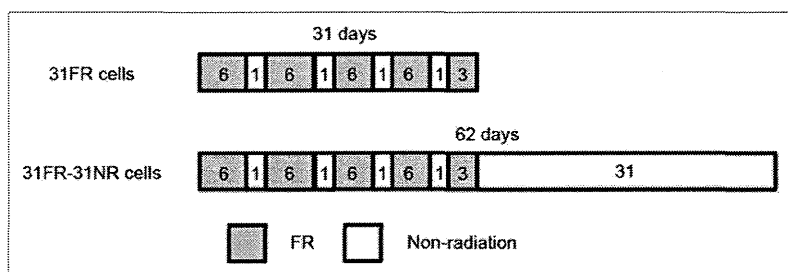


Figure 1. A fraction schedule to establish 31FR and 31FR-31NR cells. Number described in the box indicates the day. Cells were exposed to FR 6 d a week for 31 d to establish 31FR cells. Cells were further cultured without FR for 31 d to make 31FR-31NR cells.

Cyclin D1 Regulation after Irradiation

After exposure to 10 Gy or 20 Gy of SR, cyclin D1 undergoes ubiquitin-proteasome degradation for a G_1/S checkpoint and prevents entry of irradiated cells into the S phase.[3,9] ATM signaling has been shown to play a critical role in regulating cyclin D1 degradation in response to DNA damage via F-box protein 31 (FBXO31), best known for their role as the substrate recognition components of the SCF (SKP/Cullin/F-box protein) class of E3 ubiquitin ligases. ATM directly phosphorylates FBXO31 to facilitate cyclin D1 degradation.[23] *CCND1* (Cyclin D1 gene) expression is also downregulated following irradiation by inhibiting CREB binding protein (CBP)/p300 histone acetyltransferase (HAT) activities via binding of an RNA binding protein, translocated in liposarcoma (TLS), that contains non-coding RNAs.[24] Cdks inactivation by downregulating cyclin D1 expression results in Rb dephosphorylation, which then sequesters E2F to prevent its transactivating activity and to arrest cells at the G_1/S boundary.

Conversely, cyclin D1 is stabilized in HepG2 and HeLa cells after exposure to 0.5 Gy of FR for 31 d, which results in cyclin D1 overexpression.[9] The *CCND1* mRNA levels were not dramatically different before and after 31-d FR.[9] Therefore, 31 d FR-induced cyclin D1 overexpression was not due to some genetic change, such as gene amplification, but was due to the decreased protein degradation mediated by the AKT signaling pathway. AKT, a positive regulator of cyclin D1, is constitutively activated when cells are exposed to FR for >14 d (total dose is 12 Gy).[9] In contrast, transient AKT activation has been reported in HepG2, HeLa, and human umbilical vein endothelial cells after 2 or 3 Gy of SR.[9,25]

Collectively, these results suggest that AKT pro-survival signals accumulate under the situation of constitutive activation of DNA-PK and ATM due to repeated radiation exposures. There is a threshold for the changes in the AKT radioresponse from a transient activation pattern to a constitutive activation pattern approximately 14 d of FR (**Fig. 2**). AKT activation and GSK3β inactivation precede cyclin D1 overexpression, because cyclin D1 overexpression is evident 31 d after FR. In addition, pro-survival signaling via the AKT/ERK pathway is activated at lower DSB levels (<2 Gy) but not at higher DSB levels (>2 Gy).[26] Thus, the AKT pro-survival signaling pathway varies according to the magnitude of the irradiated dose and the duration of radiation exposure.

DNA-PK activates AKT in response to various genotoxic stresses, including low doses of radiation,[27] and is the upstream target of the AKT pathway in 31FR cells.[9] This epigenetic

©2013 Landes Bioscience. Do not distribute.

change in the DNA damage signaling pathway with DNA-PK/AKT/GSK3β-mediated cyclin D1 overexpression is irreversible, even after discontinuing FR for >1 mo. Cyclin D1-T286A that is mutated at the phosphorylation site on Thr286 resists radiation-induced cyclin D1 degradation by the ATM-FBXO31 pathway.[23] This demonstrated that AKT-mediated cyclin D1 dephosphorylation on Thr286 invalidated ATM/FBXO31-mediated cyclin D1 degradation after 31 d FR.

Establishment of a Positive Feedback Loop through the DNA-PK/AKT/GSK3β/Cyclin D1 Pathway by Replication-Associated DSBs Triggered by Cyclin D1 Overexpression

We previously reported that downregulation of cyclin D1 degradation resulted in persistent cyclin D1 expression during the S phase of 31FR cells.[9] Deregulation of cyclin D1 expression perturbed DNA replication by inhibiting replication fork progression.[22] Cyclin D1 has been shown to bind with the replication factor PCNA, a clamp loader of DNA polymerase.[28-30] PCNA may recruit cyclin D1 to replication forks, and cyclin D1 binding to PCNA may inhibit replication fork movement in 31FR cells (**Fig. 3**). In response to aberrant replication forks induced by treatment with low-dose aphidicolin, an inhibitor of DNA polymerase α, DSBs were made by BLM helicase in cooperation with Mus81 nuclease for recovery.[31] We also found that Mus81 controlled DSB formation in 31FR cells (**Fig. 3**).[22] Using siRNAs, cyclin D1 or Mus81 knockdown decreased the amounts of DSBs in cyclin D1-overexpressing cells, whereas Cdk4 inactivation by siRNA or a CDK4 inhibitor of Cdk4-I had no effect.[9,22] These results demonstrated that DSBs were mediated by cyclin D1 itself and did not require the activity of cyclin D1/Cdk4.

In contrast, overexpressing cyclin D1 using a cyclin D1 expression vector triggered DNA re-replication and induced DSBs in a Cdk4-dependent manner.[21] Therefore, FR-induced DSBs were not induced by DNA re-replication, but were induced by suppressing replication fork movement. In addition to cyclin D1, cyclin A overexpression induces DSBs in human and mouse fibroblasts.[32] Cyclin D1-mediated DSBs are also associated with induction of genomic instability in normal cells and increases cancer risk.

