Chapter 17
Preparation and Genome Analysis of Immortalized Cells Derived from Wild Macaques Affected by the Fukushima Daiichi Nuclear Power Plant Accident

Tomokazu Fukuda

Abstract The evaluation of the potential biological effect of radiation is important for human health. We previously reported the deposit of radionuclides in animals from the ex-evacuation zone of the FNPP accident. In case of internal exposure, the dose of the radiation is largely affected by the metabolism of the radionuclides. We can assume that the radiation-exposed tissue is the mixed population of cells, which have genetic mutations in multiple and different sites of the genome. To detect the mutations of each cell, we need a new technology which allows us to detect the genetic alternation of the genome at the single cell level. We previously found that the expression of human-derived mutant CDK4, and overexpression of Cyclin D1 and TERT allow us to immortalize cells derived from multiple species. By applying this immortalization method to radiation-exposed tissues, we can obtain the multiple immortalized cell lines from the primary tissues. Since each cell line is expected to be derived from a single cell of the tissue, each cell line is expected to keep mutation spectrum of the genome occurring in the cell of origin after irradiation.

Keywords Fukushima Daiichi Nuclear Power Plant accident · Low-dose radiation · Mutation · Cell immortalization · Cyclin dependent kinase 4 · Cyclin D · Telomere reverse transcriptase

17.1 Background

The evaluation of the potential biological effect of radiation is important for human health. Especially, in Japan, the Fukushima Daiichi Nuclear Power Plant (FNPP) accident leads public to recognize the importance of risk assessment and radiation

T. Fukuda (✉)
Graduate School of Science and Engineering, Iwate University, Morioka, Japan

Soft-Path Engineering Research Center (SPERC), Iwate University, Morioka, Japan

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safety [1]. We previously reported the deposit of radionuclides in animals from the ex-evacuation zone of the FNPP accident [2–9]. In general, radiation exposure can be classified into the two aspects, internal and external exposure. Although radiation effects on human have been analyzed by epidemiological studies on health of the atomic bomb survivors of Hiroshima and Nagasaki (Hibakusha), such as solid cancer incidence [10], these studies are mainly based on the result of acute and external exposure. However, in case of chronic and internal exposure, radiation dose is largely affected by the metabolism of the radionuclides.

The cellular effect caused by radiation exposure is represented mainly by DNA damage, such as strand breaks. Although the damaged DNA can be repaired through several biological pathways, such as homologous recombination and/or nonhomologous end joining [11], the damage of DNA caused by radiation exposure has a risk to induce the alteration of sequence information of genome, including base substitutions and nucleotide deletion. Exposure to 1 Gy of X-ray radiation reportedly induces 1,000 single strand breaks in an irradiated cell [12]. Furthermore, the clustered damaged sites on the genome can lead multiple nucleotide substitutions in the genome [12, 13]. As far as we know, the position of nucleotide substitutions or insertion/deletion mutations is random, and the sensitive area is not reported yet [14]. Therefore, in in vivo tissues, we can assume that the radiation-exposed tissue is the mixed population of cells, which have genetic mutations in multiple and different sites of the genome. To detect mutations of each cell, we need a new technology which allows us to detect genetic alternations at the single cell level.

17.2 Immortalization of Wild Macaque-Derived Cell with the Expression of Mutant Cyclin-Dependent Kinase and Cyclin D and Telomerase

Primary cells cannot proliferate infinitely due to cellular stress and senescence during the cell culture [15]. However, the expression of oncogenic proteins such as SV40 large T or E6/E7 proteins of human papilloma virus (HPV) allows us to grow a cell, which is close to immortalization [16, 17]. Although the immortalization by these oncogenic molecules is quite reproducible and efficient, the genomic and chromosomal status becomes instable and sometimes causes abnormalities. Especially, the expression of E6/E7 causes polyploid abnormality of the genome [18]. Furthermore, these methods induce the inactivation of p53 protein which is one of the most important molecules to keep the integrity of the genome and is even called as the guardian of the genome. Furthermore, the combination of shRNA of p16 and c-Myc oncogene was reported to induce the immortalization of human mammary epithelial cells (HMEC) [19]. However, even in this method, additional chemical treatment of benzo(a)pyrene to HMEC was required for the immortalization, which possibly explained by the additional genetic alteration is required for the infinite cell proliferation [19].

