Targeted Gene Therapy of Xeroderma Pigmentosum Cells Using Meganuclease and TALENT™

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Abstract
Xeroderma pigmentosum group C (XP-C) is a rare human syndrome characterized by hypersensitivity to UV light and a dramatic predisposition to skin neoplasms. XP-C cells are deficient in the nucleotide excision repair (NER) pathway, a complex process involved in the recognition and removal of DNA lesions. Several XPC mutations have been described, including a founder mutation in North African patients involving the deletion of a TG dinucleotide (ΔTG) located in the middle of exon 9. This deletion leads to the expression of an inactive truncated XPC protein, normally involved in the first step of NER. New approaches used for gene correction are based on the ability of engineered nucleases such as Meganucleases, Zinc-Finger nucleases or TALE nucleases to accurately generate a double strand break at a specific locus and promote correction by homologous recombination through the insertion of an exogenous DNA repair matrix. Here, we describe the targeted correction of the ΔTG mutation in XP-C cells using engineered meganuclease and TALENT™. The methylated status of the XPC locus, known to inhibit both of these nuclease activities, led us to adapt our experimental design to optimize their in vivo efficiencies. We show that demethylating treatment as well as the use of TALENT™ insensitive to CpG methylation enable successful correction of the ΔTG mutation. Such genetic correction leads to re-expression of the full-length XPC protein and to the recovery of NER capacity, attested by UV-C resistance of the corrected cells. Overall, we demonstrate that nuclease-based targeted approaches offer reliable and efficient strategies for gene correction.

Introduction
Xeroderma pigmentosum (XP) is a rare, autosomal, recessive syndrome characterized by hypersensitivity to UV light [1]. It is also associated with a dramatic predisposition to skin neoplasms. Thus, risk of melanoma and non-melanoma skin cancers has been reported to be increased 2 to 10 thousand-fold, respectively [2]. XP cells are deficient in the nucleotide excision repair (NER) pathway, a complex process involved in the recognition and removal of DNA lesions induced by UV light (cyclobutane pyrimidine dimers and pyrimidine 6-4 pyrimidone photoproducts) [3]. Seven different genes named XPA to XPG are involved in that process. Mutations within the XPC gene are by far the most common genetic alteration found in European and North African XP patients. Among the known genetic alterations, a founder mutation within exon 9 has been described in almost 90% of Maghrebian XP-C patients [4] and corresponds to the deletion of a TG dinucleotide leading to the expression of an inactive and undetectable XPC truncated protein. This lack of NER activity allows UV-dependent DNA damage to accumulate and is responsible for the development of high numbers of skin cancers. Today, there is no curative treatment for XP-C patients and their cancer-free survival relies solely on full body protection from light and/or surgical resections of skin tumors. Autologous grafts have been performed using UV sensitive cells, but the benefit of such treatment is transient [5]. A major advance in cancer prevention would be to engraft patient skin produced ex vivo with cells corrected for XPC mutation.

Recently, the stable trans-complementation of XPC deficiency has been reported [6]. Using a retrovirus-based strategy, Warrick et al. were able to transduce the wild-type XPC gene into human primary XP-C keratinocyte stem cells and reconstitute their full NER capacity resulting in UV resistance. Although successfully validated in vivo and in a relevant cell line, this complementation strategy is nonetheless liable to generate potential adverse effects due to uncontrolled random integrations of the transgene. Indeed, these undesirable effects have been reported in several complemented cells for disease treatment, especially in the hematopoietic system [7,8]. In view of this result, genetically modified skin could lead to skin tumor development following engraftment. In addition, because of the ectopic expression of the XPC transgene, this strategy prevents physiological regulations of the XPC.
transcription, the importance of which has been described in other studies [9,10]. Thus, an alternative and safer approach to curing XP-C defective cells is highly desirable.

In the past few years, several studies have demonstrated the tremendous potential of nuclease-based targeted approaches for gene correction [11–14]. These approaches rely on the ability of engineered nucleases known as Meganucleases, Zinc Finger nucleases, and TALE nucleases to generate a precise double-strand break at a specific locus and promote targeted homologous recombination (HR) with an exogenous DNA repair matrix [15–17].

