RESEARCH ARTICLE

Cell migration is impaired in XPA-deficient cells

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Abstract

Xeroderma pigmentosum (XP) is a hereditary disorder characterized by photosensitivity, predisposition to skin cancers, and neurological abnormalities including microcephaly and progressive neurodegeneration. A lack of nucleotide excision repair (NER) in patients with XP can cause hypersensitivity to the sun, leading to skin cancer, whereas the etiology of the neuronal symptoms of XP remains ambiguous. There are various neurological disorders that perturb neuronal migration, causing mislocalization and disorganization of the cortical lamination. Here, we investigated the role of the XP group-A (*Xpa*) gene in directed cell migration. First, we adopted an in utero electroporation method to transduce shRNA vectors into the murine embryonic cerebral cortex for the in vivo knockdown of *Xpa*. *Xpa*-knockdown neurons in the embryonic cerebral cortex showed abnormal cell migration, cell cycle exit, and differentiation. The genotype–phenotype relationship between the lack of XPA and cell migration abnormalities was confirmed using both a scratch assay and time-lapse microscopy in XP-A patient-derived fibroblasts. Unlike healthy cells, these cells showed impairment in overall mobility and the direction of motility. Therefore, abnormal cell migration may explain neural tissue abnormalities in patients with XP-A.

Seiji Takeuchi and Takeshi Fukumoto contributed equally to this work.

1 | INTRODUCTION

Xeroderma pigmentosum (XP) is a hereditary disorder characterized by photosensitivity, predisposition to skin cancer, and various neurological symptoms.[1,2] XP is classified into 8 molecular types, XP-A through XP-G, nucleotide excision repair-deficient type, and XP-V. Cells from patients with XP-A (XP-A cells) lack both subpathways of nucleotide excision repair (NER), namely, the global genome NER (GG-NER) and transcription-coupled NER (TC-NER). XP-A cells exhibit the lowest DNA repair capacity, and patients with XP-A have the most severe cutaneous and neurological symptoms among XP.[1]

XP-A results from a mutation in the *XPA* gene. In Japan, patients with XP-A account for approximately half of all patients with XP,[3–5] due to a founder mutation of the *XPA* gene. Advances in medicine have greatly contributed to a reduction in cutaneous skin cancers through early diagnosis and early excisional treatments and prevention by strict sun protection, although the patients continue to stay away from the sun for their whole life. In contrast, no preventive measures or treatment methods have been established to tackle the neurological symptoms associated with XP such as microcephaly, mental retardation, cerebellar ataxia, sensorineural hearing impairment, and axonal peripheral polyneuropathy.[2,6] These complex neurological abnormalities are not related to sunlight exposure but could be caused by developmental defects as well as faulty repair of DNA damage in neuronal cells. Although the cause of neurological symptoms is unclear, several explanations have been proposed. One possibility is that neurological symptoms are caused by neurodegeneration due to oxidative DNA damage, such as the formation of cyclopurine, which is produced through endogenous metabolism and/or other environmental damage and is known to be repaired by NER.[7–9] The second possibility is that neurological symptoms are caused by abnormal mitophagy,[10] which occurs during energy metabolism in neuronal cells. The third possible cause for the symptoms could be a disturbance of transcription because only patients with XP of the TC-NER deficient type but not the GG-NER deficient type exhibit neurological symptoms.[11] However, the main neuropathology observed in patients with XP is primarily neuronal degeneration, while reduced myelination of the brain is observed in other TC-NER-related disorders like Cockayne syndrome (CS) and trichothiodystrophy (TTD). To determine the treatment strategy, it is crucial to unravel the molecular etiology of the genotype–phenotype relationships in XP symptoms.

Directed cell migration is a fundamental process underlying diverse physiological and pathophysiological phenomena, including wound healing, induction of an immune response, and development of the brain.[12] During radial migration, immature excitatory neurons migrate from the ventricular zone (VZ) toward the apical surface in the neonatal mammalian neocortex. Migrating neurons display various morphologies and modes of migration that are tightly regulated by signaling and cytoskeletal molecules. Excitatory neurons newly generated at the VZ first exhibit multipolar morphology in the subventricular zone (SVZ) and the lower intermediate zones (IZ) and subsequently transform into bipolar locomotory neurons with a leading process consisting of a six-layer structure with an inside-out pattern, which is formed by the radial migration of neuroblasts that continuously bypass the preceding differentiated and migrated neurons. Disturbance of radial migration can cause mislocalization of neurons and result in disorganized cortical lamination, a defect frequently observed in various neurological disorders, such as mental retardation and epilepsy.[13]

In the present study, we investigated whether XPA-deficient murine nubroblasts or human primary fibroblasts exhibit disrupted mobility. This is the first study to point out that XPA-deficient cells exhibit abnormal migration, which may be a plausible explanation for the neurological phenotypes of XP, such as delayed neuronal development and microcephaly, which cannot be explained solely by the deficiency of NER.