Figure 4 shows a schematic representation of DDR involving cyclin D1 in response to SR or 31 d FR. After SR with high doses, cyclin D1 is degraded by the ATM/FBXO31 pathway to inactivate Cdks. Cells are arrested at the G_1/S boundary because of activation of a G_1/S checkpoint. In contrast, cyclin D1 is stabilized after FR for 31 d by DNA-PK/AKT-mediated downregulation of its proteolysis. Persistent cyclin D1 expression during the S phase perturbs DNA replication and induces DSBs during the processing of abnormal DNA replication forks (**Fig. 3**). Cyclin D1-induced DSBs again activate the DNA-PK/AKT pathway, thus establishing a positive feedback loop of cyclin D1 overexpression cycle. Cyclin D1-induced DSBs are also associated with genomic instability and tumorigenesis in 31FR cells.

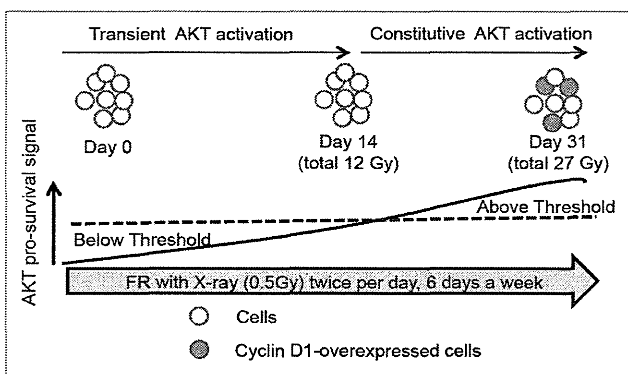


Figure 2. AKT radioresponse after 31-d FR. AKT pro-survival signals accumulate during exposure to FR. When these signals cross a threshold, the AKT response is changed from transient activation to constitutive activation after irradiation. Cyclin D1 is overexpressed in 31FR cells in which AKT is constitutively activated because of cumulative AKT pro-survival signals.

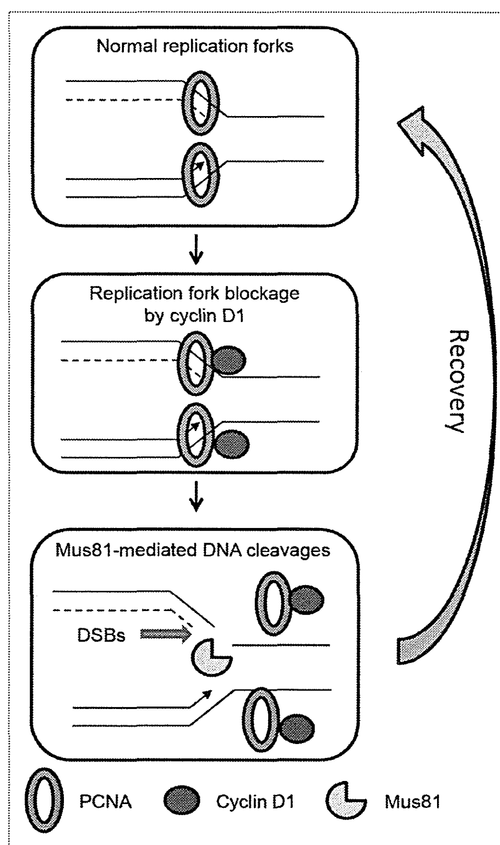


Figure 3. Induction of DSBs during the recovery of cyclin D1-mediated aberrant replication forks. PCNA, a clamp loader, is involved in the formation of the replication fork complex and regulates replication fork progression. Cyclin D1 binding to PCNA in a replication fork complex inhibits replication fork progression. Mus81 creates DSBs at a replication site to remove aberrant replication forks for the recovery from replication stress mediated by cyclin D1 overexpression. Replication forks are then reconstructed to resume DNA replication.

©2013 Landes Bioscience. Do not distribute.

Cyclin D1 as a Marker of Long-Term Exposure to Radiation

A biomarker is an indicator of a biological state that is defined as any measurement reflecting an interaction between a biological system and a genotoxic stress and is used to evaluate biological effects.[33] Chromosomal aberrations are commonly used as IR biomarkers for radiation dose estimations, although the detection limit with this assay is <100 mGy. Radiation-induced DSBs are assessed by phosphorylation of H2AX (γ-H2AX), which can be detected with <20 mGy.[34] However, it should be noted that DSBs are not unique to IR and can be induced by aging, oxidative stress, smoking, and certain chemicals. In addition, γ-H2AX has a disadvantage as a biomarker of long-term exposure to radiation, because it disappears when DSBs are repaired. Thus, another stable biomarker is needed to evaluate the effects of long-term exposure to radiation.

We are investigating suitable IR biomarkers from among molecules that are associated with DDRs of FR. Cyclin D1 is one candidate marker of FR, because cyclin D1 overexpression occurs after 31 d FR but not after SR and is long lasting even after FR is stopped. Further evaluation is needed to determine if cyclin D1 can be used as a biomarker of FR, especially by using normal human cells after long-term exposure to low doses of radiation.

A Role of Cyclin D1 Overexpression

Genetic aberrations in cell cycle regulators are frequently noted in human cancer and are linked to cancer development.[35,36] Cyclin D1 overexpression is one of the most commonly observed alterations involving chromosomal translocations, gene amplification, and polymorphisms in various types of cancer cells.[37] Cyclin D1 plays a role in controlling the transcription of several genes that regulate cell differentiation and proliferation by modulating the activities of transcription factors, co-activators, and co-repressors.[38,39] Cyclin D1 is recruited to chromatin along with chromatin remodeling factors and alters acetylation and methylation of histones, which modulates the accessibility of transcription factors to chromatin. Cyclin D1 overexpression induces chromosomal instabilities, by interfering with the transcription of genes that govern the mitotic phase, and results in tumorigenesis.[40,41]