In this study, we used engineered meganuclease and TALE nuclease to promote the targeted correction of XPC mutation in the XP4PA cell line, which carries the homozygote ΔTG mutation in the XPC gene. The presence of methylated cytosines (5 mCs) in the XPC locus led us to adapt the design of these tools, as well as our experimental conditions, to optimize their in vivo efficacy. We showed that treatment with a demethylating agent or the use of 5 mC insensitive nuclease allowed successful XPC gene correction without requiring selection marker. Such genetic correction enabled re-expression of the full-length XPC protein and full recovery of wild-type UV resistance in the XP4PA cell line. We demonstrate that nuclease-based targeted approaches constitute a robust and reliable strategy for XPC gene therapy.

Results

The recent development of engineered nucleases able to introduce a DSB and stimulate HR at a specific locus [18,19] has opened up new opportunities for XPC gene correction. In order to induce a high frequency of HR at an endogenous locus, it is crucial to generate specific and efficient nucleases. For this study, two types of engineered nucleases were developed to target a DNA sequence located 100 bp upstream from the ΔTG XPC founder mutation (Figure S1A). The first nuclease was a single-chain meganuclease named XPCm, derived from I-CreI endonuclease [15]. The second was a TALEN™ named XPCT1 and derived from TALE AvrBs3 [20].

An Engineered Meganuclease (XPCm) Specifically Designed to Target the XPC Locus

The engineered meganuclease XPCm has been previously described [15]. Its intrinsic activity was first determined by a single-strand annealing (SSA) extrachromosomal assay in CHO-K1 cells [Materials and Methods S1] [21]. XPCm showed high activity similar to that of the meganuclease RAG1m, used here as a positive control, and better than that of I-SceI (Figure S1B). We then assessed the ability of XPCm to cleave the endogenous XPCt sequence in 293-H cells by quantifying the frequency of targeted mutagenesis (TM) induced by XPCm at its endogenous locus. TM consists of small insertions or deletions of nucleotides occurring at the DSB site. TM was quantified by specific PCR (Figure 1A). In order to determine whether demethylating treatment directly affected the cleavage activity of the nuclease, we used LM-PCR to monitor the non-processed DNA ends generated upon cleavage by the meganuclease (Materials and Methods S1). The number of free DNA ends at the XPC locus was increased up to 7-fold in the presence of 5-aza-dC versus untreated cells (P<0.05) (Figure S2C). Although our protocol can only quantify non-processed DNA ends, this result strongly suggests that at least a substantial portion of TM stimulation results directly from higher cleavage activity of the meganuclease on unmethylated sequences.

We then evaluated the impact of 5-aza-dC on the ability of XPCm to trigger HGT in 293-H cells. The DNA repair matrix was composed of two homology arms interrupted by an exogenous DNA sequence (29 bp) specifically designed to screen and identify the HGT events by PCR. Because 5-aza-dC had a major impact on cellular proliferation at a dose of 1 μM, this experiment was performed with dose of 0.2 μM. As regards TM, our results showed that 5-aza-dC treatment increased HGT frequency 12-fold, leading to up to 12% of corrected cells (P<0.001) (Figure 1C). Altogether, our data indicated that the presence of an epigenetic effector such as a 5-aza-dC significantly enhanced meganuclease-assisted TM and HGT at the XPC locus.

XPC Gene Correction in XP4PA Cells using XPCm Meganuclease and a Demethylating Agent

The XP4PA cell line was derived from dermal fibroblast obtained from a patient bearing the homozygote mutation matching the TG deletion in exon 9 of the XPC gene [23]. These cells’ impairment in NER has been already described, as well as the possibility of complementation using plasmid or recombinant retroviruses expressing wild type XPC cDNA [24]. As in the 293-H cell line, the XPCt DNA sequence appeared to be fully methylated in XP4PA cells. Treatment of the cells with 0.2 μM 5-aza-dC led to 50% demethylation of XPCt (Figure 2A) and increased TM frequency 18-fold with respect to untreated cells (P<0.05) (Figure 2B). In order to perform gene correction experiments, we designed a new DNA matrix able to restore the
XPC open reading frame. It contained two arms of 1.5 and 1.8 Kb respectively, homologous to the wild type XPC sequences surrounding the cleavage site. To avoid any possible cleavage of the DNA repair matrix by XPCm, silent mutations were introduced within the meganuclease-recognizing site (Figure 2C). XP4PA cells were transfected with the DNA repair matrix and the meganuclease XPCm expression vector. Three days later, cells were seeded at a density of 100 cells per well in a 96-well plate. Each well was then screened for locus-specific integration using specific primers. While no positive wells were found in untreated conditions, 3 out of 480 wells were identified as positive after 5-aza-dC treatment. Further DNA sequencing confirmed that the initial XPC mutation was corrected in 2 out of these 3 cell populations (Figure 2D). Furthermore, the presence of all silent mutations, present only on the matrix plasmid, together with the corrected mutation (100 bp from cleavage site) indicated that these were transferred from the DNA matrix to the genomic DNA, and confirmed the homologous recombination process (data not shown). One corrected population was then sub-cloned. After amplification, we isolated three clones positive for the PCR-detected HGT event and used them for further phenotypic characterizations.