2 | MATERIALS AND METHODS

2.1 | Animals

C57BL/6 mice were purchased from a local vendor (SLC, Hamamatsu, Japan), housed at constant temperature and humidity, and provided with food and water ad libitum. Embryonic day 0 (E0) was defined as the day the vaginal plug was confirmed. All pregnant animals were anesthetized intraperitoneally. The animal experiments were approved by the Institutional Review Board of the Ethics Committee of the University of Fukui. All animal experiments were conducted in accordance with the Ethics Committee of the University of Fukui, School of Medicine, the Declaration of Helsinki, and the Guidelines for Use of Laboratory Animals of the University of Fukui. Efforts were made to minimize the number of animals used and their suffering.

2.2 | RNA interference

Three different constructs of *Xpa* siRNAs were prepared using the mU6pro vector.[14] The short hairpin RNAs 5′-GGCCCGUCAUGGAGUUUGAUuucaagagaAUCAAAC UCCAUGACGGGCC-3′, 5′-GACAGAAGCGAAGCAA

GAGUACCuucaagagaGGUACUCUUGCUUCGCUUCU
GUC-3′, and 5′-GACAGAAGCGAAGCAAGAGUACCu
ucaagagaGGUACUCUUGCUUCGCUUCUGUC-3′ targeted nucleotides 372–391, 511–533 of mouse *Xpa* cDNA (GenBank accession number NM 011728.2) and were termed *Xpa* R-1, and *Xpa* R-2, respectively. The sequences indicated in lower case correspond to the loop region. To assess the effects of these siRNAs, an expression vector of *Xpa*-EGFP was co-transfected into COS-7 cells with either the siRNA-expressing vector or the mU6pro vector, and the amount of Xpa-EGFP in the cells was compared by immunoblotting using an anti-EGFP antibody.

2.3 | In utero electroporation gene transfer

Plasmids were transfected by in utero electroporation using previously described methods.[15] Briefly, E14 pregnant female C57BL/6 mice were anesthetized by an intraperitoneal injection of 2,2,2-tribromoethanol (200–300 mg/kg body weight) prior to the experiments. A total of 1 μg of plasmid was injected by transuterine pressure microinjection into the lateral ventricle of the embryos by delivering five electrical pulses (40 V, 50 ms duration) at intervals of 950 ms using a square-pulse electroporator (CUY21EDIT; Nepa Gene) and a tweezer-type electrode with disc electrodes (5 mm in diameter) at the tip (CUY650-5; Nepa Gene, Chiba, Japan). For the analysis of migration and cell shape, the brains of mice were fixed with 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4), cut coronally into 100 μm slices with a Vibratome (VT1000S; Leica Microsystems), and imaged using a laser-scanning confocal microscope (LSM 5 PASCAL; Carl-Zeiss).

2.4 | Immunostaining and immunohistochemistry

Frozen sections of *Xpa*-knockdown brains were prepared and sliced into 14-μm sections. Immunohistochemical staining was performed on these sections using antibodies against MAP2 (AP20, Chemicon) and Ki-67 (Rabbit monoclonal antibody (SP6), Lab vision). For MAP2 samples, 0.1% Triton-X in phosphate-buffered saline (PBS) was used for permeabilization, and Ki-67 samples were heat-treated in a microwave. The VECTOR MOM Immunodetection Kit (Vector Laboratories, Newark, CA, USA) was used as a blocking reagent for immunostaining of MAP2 according to the manufacturer's protocol, and overnight blocking with 1% bovine serum albumin (BSA) in PBS was performed for Ki-67. After washing with PBS, the primary antibody of MAP2 was diluted with the attached MOM

diluent (1/100) and incubated with samples at room temperature for 1 h. Ki-67 was diluted to 1/100 with 1% BSA in PBS and incubated with samples at room temperature for 1 h. Subsequently, Alexa Fluor 594-conjugated anti-mouse or anti-rabbit IgG (H + L) antibody (Cell Signaling Technology, Danvers, MA, USA) was added to MAP2 or Ki-67 samples, respectively, and incubated at room temperature for 1 h. After washing with PBS, Hoechst counterstaining was performed using VECTASHIELD (Vector Laboratories).