Overexpression of wild-type cyclin D1 has been shown to be insufficient for inducing cell transformation, as nuclear export and subsequent cytoplasmic proteolysis reduce the oncogenic potential of this protein.[16,42] In contrast, overexpression of non-degradable cyclin D1-T286A results in tumorigenesis in transgenic mice.[16,43-45] Thus, nuclear accumulation of cyclin D1 is associated with tumor-initiation event. Oncogene activation results in constitutive activation of the ATM-regulated DDR.[46] ATM and DNA-PK activation due to cyclin D1 overexpression was also observed in 31FR and 31FR-31NR cells.[9,22] Oncogene activation perturbs DNA replication and causes DSB accumulation because of replication stress and genomic instability in non-malignant cells.[47-49] Therefore, cyclin D1 nuclear accumulation after exposure to 31 d FR possibly leads to the progression of malignancy in normal cells caused by the induction of genomic instability triggered by cyclin D1-dependent DSBs. On the other hand, cyclin D1 overexpression is considered as a marker of cellular senescence. It was reported that senescent cells express high levels of cyclin D1 in normal human fibroblasts.[50-53]

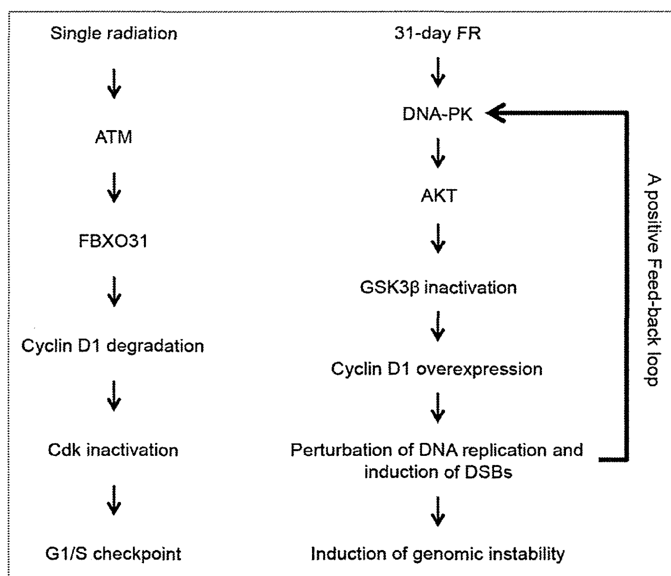


Figure 4. Cyclin D1 overexpression cycling in response to 31-d FR. After SR, cyclin D1 degradation causes Cdks inactivation as a G_1/S checkpoint. In contrast, DNA-PK/AKT/GSK3β-mediated cyclin D1 overexpression results in perturbation of DNA replication and induction of DSBs. Cyclin D1-dependent DSBs establish a positive feedback loop for cyclin D1 overexpression cycling and the subsequent induction of genomic instability in 31FR cells.

Targeting Cyclin D1 for Radioprotection

Accumulating evidence suggests that the PI3K/AKT signaling pathway regulates cell proliferation and survival processes that contribute to tumor progression.[12,13,15] This pathway is upregulated after irradiation and is strongly correlated with radiosensitivity of irradiated cells. AKT can block apoptotic pathways by regulating various target molecules, including pro-apoptotic and anti-apoptotic proteins.[15,54,55] Activated AKT, a common mediator of cell survival signals induced by radiation through multiple intracellular signaling pathways, modulates apoptosis and increases the apoptotic threshold.[56,57] Thus, constitutive activation of the AKT/GSK3β/cyclin D1 pathway due to the accumulation of AKT survival signals results in radioresistance of 31FR cells.[9]

Cyclin D1 is considered a potential therapeutic target for cancer treatment.[58,59] Aberrant cyclin D1 expression is often detected in premalignant and malignant tissues. Cyclin D1 overexpression is strongly correlated with a poor prognosis in oral carcinoma and head and neck squamous cell carcinoma after radiotherapy or chemoradiotherapy.[60,61] Cyclin D1 levels are believed to be worth monitoring during the course of treatment to assess clinical responses. In addition to being a molecular target for cancer treatment, cyclin D1 is a selective molecular marker of long-term exposure to radiation in

©2013 Landes Bioscience. Do not distribute.

irradiated cells, where genomic instability is induced by cyclin D1 overexpression. API-2 was originally identified as a highly selective AKT inhibitor during screening by the National Cancer Institute and is considered an anticancer drug.[62] Phase I and II clinical trials for API-2 have been conducted for advanced tumors.[63,64] We also confirmed the efficacy of API-2 for suppressing the radioresistance of tumors derived from HepG2 and HeLa cells in animal experiments.[65] We previously showed that inactivation of AKT/GSK3β-mediated cyclin D1 overexpression using an AKT inhibitor of API-2 abrogated cyclin D1 overexpression and rendered 31FR cells susceptible to radiation with increased apoptosis.[9,65] Because API-2 facilitates GSK3β-mediated cyclin D1 proteolysis in 31FR and 31FR-31NR cells,[9] it also can suppress tumoregenesis that is induced by cyclin D1 overexpression in normal cells. Therefore, targeting cyclin D1 is likely to cancel the effects of long-term exposure to FR that are caused by cyclin D1 overexpression.

Conclusions

We have described a unique DDR involving cyclin D1 after 31 d FR and its biological effects. Cyclin D1 overexpression generates DSBs during DNA replication and is associated with tumor initiation by inducing genomic instability in cells. Determination of cyclin D1 expression is proved to be a marker for monitoring long-term exposure of radiation. Furthermore, cyclin D1 is a molecular target for reducing the cancer risk posed by 31 d FR for radioprotection of humans.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Prof Satish Kumar Adiga for his critical reading of the paper. The authors were supported by research grants from the Japanese Ministry of Education and Science Kiban C (24510063). This work was conducted at the Joint Usage/ Research Center (RIRBM), Hiroshima University.

Sources of Support

Research grants from the Japanese Ministry of Education and Science Kiban C (24510063). This work was performed at the Joint Usage/ Research Center (RIRBM), Hiroshima University.