XPC Gene Correction in XP4PA Cells using XPCT1 TALEN™

We recently reported the design of methylation-insensitive TALE nuclease using the unconventional TALE repeat N* [20]. In order to determine whether this approach could be used for the gene correction of XP4PA cells, we used the TALEN™ (XPCT1) containing the N* residue previously described to induce up to 17% of TM frequency at the methylated XPCT1t in 293-H cells. As XPCT1 targets a genomic sequence (XPCT1t) overlapping the previous meganuclease target (Figure S1A), HGT experiments were performed with the same DNA repair matrix and therefore the same screening design as for gene correction experiment using meganuclease. XP4PA cells were transfected with the DNA matrix and a plasmid encoding the XPCT1. Three days post-transfection, a fraction of cells was recovered to verify the expression of TALEN™ by western blot (Figure 3A), seeded the remaining cells at a density of 20 cells/well and characterized them three weeks later. Specific PCR screening for HGT events revealed that HGT occurred in 6.25% ± 0.95 (respectively 56/192, 69/192) of transfected cells, taking into account the number of cells per well and the efficiencies of transfection and cloning (Figure 3B).

A second series of three independent experiments was then performed in which transfected cells were seeded at low density in
Petri dishes allowing for clonal cell expansion. Independent clones were isolated and characterized. Under these conditions, 4.9% ± 2.5 (respectively, 33/493, 49/796, 16/619) of clones were positive for HGT events (Figure 3B). As expected, no positive clone was obtained when cells were co-transfected with a non-targeting TALEN and the DNA matrix. In order to determine the frequency of ΔTG correction, DNA sequencing of the genomic XPC gene in 45 randomly chosen HGT-positive clones was performed. The presence of the wild-type sequence (i.e. the corrected sequence) was observed in 53% (24/45) of the HGT corrected clones resulting from transfection with the TALEN™ XPC-T1. Expression level was analyzed over 3 months at population doubling (PD) 35, 63, 95 and 125 (Figure S3). The full-length XPC protein was steadily expressed over time, demonstrating the stability of the genetic correction. Finally, NER rescue was confirmed by the cell survival rate following different UV exposures (Figure 4B). One third of corrected clones expressing the XPC protein were tested for sensitivity to UV-C and showed a clear improvement in survival rate, with a behavior similar to that of the MRC5 control cell line, proficient for XPC (Figure 4B). This UV-C survival rescue was confirmed in corrected clones obtained from 3 independent experiments (Figure S4). As expected, clones negative for HGT events and TG correction displayed high sensitivity to UV-C. Taken together, these results provided the first demonstration of a stable correction of XPC mutation using sequence-specific engineered nucleases.

**Discussion**

In this study, we showed that both meganuclease- and TALEN™-assisted targeted approaches allowed efficient correction of the XPC founder mutation in an X4PA cell line derived from the fibroblasts of XP-C patients. The successful correction of XPC, enabled stable re-expression of the full-length XPC protein and allowed X4PA cells to recover their fully functional NER pathway. Because of the methylation status of the sequences surrounding the XPC mutation, we opted for two independent strategies to overcome the nucleases’ sensitivity to methylation and enhance their activity in vivo.