2.5 | Modified scratch assay

The primary fibroblasts were inoculated in a collagen-coated 35 mm glass bottom dish (Matsunami Glass, Osaka, Japan) and incubated in a confluent state. The cells were peeled off in one direction with a small scraper and moved to a space without cells. Dulbecco's modified Eagle medium (DMEM) containing 0.5% fetal bovine serum (FBS) was used to reduce the effects of cell division. On the 3rd day after scraping, the cells were fixed with 10% formalin, stained with crystal violet, and observed under a phase-contrast microscope, and the distance traveled was measured. To confirm the results of the static analysis, in a confluent state, cells were peeled off with a scraper as described above, and live imaging was performed using an incubator microscope (BZ-9000, Keyence JAPAN). Live images were edited using Final Cut Pro (Adobe, Mountain View, CA, USA).

2.6 | Digital holographic microscopy

Digital holographic microscopy was performed as previously described.[16] The HoloMonitor® M4 microscope (Phase Holographic Imaging AB, Lund, Sweden) was used for imaging and tracking healthy control (HC) and XP-A cell movements. 3D holographic cell images were captured with a 20× magnification objective and a low-power 635 nm diode laser. Images were converted from wavelength interactions to cellular representations using a computer algorithm (AppSuite, phase holographic imaging). HC and XP-A cells (1×10^4 cells) in a 6-well plate and allowed to adhere for 24 h. Fibroblasts were treated with DMEM containing 0.5% FBS and images were captured every 10 min for 72 h.

2.7 | Cell lines and cell culture

A 1-year-old male patient with XP-A, designated XP101KO,[17] harbored a homozygous G-to-C mutation in the last nucleotide of intron 3 of *XPA*, which is a founder

mutation observed in Japanese patients with XP-A. The patient, XP101KO, and a 28-year-old male healthy control cooperated to establish primary skin fibroblasts after providing written informed consent, and all experiments were conducted after the approval of the ethical committee of the institutional review board of Kobe University. Fibroblasts were cultured in DMEM supplemented with 15% FBS in a humidified 37°C atmosphere containing 5% CO_2.

3 | RESULTS

3.1 | Knockdown of the *Xpa* gene delays neuronal radial migration in the developing cortex

In order to investigate the function of XPA in the developmental stage of the central nervous system, we knocked down the *Xpa* gene in utero, using an electroporation method to transduce shRNA vectors into the murine embryonic cerebral cortex. The shRNA vector with oligonucleotides targeting *Xpa* was transfected into the embryonal telencephalon of 14-day-old C57/BL6 mice (E14). EGFP was co-transfected with the shRNA vector, which enabled the transfected neurons to be marked in green. At E17, 3 days after electroporation, we compared the distribution of GFP-positive cells in the cerebral cortex between control and *Xpa* knockdown (*Xpa*-KD) mice (Figure 1A, B) after the confirmation of the knockdown efficiency (Figure S1). The knockdown efficiency is partial, and the knocked-down COS7 cells retain exogenous mouse XPA protein to some extent. We adopted *Xpa*-R1 as a shRNAi vector in the subsequent experiments. In the control brain, many GFP-positive cells reached the cortical plate (CP); on the contrary, in the *Xpa*-KD cells, few GFP-positive cells were distributed in the CP (Figure 1). Despite the obvious E14–E17 result, there was no clear difference in migration between control and *Xpa*-KD samples 2 days after electroporation (at E14–E16, Figure D, E). For further prolonged observation, we compared the control and *Xpa*-KD neurons at a later stage (E14-P0, Figure 1F, G). Consistent with the result at E14-E17, delayed migration was observed in *Xpa*-KD neurons at P0. In both *Xpa*-KD and control samples, the GFP-positive cells could reach the apical surface compared with the result at E17, suggesting that *Xpa*-KD neurons could have survived during the observation period (E14–P0).

To rule out the possibility that the delayed migration in *Xpa*-KD neurons was due to an off-target effect, the human *XPA* gene was co-transfected with the shRNA vector for the *Xpa* gene (Figure 1C) Four out of 20 target bases in the human *XPA* gene are different from those in mice; thus, knockdown failure is possible. The delayed migration in the *Xpa*-KD neurons was partially complemented,

as the human-*XPA*-transfected cells reached the cortical surface, unlike the *Xpa*-KD neurons. This result indicated that the effect of delayed migration could be caused by XPA deficiency.