We previously found that the combination of expression of R24C mutant type of cyclin-dependent kinase 4 (*CDK4*), *Cyclin D1* and enzymatic complex of telomerase (*TERT*) allows us to bypass the negative feedback of the senescence protein, p16 [20]. To be noted, the amino acid sequence of the cell cycle regulators, such as CDK4 and Cyclin D are quite conservative among species. Based on this evolu-

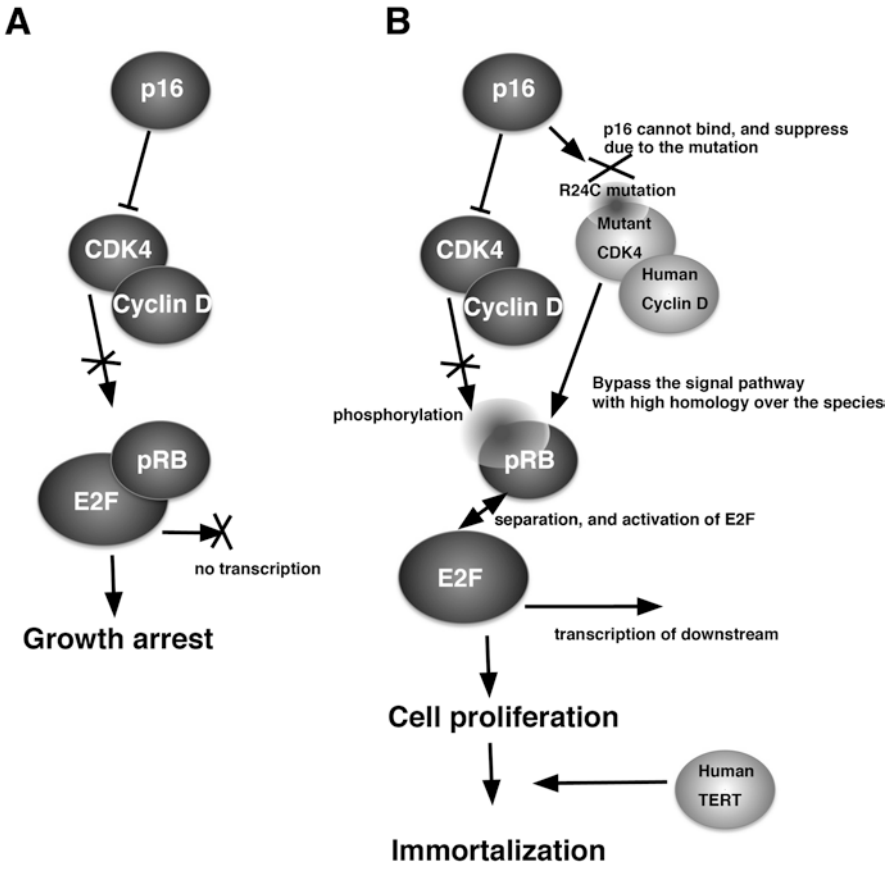

Fig. 17.1 Expected accelerated cell growth mechanism of mutant human-derived CDK4, Cyclin D and TERT over the multiple species. (**a**) Cell growth arrest under the cellular senescence and/or cellular stress. The protein level of p16 increases under the senescence. The p16 protein binds to the pocket of the CDK4 and negatively regulates the activity of CDK4-Cyclin D complex. The inactivated CDK4-Cyclin D complex cannot induce the phosphorylation of pRB resulting in its inactivation. Under the intact condition of pRB, E2F is not released from the binding status, resulting in no transcription of the downstream genes and growth arrest of the cells. (**b**) Enhanced cellular proliferation with mutant *CDK4* and *Cyclin D1* and *TERT* overexpression. Due to the R24C mutation of the human-derived CDK4, p16 protein cannot suppress the activity of protein complex of mutant CDK4 and Cyclin D. The exogenously introduced human-derived mutant CDK4-Cyclin D complex with endogenous pRB phosphorylates pRB. Due to the phosphorylation and inactivation of pRB, the transcription factor, E2F would be released from the complex and induce cell proliferation. This figure was reproduced from our previous publication with slight modification [22]