In the case of the meganuclease XPCm, we used the epigenetic drug 5aza-dC to demethylate the XPC locus and rescue its nuclease activity. As expected, such treatments led to a significant enhancement of HGT frequency in 293-H cells (12-fold, 12% HGT, figure 1D). Although to a much lower extent, an increase of HGT frequency was also observed in X4PA patient cell line as HGT-positive clones were obtained only after 5-aza-dC treatment. In addition, the X4PA cells treated with 5-aza-dC displayed an altered cellular proliferation compared to their untreated counterparts. These two results might be explained by the co-existence of two deleterious factors in our experiment: the NER deficiency of X4PA cell line and the well known ability of 5-aza-dC to alter DNA structure, especially by being inserted into DNA as a nucleotide analogue and by promoting DSB, gene expression modification, cell cycle disruption [26]. Indeed, we hypothesized that due to their NER deficiency, X4PA cells would be unable to proficiently process altered DNA structures generated by 5-aza-dC, leading to significant cell death. Because the vast majority of corrected cells came from cells with a high level of demethylation (Figure 1B), we made the assumption that a high proportion of corrected cells were dying. Thereby, due to its deleterious effect, the use of 5-aza-dC, at least at the dose used in this study, may represent a hurdle for its application in primary keratinocytes, the relevant primary cells for genetic correction.

An alternative strategy was to use an engineered TALEN™ (XPC-T1) insensitive to 5mC. This TALEN™, previously described to induce up to 17% of TM frequency at the methylated XPC locus in 293-H cells [20], induced about 2.5% of genetically corrected cells.
corrected XP4PA cells in the presence of a repair matrix lacking selection marker. Because XPC is an autosomal recessive disease, a monoallelic correction of only few keratinocytes may be sufficient for clinical application providing a safe selection method is available [27].

Overall, with these two independent strategies, the TALENTM and the meganuclease succeeded at correcting the XPC locus and restoring the full NER pathway of XP4PA cells, as shown by an UV-C survival equivalent to that of MRC5 cells. Interestingly, among the HGT-positive clones identified by HGT-specific PCR, we found that 47% still exhibited the ΔTG mutation. Such a peculiar result could be explained by the fact that the length of the conversion tract during the homologous recombination mechanism is a function of the distance from the cleavage site [28]. For a

Figure 3. Homologous gene targeting (HGT) induced by the TALENTM (XPCT1) in XP4PA cells. (A) Western blot performed on protein extracts from cells transfected with XPCT1 (+) or empty vector (−). Each monomer, XPCT1R and XPCT1L was tagged with S-tag and HA-tag, respectively. (B) HGT frequency was determined from XP4PA cells transfected with XPCT1 (+) or non-related TALENTM (−) in the presence of the DNA correction matrix described in Figure 2. The transfected cells were seeded at a density of 20 cells/well or lower, enabling the formation of individual clones.
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Figure 4. Phenotypic correction of XP4PA cells. (A) Western blot performed on protein extracts from clones derived from transfection with the meganuclease XPCm in the presence of demethylating treatment (left panel) or from transfection with the TALENTM XPCT1 (right panel). XPC expression of corrected clones (Corr) was compared to negative controls, XP4PA (1), to uncorrected ΔTG clones (3), and to a positive control MRC5, proficient for XPC (2). In the left panel, an additional band is revealed by the XPC antibody. This band is most probably due to the non-specific binding of the antibody. Furthermore, this could be heightened by the 5-aza-dC treatment, as the band seems to appear only in treated samples. (B) UV-C survival assay on clones derived from gene correction experiment using XPCm (left panel) or using XPCT1 (right panel). The percentage of cell survival after exposure to UV-C of XPC corrected clones (closed triangle and lozenge) was compared to two negative controls, XP4PA and uncorrected ΔTG clone (open triangle and lozenge, respectively) and one positive control MRC5 (closed square).
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gene correction purpose, one can anticipate that the further the mutation to correct is from the cleavage site, the lower the correction frequency will be. To optimize the frequency of gene correction using nuclease-assisted targeted approaches, the engineered nuclease should be designed to cut as close as possible to the mutation to correct. Another way to improve the frequency of gene correction would be to modulate effectors such as MMR proteins, which have already been reported to decrease the gene conversion efficacy [29]. The downregulation of hMLH1 or MSH2 should increase the frequency of HGT. However, as for the use of a demethylating agent, it could be considered whether the benefits are greater than the potential adverse effects induced by MMR deficiency.