3.2 | Delayed migration of Xpa-KD neurons affects cell cycle termination and differentiation from neural progenitor cells

During brain development, neural progenitor cells are in the cell cycle stage before migrating to the CP and performing an elevator movement. During migration to the CP, neural progenitor cells may exit the cell cycle stage and differentiate into mature neurons. To investigate the effect of delayed migration on cell cycle exit in *Xpa*-KD neurons, the cell cycle marker Ki-67 was used to examine their immunopositivity. In the control sample (E14–E17), Ki-67-positive cells were scarcely observed from the SVZ to the IZ, but no Ki-67-positive cells were observed in the CP (Figure 2A, B). In the *Xpa*-KD sample, Ki-67-positive cells were frequently observed from the SVZ to the IZ (Figure 2C, D). At E18.5, no Ki-67-positive cells were found in the CP in either the *Xpa*-KD or control samples (Figure 2E-H). These results imply cell cycle exits after neural progenitor cells migrate to the CP regardless of the genetic status of *Xpa*.

Next, to investigate the effect of delayed migration on the differentiation of *Xpa*-KD cells into mature neurons, immunohistochemical staining was performed and analyzed using a neuronal marker for differentiation, MAP2. In the control sample, all GFP-positive cells that reached the CP were MAP2-positive and differentiated normally into mature neurons (Figure 2I–L). On the contrary, in the E14-E17 *Xpa*-KD sample, GFP-positive cells remained in the VZ, SVZ, and IZ, and all of them were MAP2-negative (Figure 2M–P). This result indicated that the *Xpa*-KD cells displaying a delay in migration did not differentiate into neurons. Furthermore, we observed hypoplasia of 2–3 layers of the CP. This result indicated that abnormal cell migration of the *Xpa*-KD cells resulted in a failure to form the normal cerebral cortex. Similar results were obtained in the Ki-67 experiment, where it was observed that the progression of differentiation also depends on where the neurons reside and are not determined by the time after birth.

3.3 | Cell migration in fibroblasts from patients with XP-A was impaired in the modified migration assay

The results thus far indicate that *Xpa* knockdown in neurons causes delayed cell migration during cerebral
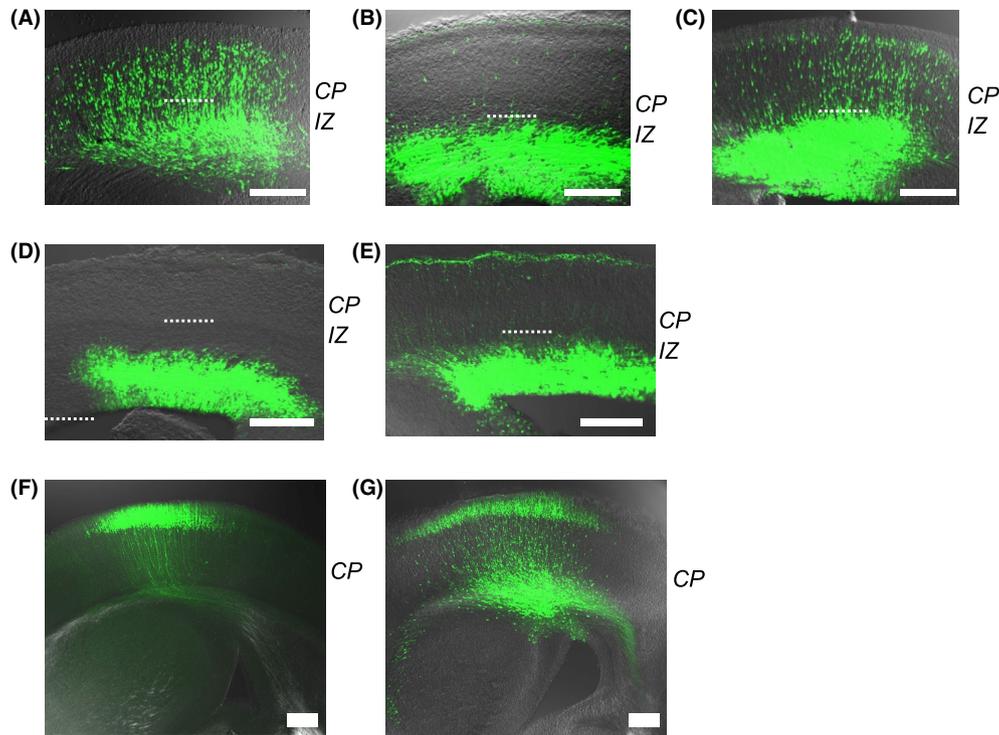
FIGURE 1 Delayed migration of embryonic neurons in mice in which *Xpa* was knocked down. Using an in utero electroporation method, shRNAi (Xpa-R1) targeting *Xpa* was introduced into the lateral ventricles of E14 C57BL/6 mice. Green fluorescence identifies the neurons into which EGFP was introduced. Gray signals indicate differential interference contrast images. EGFP-induced cortices were removed from E17 embryos and cerebral slices were observed using a confocal microscope. (A) Control mice; (B) Xpa-R1-introduced mice; (C) Xpa-R1+ h*XPA*-introduced mice. After introducing Xpa-R1 into the fetal mouse lateral ventricle at E14, embryonic cortices at E16 were removed, and the cerebral slices were observed with a confocal microscope (D, E: Control and Xpa-R1, respectively). Embryonic cortices were removed from P0 (F, G: Control, Xpa-R1). CP: cortical plate, IZ: intermediate zone, Dotted line: border between CP and IZ. Scale bar = 200 μm.