References

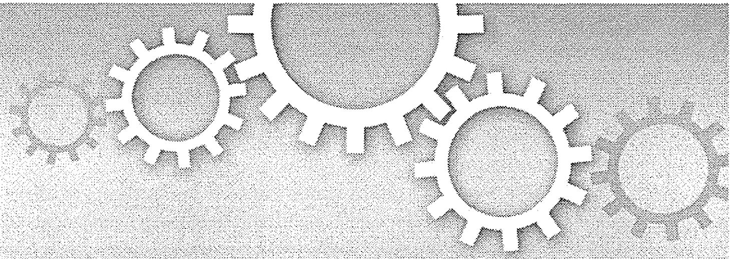
1. Elkind MM. Repair processes in radiation biology. Radiat Res 1984; 100:425-49; PMID:6390488; http://dx.doi.org/10.2307/3576409
2. Ciccia A, Elledge SJ. The DNA damage response: making it safe to play with knives. Mol Cell 2010; 40:179-204; PMID:20965415; http://dx.doi.org/10.1016/j.molcel.2010.09.019
3. Agami R, Bernards R. Distinct initiation and maintenance mechanisms cooperate to induce G1 cell cycle arrest in response to DNA damage. Cell 2000; 102:55-66; PMID:10929713; http://dx.doi.org/10.1016/S0092-8674(00)00010-6
4. Cann KL, Hicks GG. Regulation of the cellular DNA double-strand break response. Biochem Cell Biol 2007; 85:663-74; PMID:18059525; http://dx.doi.org/10.1139/O07-135
5. Harper JW, Elledge SJ. The DNA damage response: ten years after. Mol Cell 2007; 28:739-45; PMID:18082599; http://dx.doi.org/10.1016/j.molcel.2007.11.015
6. Lakin ND, Jackson SP. Regulation of p53 in response to DNA damage. Oncogene 1999; 18:7644-55; PMID:10618704; http://dx.doi.org/10.1038/sj.onc.1203015
7. Zhou L, Yuan R, Serggio L. Molecular mechanisms of irradiation-induced apoptosis. Front Biosci 2003; 8:d9-19; PMID:12456331; http://dx.doi.org/10.2741/927
8. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol 2007; 35:495-516; PMID:17562483; http://dx.doi.org/10.1080/01926230701320337
9. Shimura T, Kakuda S, Ochiai Y, Nakagawa H, Kuwahara Y, Takai Y, Kobayashi J, Komatsu K, Fukumoto M. Acquired radioresistance of human tumor cells by DNA-PK/AKT/GSK3beta-mediated cyclin D1 overexpression. Oncogene 2010; 29:4826-37; PMID:20562919; http://dx.doi.org/10.1038/onc.2010.238
10. Shimura T. Acquired radioresistance of cancer and the AKT/GSK3β/cyclin D1 overexpression cycle. J Radiat Res 2011; 52:539-44; PMID:21881296; http://dx.doi.org/10.1269/jrr.11098
11. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev 1998; 12:3499-511; PMID:9832503; http://dx.doi.org/10.1101/gad.12.22.3499
12. Vivanco I, Sawyers CL. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer 2002; 2:489-501; PMID:12094235; http://dx.doi.org/10.1038/nrc839
13. Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. Cell 2007; 129:1261-74; PMID:17604717; http://dx.doi.org/10.1016/j.cell.2007.06.009
14. Filmus J, Robles AI, Shi W, Wong MJ, Colombo LL, Conti CJ. Induction of cyclin D1 overexpression by activated ras. Oncogene 1994; 9:3627-33; PMID:7970723
15. Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002; 14:381-95; PMID:11882383; http://dx.doi.org/10.1016/S0898-6568(01)00271-6
16. Alt JR, Cleveland JL, Hannink M, Diehl JA. Phosphorylation-dependent regulation of cyclin D1 nuclear export and cyclin D1-dependent cellular transformation. Genes Dev 2000; 14:3102-14; PMID:11124803; http://dx.doi.org/10.1101/gad.854900
17. Massagué J. G1 cell-cycle control and cancer. Nature 2004; 432:298-306; PMID:15549091; http://dx.doi.org/10.1038/nature03094
18. Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM, Koff A. p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. Genes Dev 1994; 8:9-22; PMID:8288131; http://dx.doi.org/10.1101/gad.8.1.9
19. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999; 13:1501-12; PMID:10385618; http://dx.doi.org/10.1101/gad.13.12.1501
20. Yang K, Guo Y, Stacey WC, Harwalkar J, Fretthold J, Hitomi M, Stacey DW. Glycogen synthase kinase 3 has a limited role in cell cycle regulation of cyclin D1 levels. BMC Cell Biol 2006; 7:33; PMID:16942622; http://dx.doi.org/10.1186/1471-2121-7-33
21. Aggarwal P, Lessie MD, Lin DI, Pontano L, Gladden AB, Nuskey B, Goradia A, Wasik MA, Klein-Szanto AJ, Rustgi AK, et al. Nuclear accumulation of cyclin D1 during S phase inhibits Cul4-dependent Cdt1 proteolysis and triggers p53-dependent DNA rereplication. Genes Dev 2007; 21:2908-22; PMID:18006686; http://dx.doi.org/10.1101/gad.1586007
22. Shimura T, Ochiai Y, Noma N, Oikawa T, Sano Y, Fukumoto M. Cyclin D1 overexpression perturbs DNA replication and induces replication-associated DNA double-strand breaks in acquired radioresistant cells. Cell Cycle 2013; 12:773-82; PMID:23388457; http://dx.doi.org/10.4161/cc.23719
23. Santra MK, Wajapeyee N, Green MR. F-box protein FBXO31 mediates cyclin D1 degradation to induce G1 arrest after DNA damage. Nature 2009; 459:722-5; PMID:19412162; http://dx.doi.org/10.1038/nature08011
24. Wang X, Arai S, Song X, Reichart D, Du K, Pascual G, Tempst P, Rosenfeld MG, Glass CK, Kurokawa R. Induced ncRNAs allosterically modify RNA-binding proteins in cis to inhibit transcription. Nature 2008; 454:126-30; PMID:18509338; http://dx.doi.org/10.1038/nature06992
25. Tan J, Geng L, Yazlovitskaya EM, Hallahan DE. Protein kinase B/Akt-dependent phosphorylation of glycogen synthase kinase-3beta in irradiated vascular endothelium. Cancer Res 2006; 66:2320-7; PMID:16489037; http://dx.doi.org/10.1158/0008-5472.CAN-05-2700
26. Hawkins AJ, Golding SE, Khalil A, Valerie K. DNA double-strand break - induced pro-survival signaling. Radiother Oncol 2011; 101:13-7; PMID:21726915; http://dx.doi.org/10.1016/j.radonc.2011.05.074
27. Bozulic L, Surucu B, Hynx D, Hemmings BA. PKBalpha/Akt1 acts downstream of DNA-PK in the DNA double-strand break response and promotes survival. Mol Cell 2008; 30:203-13; PMID:18439899; http://dx.doi.org/10.1016/j.molcel.2008.02.024
28. Fukami-Kobayashi J, Mitsui Y. Cyclin D1 inhibits cell proliferation through binding to PCNA and cdk2. Exp Cell Res 1999; 246:338-47; PMID:9925749; http://dx.doi.org/10.1006/excr.1998.4306