The targeted nature of nuclease-assisted gene correction offers a major advantage compared to conventional gene therapy strategies relying on retrovirus-assisted gene complementation. Because it is corrected in situ, the functional gene benefits from the natural chromosomal context and regulatory regions (endogenous promoter, terminator, and enhancer), known to play key roles in the fine tuning of gene expression. This is particularly true for the XPC promoter region, shown to contain regulatory elements located 1,700 bp upstream from the Transcription Start Site (TSS) [30]. XPC is induced following UV radiation, and the response seems to be substantial after repeated daily exposures. Likewise, our data showed that gene therapy using nucleases enabled the full-length XPC protein to be re-expressed at a level compatible with normal UV-dependent DNA damage repair. Physiological level of XPC expression is finely regulated and maintained at low background level. When XPC-GFP or HA-RAD4 were overexpressed in murine fibroblasts or in yeast respectively, a rapid degradation of these proteins by the proteasome was observed [31,32]. Overexpression of XPC could be detrimental due to its versatile capacity to recognize physiological distortions in the DNA double helix and to bind to DNA mismatch with high affinity. Finally, we observed a steady expression of the protein at least up to 125 population doubling (Figure S4), which indicated that the expression of the corrected XPC was not down-regulated with time. This suggests that targeted nuclease approaches are unlikely to trigger epigenetic silencing of the corrected gene, as reported in complementation using retroviral approaches [33].

In summary, our work provides the first evidence that nuclease-assisted targeted approaches promote successful correction of the XPC founder mutation and enable restoration of the NER pathway in XP4PA cells. This study represents a strong framework for further research into xeroderma pigmentosum gene therapy.

Materials and Methods

Engineered Nucleases

The XPCm, RAG1m, and CAPNS1m meganucleases used in this study are derived from I-CreI and were engineered as described previously [15]. They are designed to recognize sequences within the genes XPC (NM_004626), RAG1 (NM_000448.2) and CAPNS1 (NM_001749.2), respectively. The XPCm, RAG1m and CAPNS1m meganucleases were cloned in a circular plasmid. Three days post-transfection, cells were co-transfected with 3 μg of meganuclease expression vector or with 5 μg of each monomer of TALEN™ expression vector. As a control, cells were transfected with empty vector or non related TALEN™ (targeting a different genomic locus). Three days post-transfection, genomic DNA was extracted and the study targets were amplified using specific primers (Table S1). PCR amplicons were analyzed via regular or deep sequencing using specific primers (Table S1).

Monitoring of Nuclease Activity at Endogenous Loci

In order to evaluate the ability of nucleases to induce TM, 293-H or XP4PA cells were transfected with 3 μg of meganuclease expression vector or with 5 μg of each monomer of TALEN™ expression vector. As a control, cells were transfected with empty vector or non related TALEN™ (targeting a different genomic locus). Three days post-transfection, genomic DNA was extracted and the study targets were amplified using specific primers (Table S1). Flanking specific adapters needed for HTS sequencing, as described in Daboussi et al [15]. An average of 10,000 sequences per sample were analyzed. To evaluate the ability of nucleases to induce HGT at the XPC endogenous locus, cells were co-transfected with 3 μg of meganuclease expression vector and 2 μg of DNA circular matrix, or with 5 μg of each monomer of TALEN™ expression vector and 5 μg of DNA circular matrix. In 293-H cells, the matrix was composed of two homologous arms (980 bp and 1,000 bp) separated by 29 bp of an exogenous sequence. In XP4PA cells, the matrix was composed of two arms of 1.3 and 1.5 Kb homologous to the XPC sequences, separated by the meganuclease-recognizing site modified via silent mutation to avoid any cleavage of the matrix by XPCm (Figure 2C). The matrix was cloned in a circular plasmid. Three days post-transfection, cells were seeded at low density to form individual clones. Two weeks later, the colonies were picked up and transferred into 96-well plates for screening. DNA extraction was performed using the ZR-96 genomic DNA kit (Zymo research) according to the supplier’s protocol. The detection of targeted integration was performed via specific PCR amplification using one primer located within the heterologous insert of the DNA repair matrix and another located on the genomic sequence outside the matrix homology arms (Table S1). In pool experiments (20 cells/well or 100 cells/well), HGT frequencies were normalized to plating efficiencies (20%). Sequences of the primers used are presented in Table S1.