development, and their positional abnormalities affect the differentiation of neurons and cell cycle exit. To further strengthen the genotype–phenotype relationship between the lack of XPA and cell migration abnormalities, we examined both quantitative and qualitative aspects of the migration of fibroblasts derived from patients with XP-A diagnosed at Kobe University, designated XP101KO cells,[17] using a modified scratch-assay method. In this assay, when scratching confluent fibroblasts, the cells moved directionally into cell-free space (Figure 3A) and the behavior of cell migration was analyzed. We found that the cells from patients with XP-A had lower overall cell mobility than cells derived from healthy subjects. The number of XP-A cells that migrated farther was less than that of HC cells (Figure 3B). In addition, we performed live imaging using time-lapse microscopy to visualize cell migration. After creating a scratch, control cells initiated a movement in a directional manner into the cell-free space (Figure S2). Compared to control cells, the XP-A cells initially moved directionally into a cell-free space, but subsequently moved in a way that caused them to lose direction (Figure S3). The migration phenotypes observed in *Xpa*-KD neurons were also observed in XP-A cells (XP101KO) lacking XPA protein, indicating the role of XPA deficiency

in abnormal cell migration. Taken together, we determined that in addition to displaying defects in cell movement, these cells lost directionality.

3.4 | Live imaging analysis to study the impairment in XPA patient fibroblasts

Next, we compared the motility of fibroblasts derived from a healthy individual with that of XP101KO cells using a Holo Monitor®. We measured the total distance traveled and found that the XP101KO cells traveled shorter distances than HC fibroblasts. Moreover, the average motility of XP101KO cells was lower than that of healthy fibroblasts (Figure 4A). These results are consistent with those from our scratch assay, confirming that XPA deficiency impairs motility. To test whether these cells demonstrated additional defects, we selected several cells and tracked them for 72 h (Figure 4B). Interestingly, while a healthy person's fibroblasts moved equivalently in various directions, the XP-A cells moved in only limited directions (Figure 4B), contrary to the movement exhibited by XP-A cells in the previous section. This apparent contradiction can be explained by differences in the two assay systems,
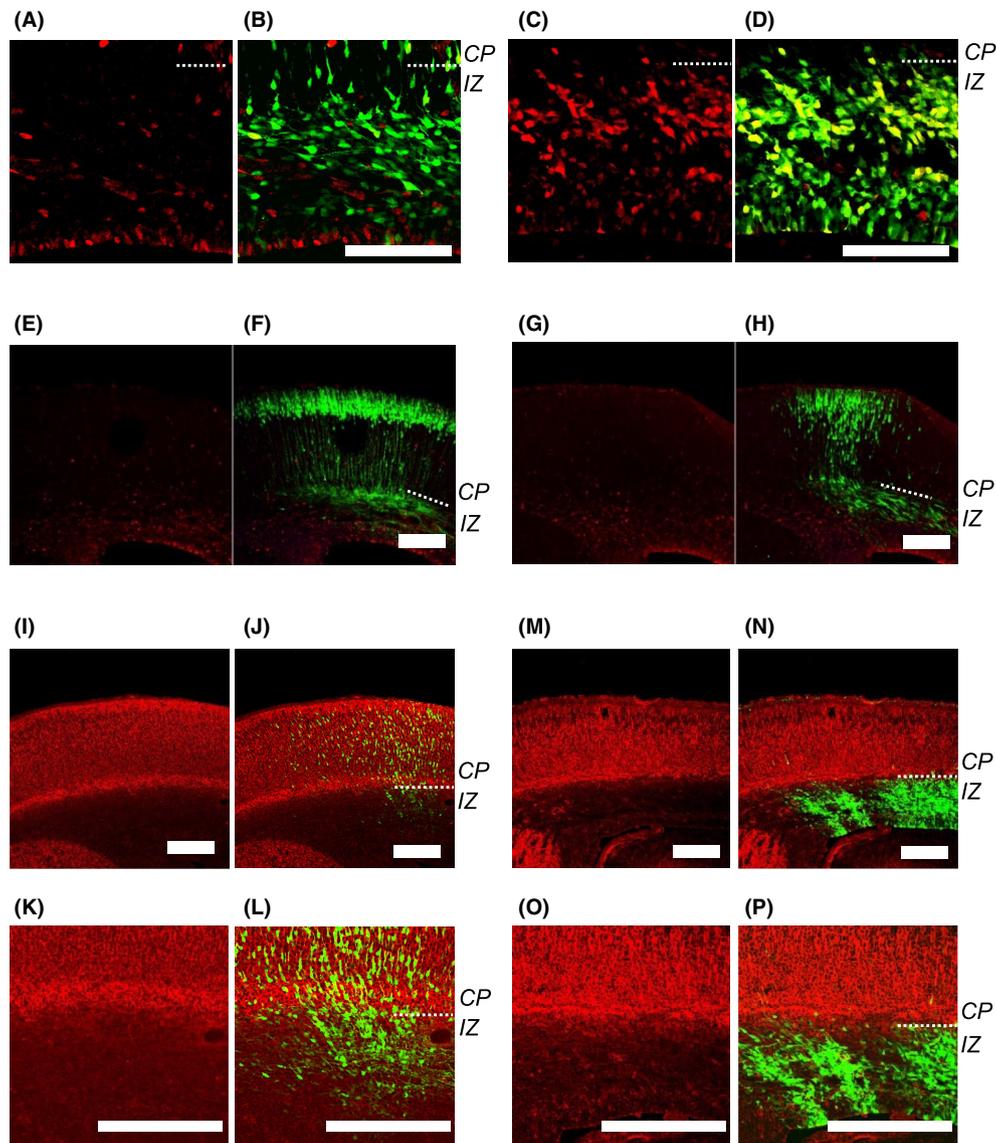
FIGURE 2 Delayed migration of *Xpa* knockdown neurons affects cell cycle termination and differentiation. Cell cycle and neural differentiation in *Xpa* knocked down neurons were analyzed by immunohistological analysis using Ki-67 antibody (A-H) or MAP2 antibody (I-P). CP: cortical plate, IZ: intermediate zone, Dotted line: border between CP and IZ. Green indicates EGFP signals and red signals indicate Ki-67 positive cells (A, B: Control E14-E17 C, D: Xpa-R1 E14-E17, E, F: Control E14-E18.5 G, H: Xpa-R1 E14-E18.5). Neurological differentiation was analyzed by immunohistological analysis using the MAP2 antibody. Green indicates EGFP signals and red signals indicate MAP-2 positive cells (I, J: Control E14-E17 low-magnification, K, L: Control E14-E17 high-magnification, M, N Xpa-R1 E14-E17 low-magnification, O, P: Xpa-R1 E14-E17 high-magnification). Scale bar = 200 μm.

with fibroblasts demonstrating a directed mode of migration in the modified migration assay while migrating randomly in the Holo Monitor®. Nevertheless, we conclude that the mobility of XP-A patient-derived fibroblasts is clearly lower than that of normal cells.

4 | DISCUSSION

Xpa-KD neurons in the embryonic cerebral cortex showed abnormal cell migration, cell cycle exit, and abnormal differentiation. Furthermore, cells derived from a patient