©2013 Landes Bioscience. Do not distribute.

29. Prosperi E, Scovassi AI, Stivala LA, Bianchi L. Proliferating cell nuclear antigen bound to DNA synthesis sites: phosphorylation and association with cyclin D1 and cyclin A. Exp Cell Res 1994; 215:257-62; PMID:7982468; http://dx.doi.org/10.1006/excr.1994.1341
30. Xiong Y, Zhang H, Beach D. D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell 1992; 71:505-14; PMID:1358458; http://dx.doi.org/10.1016/0092-8674(92)90518-H
31. Shimura T, Torres MJ, Martin MM, Rao VA, Pommier Y, Katsura M, Miyagawa K, Aladjem MI. Bloom's syndrome helicase and Mus81 are required to induce transient double-strand DNA breaks in response to DNA replication stress. J Mol Biol 2008; 375:1152-64; PMID:18054789; http://dx.doi.org/10.1016/j.jmb.2007.11.006
32. Tane S, Chibazakura T. Cyclin A overexpression induces chromosomal double-strand breaks in mammalian cells. Cell Cycle 2009; 8:3900-3; PMID:19901524; http://dx.doi.org/10.4161/cc.8.23.10071
33. Pernot E, Hall J, Baatout S, Benotmane MA, Blanchardon E, Bouffler S, El Saghire H, Gomolka M, Guertler A, Harms-Ringdahl M, et al. Ionizing radiation biomarkers for potential use in epidemiological studies. Mutat Res 2012; 751:258-86; PMID:22677531; http://dx.doi.org/10.1016/j.mrrev.2012.05.003
34. Lassmann M, Hänscheid H, Gassen D, Biko J, Meineke V, Reiners C, Scherthan H. In vivo formation of gamma-H2AX and 53BP1 DNA repair foci in blood cells after radioiodine therapy of differentiated thyroid cancer. J Nucl Med 2010; 51:1318-25; PMID:20660387; http://dx.doi.org/10.2967/jnumed.109.071357
35. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G. Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining. Cancer Res 1994; 54:1812-7; PMID:8137296
36. Russell A, Thompson MA, Hendley J, Trute L, Armes J, Germain D. Cyclin D1 and D3 associate with the SCF complex and are coordinately elevated in breast cancer. Oncogene 1999; 18:1983-91; PMID:10208420; http://dx.doi.org/10.1038/sj.onc.1202511
37. Diehl JA. Cycling to cancer with cyclin D1. Cancer Biol Ther 2002; 1:226-31; PMID:12432268
38. Fu M, Rao M, Bouras T, Wang C, Wu K, Zhang X, Li Z, Yao TP, Pestell RG. Cyclin D1 inhibits peroxisome proliferator-activated receptor gamma-mediated adipogenesis through histone deacetylase recruitment. J Biol Chem 2005; 280:16934-41; PMID:15713663; http://dx.doi.org/10.1074/jbc.M500403200
39. Hulit J, Wang C, Li Z, Albanese C, Rao M, Di Vizio D, Shah S, Byers SW, Mahmood R, Augenlicht LH, et al. Cyclin D1 genetic heterozygosity regulates colonic epithelial cell differentiation and tumor number in ApcMin mice. Mol Cell Biol 2004; 24:7598-611; PMID:15314168; http://dx.doi.org/10.1128/MCB.24.17.7598-7611.2004
40. Casimiro MC, Pestell RG. Cyclin d1 induces chromosomal instability. Oncotarget 2012; 3:224-5; PMID:22538871
41. Lung JC, Chu JS, Yu JC, Yue CT, Lo YL, Shen CY, Wu CW. Aberrant expression of cell-cycle regulator cyclin D1 in breast cancer is related to chromosomal genomic instability. Genes Chromosomes Cancer 2002; 34:276-84; PMID:12007188; http://dx.doi.org/10.1002/gcc.10072
42. Quelle DE, Ashmun RA, Shurtleff SA, Kato JY, Bar-Sagi D, Roussel MF, Sherr CJ. Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. Genes Dev 1993; 7:1559-71; PMID:8339933; http://dx.doi.org/10.1101/gad.7.8.1559
43. Benzeno S, Lu F, Guo M, Barbash O, Zhang F, Herman JG, Klein PS, Rustgi A, Diehl JA. Identification of mutations that disrupt phosphorylation-dependent nuclear export of cyclin D1. Oncogene 2006; 25:6291-303; PMID:16732330; http://dx.doi.org/10.1038/sj.onc.1209644
44. Gladden AB, Diehl JA. Location, location, location: the role of cyclin D1 nuclear localization in cancer. J Cell Biochem 2005; 96:906-13; PMID:16163738; http://dx.doi.org/10.1002/jcb.20613
45. Lin DI, Lessie MD, Gladden AB, Bassing CH, Wagner KU, Diehl JA. Disruption of cyclin D1 nuclear export and proteolysis accelerates mammary carcinogenesis. Oncogene 2008; 27:1231-42; PMID:17724472; http://dx.doi.org/10.1038/sj.onc.1210738
46. Mallette FA, Gaumont-Leclerc MF, Ferbeyre G. The DNA damage signaling pathway is a critical mediator of oncogene-induced senescence. Genes Dev 2007; 21:43-8; PMID:17210786; http://dx.doi.org/10.1101/gad.1487307
47. Bartkova J, Rezaei N, Liontos M, Karakaidos P, Kletsas D, Issaeva N, Vassiliou LV, Kolettas E, Niforou K, Zoumpourlis VC, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. Nature 2006; 444:633-7; PMID:17136093; http://dx.doi.org/10.1038/nature05268
48. Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, Schurra C, Garre' M, Nuciforo PG, Bensimon A, et al. Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. Nature 2006; 444:638-42; PMID:17136094; http://dx.doi.org/10.1038/nature05327
49. Murfuni I, Nicolai S, Baldari S, Crescenzi M, Bignami M, Franchitto A, et al. The WRN and MUS81 proteins limit cell death and genome instability following oncogene activation. Oncogene 2013; 32:610-20; PMID:22410776; http://dx.doi.org/10.1038/onc.2012.80
50. Dulić V, Drullinger LF, Lees E, Reed SI, Stein GH. Altered regulation of G1 cyclins in senescent human diploid fibroblasts: accumulation of inactive cyclin E-Cdk2 and cyclin D1-Cdk2 complexes. Proc Natl Acad Sci U S A 1993; 90:11034-8; PMID:8248208; http://dx.doi.org/10.1073/pnas.90.23.11034
51. Lucibello FC, Sewing A, Brüsselbach S, Bürger C, Müller R. Deregulation of cyclins D1 and E and suppression of cdk2 and cdk4 in senescent human fibroblasts. J Cell Sci 1993; 105:123-33; PMID:8360268
52. Atadja P, Wong H, Veillete C, Riabowol K. Overexpression of cyclin D1 blocks proliferation of normal diploid fibroblasts. Exp Cell Res 1995; 217:205-16; PMID:7698220; http://dx.doi.org/10.1006/excr.1995.1080
53. Leontieva OV, Lenzo F, Demidenko ZN, Blagosklonny MV. Hyper-mitogenic drive coexists with mitotic incompetence in senescent cells. Cell Cycle 2012; 11:4642-9; PMID:23187803; http://dx.doi.org/10.4161/cc.22937
54. Duronio V. The life of a cell: apoptosis regulation by the PI3K/PKB pathway. Biochem J 2008; 415:333-44; PMID:18842113; http://dx.doi.org/10.1042/BJ20081056
55. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 2005; 9:59-71; PMID:15784165; http://dx.doi.org/10.1111/j.1582-4934.2005.tb00337.x
56. Dent P, Yacoub A, Contessa J, Caron R, Amorino G, Valerie K, Hagan MP, Grant S, Schmidt-Ullrich R. Stress and radiation-induced activation of multiple intracellular signaling pathways. Radiat Res 2003; 159:283-300; PMID:12600231; http://dx.doi.org/10.1667/0033-7587(2003)159[0283:SARIAO]2.0.CO;2
57. Schmidt-Ullrich RK, Contessa JN, Dent P, Mikkelsen RB, Valerie K, Reardon DB, Bowers G, Lin PS. Molecular mechanisms of radiation-induced accelerated repopulation. Radiat Oncol Investig 1999; 7:321-30; PMID:10644055; http://dx.doi.org/10.1002/(SICI)1520-6823(1999)7:6<321::AID-ROI2>3.0.CO;2-Q
58. Alao JP. The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. Mol Cancer 2007; 6:24; PMID:17407548; http://dx.doi.org/10.1186/1476-4598-6-24
59. Musgrove EA, Caldon CE, Barraclough J, Stone A, Sutherland RL. Cyclin D as a therapeutic target in cancer. Nat Rev Cancer 2011; 11:558-72; PMID:21734724; http://dx.doi.org/10.1038/nrc3090
60. Jayasurya R, Francis G, Kannan S, Lekshminarayanan K, Nalinakumari KR, Abraham T, Abraham EK, Nair MK. p53, p16 and cyclin D1: molecular determinants of radiotherapy treatment response in oral carcinoma. Int J Cancer 2004; 109:710-6; PMID:14999779; http://dx.doi.org/10.1002/ijc.20042
61. Higuchi E, Oridate N, Homma A, Suzuki F, Atago Y, Nagahashi T, Furuta Y, Fukuda S. Prognostic significance of cyclin D1 and p16 in patients with intermediate-risk head and neck squamous cell carcinoma treated with docetaxel and concurrent radiotherapy. Head Neck 2007; 29:940-7; PMID:17563903; http://dx.doi.org/10.1002/hed.20632
62. Yang L, Dan HC, Sun M, Liu Q, Sun XM, Feldman RI, Hamilton AD, Polokoff M, Nicosia SV, Herlyn M, et al. Akt/protein kinase B signaling inhibitor-2, a selective small molecule inhibitor of Akt signaling with antitumor activity in cancer cells overexpressing Akt. Cancer Res 2004; 64:4394-9; PMID:15231645; http://dx.doi.org/10.1158/0008-5472.CAN-04-0343
63. Feun LG, Blessing JA, Barrett RJ, Hanjani P. A phase II trial of tricyclic nucleoside phosphate in patients with advanced squamous cell carcinoma of the cervix. A Gynecologic Oncology Group Study. Am J Clin Oncol 1993; 16:506-8; PMID:8256767; http://dx.doi.org/10.1097/00000421-199312000-00010
64. Feun LG, Savaraj N, Bodey GP, Lu K, Yap BS, Ajani JA, Burgess MA, Benjamin RS, McKelvey E, Krakoff I. Phase I study of tricyclic nucleoside phosphate using a five-day continuous infusion schedule. Cancer Res 1984; 44:3608-12; PMID:6744283
65. Shimura T, Kakuda S, Ochiai Y, Kuwahara Y, Takai Y, Fukumoto M. Targeting the AKT/GSK3β/cyclin D1/Cdk4 survival signaling pathway for eradication of tumor radioresistance acquired by fractionated radiotherapy. Int J Radiat Oncol Biol Phys 2011; 80:540-8; PMID:21398050; http://dx.doi.org/10.1016/j.ijrobp.2010.12.065

©2013 Landes Bioscience. Do not distribute.