Demethylation Treatment

Two strategies were used: for 5-aza-dC treatment, the 293-H and XP4PA cells were pre-treated 48 hours before transfection with 0.2 μM or 1 μM of 5-aza-dC (Sigma) and the treatment was maintained 48 hours post-transfection. The medium was changed every day. Two days post-transfection, genomic DNA was extracted. The monitoring of demethylation treatment was performed via bisulfite treatment, which converts cytosine (C) but not 5-methylcytosine into Uracil, using to the DNA methylation Gold Kit (Zymo Research). DNA was then amplified via PCR using specific primers (Table S1). PCR amplicons were analyzed via regular or deep sequencing using specific primers (Table S1).
Phenotypic Characterization of XPC Corrected Clones

XPC expression was revealed by western blot using an XPC specific antibody 1:1000 (Abcam Ab62664). Actin antibody 1:10000 (Sigma A1978) was used as a loading control. For the survival assay, cells were seeded at a density of 1.10³ cells per well in 6-well plates and exposed the following day to different doses of UV-C (254nm) at a fluency of 0.3 J/m²/sec. Three days post-irradiation, cells were counted and the survival frequency was determined by the ratio between irradiated and non-irradiated cells.

Statistical Analysis

Data depicted in the Figure 1B were analysed using Chi² test. All the other statistical analysis were performed using Student’s t-test.

Supporting Information

Figure S1 Engineering of nucleases with recognition of the XPC sequence. (A) Description of the sequences targeted by the XPCm meganuclease and the XPCt TALEN™. The two CpG sequences are underlined. (B) In vivo cleavage activity of the XPCm, I-Scelm and RAG1 m engineered meganucleases monitored in an extrachromosomal SSA assay. TM (C) and HGT (D) frequencies were determined from 293-H cells transfected with XPCm or RAG1 m meganucleases. (TIF)

Figure S2 Impact of demethylating treatment on XPCt methylation status and biological consequences, in 293-H cells. (A) Chromatogram showing the impact of 5-aza-dC treatment on methylation status of XPCt. Cells were grown with 0.2 μM (+) or without (−) 5-aza-dC and transfected with empty vector under the same conditions as in TM or HGT experiments. While the CpGs present in XPCt were fully methylated under non-treated conditions, the 5-aza-dC treatment induced partial demethylation as shown by the presence of a double peak. This demethylation frequency was quantified after bisulfite treatment by deep sequencing (B). (C) Monitoring of non-processed DNA ends by LM-PCR in cells grown with 0.2 μM (+) or without (−) 5-aza-dC, and transfected with XPCm or RAG1 m. (TIF)

Figure S3 Long-term expression of the XPC protein in X4P4A corrected cells. Two corrected clones (Corr1 and Corr2) from transfection with XPCt and one clone from transfection with non-related TALEN™ (control ΔTG) were kept in culture for 3 months. Protein extracts were prepared at PD35, PD65, PD95 and PD125 following transfection and XPC protein expression was monitored by western blot. X4P4A and MRC5 were used as negative and positive controls, respectively. Beta-actin was used as a loading control. (TIF)

Figure S4 UV-C survival assay on clones derived from gene correction experiments using XPCt1. Clones corrected for TG mutation from experiments 1, 2 and 3 as well as uncorrected clones from experiments 1 and 2, parental cells X4P4A (negative control) or MRC5 cells, proficient for NER, were irradiated with UV-C. Three days post-irradiation, cells were counted. Cell survival was calculated as a ratio of number of cells counted after UV exposure to the number of cells counted in absence of exposure. This percentage was related to the percentage of survival of MRC5 cells. (TIF)

Table S1 Names and sequences of oligonucleotides used to perform bisulphite sequencing analysis of XPC locus, LM-PCR and Q-PCR of XPC and RAG1 loci and to monitor TM (Targeted Mutagenesis) and HGT (Homologous Gene Targeting) events at different endogenous loci in 293-H and X4P4A cells. (DOC)

Materials and Methods S1 Methodologies used to perform ligation-mediated PCR (LM-PCR) and to assess meganuclease and TALEN™ activities using an extrachromosomal assay. (DOC)

Author Contributions

Conceived and designed the experiments: AD JV RG AG A. Sarasin FD. Performed the experiments: AD SL JA RG CL A. Stary FP PD FD. Contributed reagents/materials/analysis tools: AD SL JA RG CL A. Stary FP PD FD. Wrote the paper: JV RG PD A. Sarasin FD.

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