with XP-A, XP101KO cells, also showed abnormal migration. To the best of our knowledge, only one study has investigated the relationship between NER and cell migration.[18] According to that study, patients with TTD showed increased expression of MMP-1, which is involved in extracellular matrix metabolism, and showed increased cell mobility compared with normal and XP cells. Our results, for the first time, revealed that XP-A cells showed reduced cell mobility, contradicting the results for TTD cells. From previous work, we speculate that the reduced mobility of XPA-deficient cells could be due to the following reasons. Retinoic acid (RA) receptor is a nuclear receptor that acts
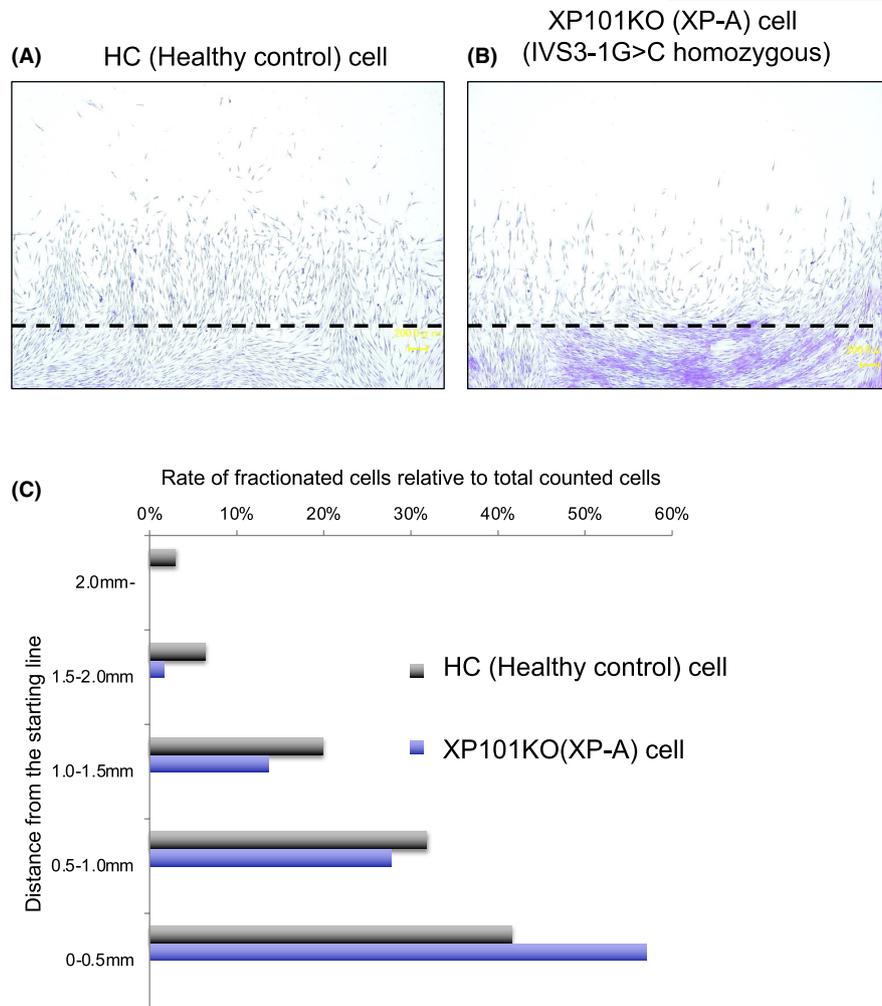
(A) HC (Healthy control) cell

(B) XP101KO (XP-A) cell
(IVS3-1G>C homozygous)

(C)

Rate of fractionated cells relative to total counted cells

- HC (Healthy control) cell
- XP101KO(XP-A) cell

Distance from the starting line

FIGURE 3 Migration of fibroblasts from the patient with XP-A was found to be impaired in the modified migration assay (A: Healthy person's fibroblast, B: XP-A patient's fibroblast). IVS3-1G > C homozygous: homozygous G-to-C mutation in the last nucleotide of intron 3 of *XPA*. The dotted lines indicate the starting line of cells made by scratching. The bar graph (C) is a quantification by measuring the number of cells in (A) and (B).

as a transcription factor. RA-mediated transactivation of RARB transcription has been suggested to depend on NER integrity.[19] In the study, the authors found that preinitiation complex formation preceded the sequential recruitment of the NER factors XPC, XPA, RPA, XPG, and XPF/ERCC1 at the promoters of inducible genes in the absence of an exogenous genotoxic attack. Consistent with this result, in XPA-deficient cells, transcripts that are normally activated by RA have been shown to be generally downregulated.[11] RA signals are important for nerve development and activated RA receptors are present in the dorsal and mid-region of the pallium during embryonic development.[20,21] A recent study showed that inhibition of RAR function delayed slate-born neuronal migration and led to a failure in maintaining neuronal cell fate via β-catenin signaling,[22] similar to the behavior observed in *Xpa*-KD neurons. Thus, we propose that the abnormal migration of XPA-deficient cells might be caused by the decreased transcriptional activity of RAR.