tional conservancy of the molecules, we found that the expression of human-derived mutant *CDK4* and overexpression of *Cyclin D1* and *TERT* allow us to immortalize cells derived from multiple species [21–23]. Furthermore, the expression of mutant type *CDK4* and *Cyclin D1* allows us to bypass the negative regulation of p16 and pRB (retinoblastoma protein) while keeping the function of p53 protein intact. Since p53 is an important molecule to maintain the genome, we confirmed that the chromosome condition of immortalized cells is intact in comparison with wild-type cells [24]. This situation led us to assume that the expression of mutant *CDK4*, *Cyclin D1* and *TERT* could immortalize a cell from the irradiated tissue. From the characters of introducing genes, we named the immortalized cells as the K4DT (mutant CDK4, Cyclin D1 and TERT) cells, and this immortalization technique is called as the K4DT method (Fig. 17.1).

Applying the K4DT method to radiation-exposed tissues, we can obtain multiple immortalized cell lines from the primary tissues. Since each cell line is expected to be derived from a single cell of the tissue, each cell line is expected to keep mutation spectrum of the genome occurring in the cell of origin after irradiation. The immortalized cells can be also obtained from the tissues of non-irradiated control animals.

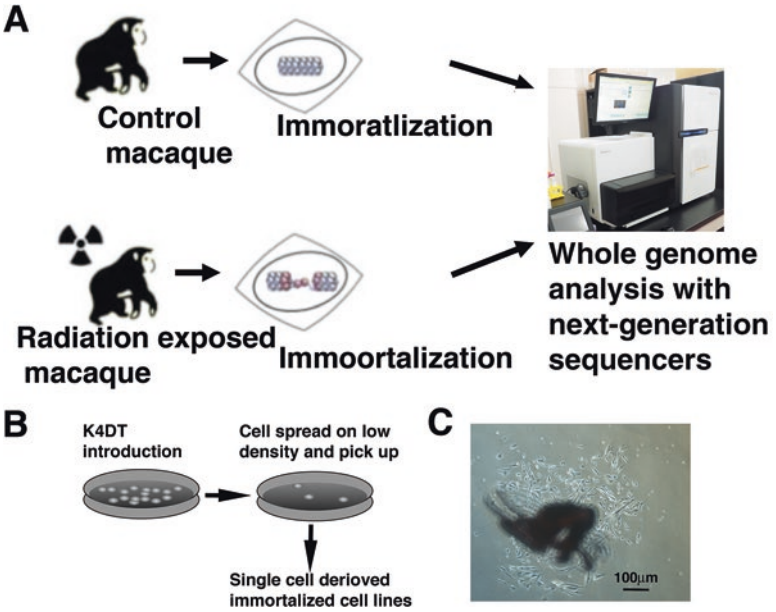

Fig. 17.2 Strategy of whole genome analysis to identify the radiation-induced genomic alteration with the next-generation sequencing. (**a**) Note that the irradiated tissue is the mixed population of cells with mutations at the random position of the genome. After the establishment of immortalized cells from a single cell, we can identify the genomic alteration caused by radiation exposure. (**b**) Strategy of a single cell-derived immortalized cells from the K4DT method. (**c**) Cell morphology which showed the proliferation as a single cell-derived colony. The colony has been marked by black marker from the bottom of the plastic dish

Based on the whole genome sequencing of cloned control and a single cell from radiation-exposed tissues, genomic mutations caused by radiation exposure could be detected. The genetic alterations at the single cell level caused by irradiation can be detected by the combination of the K4DT immortalization method and whole genome sequencing (Fig. 17.2a).

Currently, we are underway to establish immortalized cell lines derived from wild macaques with internal and external exposure to radioactive cesium from the affected area of the FNPP accident to elucidate genetic alternation caused by low-dose (LD) and low-dose-rate (LDR) radiation exposure (Fig. 17.2b). We have obtained multiple skeletal muscle-derived cell lines as part of the effort. We are interested in skeletal muscle because it is nondividing and accumulates the highest concentration of radioactive cesium in the body.

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