SCIENTIFIC REPORTS



OPEN

SUBJECT AREAS:
EXPERIMENTAL MODELS OF DISEASE
ANATOMY
REPRODUCTIVE SIGNS AND SYMPTOMS
RISK FACTORS

Received
21 January 2013

Accepted
4 September 2013

Published
8 October 2013

Correspondence and requests for materials should be addressed to M.F. (fukumoto@idac.tohoku.ac.jp)

Effects of radioactive caesium on bull testes after the Fukushima nuclear plant accident

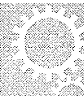
Hideaki Yamashiro[1], Yasuyuki Abe[2], Tomokazu Fukuda[3], Yasushi Kino[4], Isao Kawaguchi[5], Yoshikazu Kuwahara[6], Motoi Fukumoto[6], Shintaro Takahashi[6], Masatoshi Suzuki[6], Jin Kobayashi[7], Emi Uematsu[1], Bin Tong[1], Takahisa Yamada[1], Satoshi Yoshida[5], Eimei Sato[3], Hisashi Shinoda[8], Tsutomu Sekine[4], Emiko Isogai[3] & Manabu Fukumoto[6]

[1]Faculty of Agriculture, Niigata University, Niigata, Japan, [2]Graduate School of Science and Engineering, Yamagata University, Yamagata, Japan, [3]Graduate School of Agricultural Sciences, Tohoku University, Sendai, Japan, [4]Department of Chemistry, Tohoku University, Sendai, Japan, [5]National Institute of Radiological Sciences, Chiba, Japan, [6]Institute of Development, Aging and Cancer, Tohoku University, Sendai, Japan, [7]School of Food, Agricultural and Environmental Sciences, Miyagi University, Sendai, Japan, [8]Graduate School of Dentistry, Tohoku University, Sendai, Japan.

We aimed to investigate the effect of chronic radiation exposure associated with the Fukushima Daiichi Nuclear Plant accident on the testis from 2 bulls. Estimated dose of internal exposure in one bull was 0.7–1.2 mGy (^{134}Cs) and 0.4–0.6 mGy (^{137}Cs) and external exposure was 2.0 mGy (^{134}Cs) and 0.8 mGy (^{137}Cs) (196 days). Internal dose in the other was 3.2–6.1 mGy (^{134}Cs) and 1.8–3.4 mGy (^{137}Cs) and external dose was 1.3 mGy (^{134}Cs) and 0.6 mGy (^{137}Cs) (315 days). Sperm morphology and spermatogenesis were within normal ranges. $^{134,\,137}$Cs radioactivity was detected but Cs was not detectable in the testis by electron probe microanalysis. Thus, adverse radiation-induced effects were not observed in bull testes following chronic exposure to the above levels of radiation for up to 10 months. Since we could analyse a limited number of testes, further investigation on the effects of ionizing radiation on spermatogenesis should be extended to more animals.

Radiation accidents can result in localized or whole-body exposure and both the internal and external deposition of radioactive materials. Clinical manifestations of radiation exposure depend on the extent of penetration and the dose absorbed by various tissues[1]. After the Great East Japan Earthquake on 11 March 2011, the Fukushima Daiichi Nuclear Power Plant (FNPP) accident led to a discharge of a tremendous amount of radioactive substances[2,3]. On 22 April 2011, the evacuation zone was set to a 20-km radius surrounding the FNPP, leaving approximately 3,400 cows, 31,500 pigs, and 630,000 chickens behind within the zone. On 12 May 2011, the Japanese government ordered Fukushima prefectural government to euthanize unleashed livestock within the evacuation zone. Abandoned animals now have formed an invaluable model for studying the effects of chronic radionuclide intake. A comprehensive assessment of the effect of long-term exposure to internally deposited radionuclides on surviving domestic animals in the evacuation area is therefore urgently needed for the benefit of the livestock industry, as well as for human health. Radiobiological data from the FNPP accident could help to develop a set of internationally harmonized measures to protect domestic animals in the event of a future nuclear or radiological emergency.

Exposure to a large dose of ionizing radiation can cause irreparable damage to multiple organ systems, particularly those with highly proliferative cells, such as the skin, the hematopoietic and gastrointestinal system[4]. The testis is a relatively radiosensitive organ[5], composed of a series of spermatogenic cells such as stem cells, spermatogonia, spermatids, spermatocytes, and sperm. These different types of germ cells differ remarkably in their susceptibility to radiation-induced effects according to their level of reproductive activity[6]. The effect on reproductive organs and behaviour by chronic exposure to radionuclides is one of major concerns. Furthermore, radiation-induced genomic changes, occurring in germ cells may have hereditary effects, including carcinogenesis, congenital malformation and growth retardation in offspring. Data used for estimating the risk associated with exposure to ionizing radiation have been primarily obtained from epidemiological studies of survivors of the atomic bombing of Hiroshima and Nagasaki[7,8], the Chernobyl nuclear accident[9], and some complementary



animal experiments[10–12]. However, reports of the effect of chronic low-dose radiation on livestock animals are limited.

We recently reported radionuclide deposition in organs of abandoned cattle following the FNPP accident. The deposition occurred in an individual radionuclide and in an organ-specific manner, and radioactive caesium (Cs) was detected in all the organs examined[13]. Discharge of ^{134}Cs and ^{137}Cs that emit γ- and β-rays is of primary concern, because they were released in a large amount and have a long half-life[14]. Thus, significant questions regarding the effect of long-term exposure to radioactive Cs on human health are now being raised. The current study focused on the effect of exposure to radioactive caesium on the reproductive organs of bulls that were abandoned in the 20-km FNPP evacuation zone from 12 March to 27 September 2011 and 24 January 2012. The aim of the present study was to investigate the effect of chronic radiation exposure on bull testes to ^{134}Cs and ^{137}Cs associated with the FNPP accident.