Whether the mechanism of neurological symptom onset in patients with XP is due to abnormalities in NER or disruption of other vital functions has been debated. Magnetic resonance imaging (MRI) suggests that patients with XP-A have structural brain abnormalities in early childhood before neurological symptoms become apparent.[2] Hypothetically, abnormal cell migration may explain this neural tissue abnormality observed in XP-A. We are optimistic that our data will contribute to the development of novel therapeutic agents for patients with XP. Although it is generally accepted that the onset of neurological symptoms in XP-A occurs between 3 and 8 years of age,[23] even 1- and 2-year-old patients with XP-A show neurological abnormalities such as a decline in delayed tendon reflex and slight mental retardation, respectively.[2,5] According to parents' accounts, some patients had a history of several months' delay in initial walking. Therefore, neurological disturbances in XP may start earlier than generally believed. There could be two possible reasons: one is that the onset of neurological disturbances
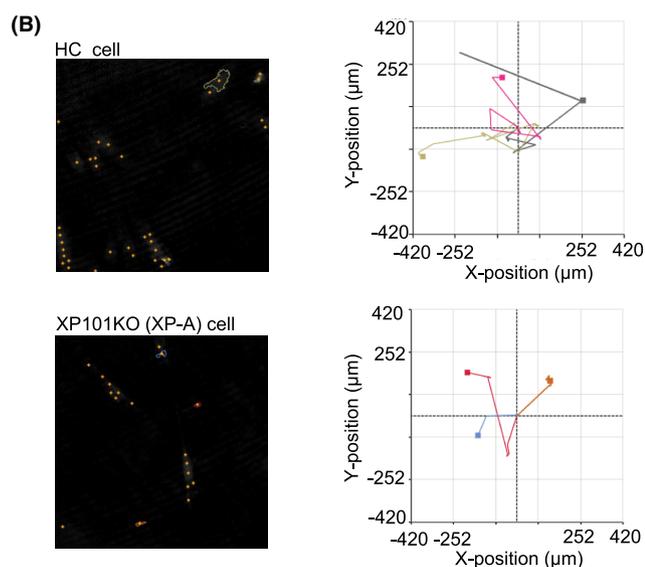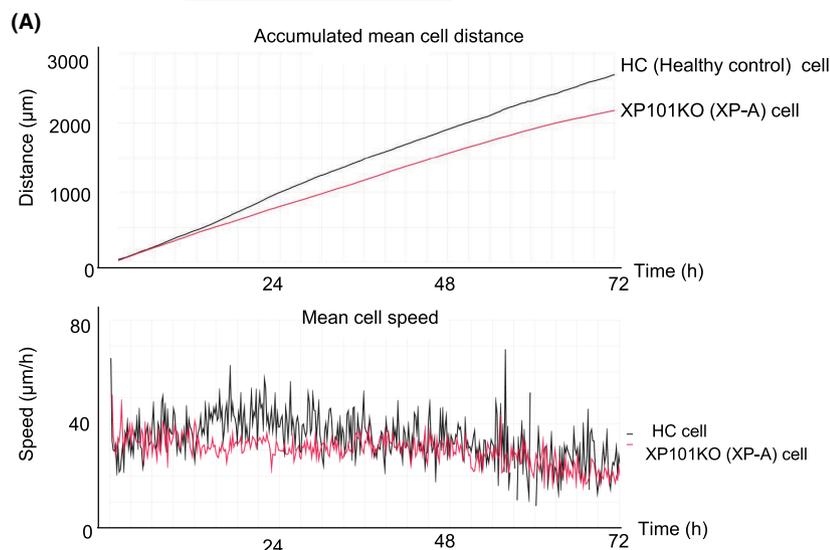
(A)

(B)

FIGURE 4 The analysis of cell motility and tracking using a HoloMonitor®M4. (A) The cellular motility distance (upper panel) and the cellular motility speed (lower panel) are shown. (B) The indicated cells were tracked for 72 h (left panels). Spatial movement graphs for selected cells (the starting position was the center) are shown in the right panel.

may insidiously start during the brain developmental stage, but is so mild that it becomes apparent only after birth but not during pregnancy in XP. The second possible explanation is that as early as embryonic stages, small amounts of oxidative DNA lesions could be produced, but the amount is too small to be identified as a neurological symptom. After birth under aerobic circumstances, additional oxidative metabolites are produced, and the progression of neurological symptoms concurrent with brain development is apparent at approximately 6 years of age.

Our results in this study indicate a likelihood of the former possibility, at least to some extent, due to the involvement of cyclopurine,[9] though we do not rule out a possible role of oxidative DNA lesions.

In conclusion, our study sheds some light on the pathomechanisms of neurological symptoms in XP, and further studies are expected to find treatment strategies for XP in the future.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All data are available in the main text or the supplementary materials.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.