Results

Radioactivity concentration of ^{134}Cs and ^{137}Cs in male bull organs and blood for liquid. Organs, including testes, and peripheral blood (PB) were collected from 12 bulls and a male foetus at different sites within the 20-km FNPP evacuation zone on different dates. PB could not be obtained from any foetus examined because they were too small. The concentration of radionuclides in these tissues was then determined (Table 1). Radioactive concentrations of ^{134}Cs and ^{137}Cs were almost similar in all the organs and PB examined for liquid. We could measure the radioactivity concentration of ^{134}Cs and ^{137}Cs of the testis from 2 bulls and 1 foetus as listed in Table 2. Organ concentration of radioactive Cs was the highest in skeletal muscles among organs examined. Testicular concentrations of ^{134}Cs and ^{137}Cs for liquid were approximately 13- to 16-fold higher in bull 1 and 17- to 18-fold higher in bull 2 than PB concentrations for liquid. The foetal organs also showed deposits of both the two radionuclides.

Calculated doses of ^{134}Cs and ^{137}Cs in bull. The average and maximum doses of internal exposure calculated are shown in Table 3. In bull 1, the estimate of the internal dose during 196 days from combined ^{134}Cs and ^{137}Cs was 1.1–1.8 mGy. In bull 2, the estimate of the internal dose for 315 days was 5.0–9.5 mGy. External exposure of bull 1 was 2.8 mGy and that of bull 2 was 1.9 mGy.

Spermatogenesis and sperm morphology are normal in irradiated bulls. Effects of long-term radiation exposure on the number and morphology of several types of germ cells present in the testis were investigated. Nuclear and acrosome morphology of sperm was assessed by DAPI and FITC-PNA staining, respectively. The sperm acrosome was located between the nucleus and the plasma membrane, and in bull, sperm envelops two-thirds of the nucleus (Figure 1). We observed that the total number and morphology of epididymal sperm from irradiated bulls were normal. In addition, relative sizes of nuclei and acrosomes were normal in almost all sperm tested.

We next examined the morphology of a series of germ cells present in the testis under a microscope. Control seminiferous tubules showed normal spermatogenesis (Figure 2A, B). Interestingly, spermatogenesis was not disrupted in the testes from radiation-exposed animals compared to controls (Figure 2C, E), indicating that radiation exposure in the present study did not affect this process. In addition, there was no difference in the number of spermatogonia, spermatocytes, spermatids, and sperm in the testes of radiation-exposed animals compared with control testes (Figure 2D, F). HE staining of foetal testes confirmed that spermatogonial cells were present in the seminiferous tubules (Figure 2G, H).

Table 1 | Radioactivity concentration of ^{134}Cs and ^{137}Cs of 12 bulls n organs and peripheral blood for liquid (Bq/kg)

Organs	^{134}Cs	^{137}Cs
Longissimus thoracis muscle	475 ± 175	480 ± 182
Tensor fasciae late muscle	382 ± 186	399 ± 195
Diaphragm	193 ± 18	186 ± 23
Heart	237 ± 104	233 ± 92
Liver	165 ± 66	175 ± 75
Kidney	253 ± 117	258 ± 118
Lung	279 ± 282	294 ± 297
Spleen	183 ± 46	185 ± 46
Blood	17 ± 9	18 ± 8

Data are presented as mean ± SD.

Table 2 | Radioactivity concentration of ^{134}Cs and ^{137}Cs in organs and peripheral blood for liquid (Bq/kg)

Bull	Organs	^{134}Cs	^{137}Cs
No.1	Longissimus thoracis muscle	284 ± 8	288 ± 7
	Tensor fasciae late muscle	356 ± 7	370 ± 6
	Diaphragm	195 ± 5	205 ± 4
	Liver	171 ± 6	181 ± 5
	Kidney	251 ± 8	259 ± 7
	Lung	164 ± 7	173 ± 6
	Spleen	218 ± 7	210 ± 6
	Testis	195 ± 8	213 ± 6
	Blood	12 ± 1	16 ± 1
No.2	Tensor fasciae late muscle	999 ± 14	1050 ± 11
	Masseter muscle	1177 ± 18	1234 ± 14
	Tongue	890 ± 9	948 ± 7
	Heart	656 ± 12	694 ± 10
	Liver	273 ± 8	276 ± 6
	Kidney	830 ± 15	897 ± 12
	Lung	537 ± 15	542 ± 11
	Spleen	257 ± 9	273 ± 7
	Testis	643 ± 23	661 ± 18
	Blood	38 ± 1	36 ± 1
Foetus	Longissimus thoracis muscle	308 ± 8	336 ± 5
	Tensor fasciae late muscle	383 ± 6	403 ± 5
	Tongue	322 ± 57	359 ± 28
	Heart	202 ± 9	232 ± 5
	Liver	163 ± 6	178 ± 7
	Kidney	166 ± 2	176 ± 6
	Lung	103 ± 6	111 ± 3
	Spleen	218 ± 6	203 ± 6
	Submandibular gland	187 ± 10	198 ± 10
	Umbilical cord	42 ± 2	44 ± 2
	Testis	187 ± 24	200 ± 20
	Blood	ND*	ND*

Data are presented as mean ± SD.
*ND: Not determined.

Table 3 | Internal and external exposure from ^{134}Cs and ^{137}Cs in the bulls

Bull	Exposure	^{134}Cs	^{137}Cs
No.1	Internal	0.7–1.2* (0.1–0.2**)	0.4–0.6* (0.09–0.1**)
	External dose (mGy)	2.0	0.8
No.2	Internal	3.2–6.1* (0.4–0.8**)	1.8–3.4* (0.2–0.4**)
	External dose (mGy)	1.3	0.6

*Estimated average dose – maximal dose (mGy).
**Estimated average dose rate – maximal dose rate (μGy/h).