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ORIGINAL ARTICLE

The GH/IGF-1 axis in a critical period early in life determines cellular DNA repair capacity by altering transcriptional regulation of DNA repair-related genes: implications for the developmental origins of cancer

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Abstract Experimental, clinical, and epidemiological findings support the concept of developmental origins of health and disease (DOHAD), suggesting that early-life hormonal influences during a sensitive period around adolescence have a powerful impact on cancer morbidity later in life. The endocrine changes that occur during puberty are highly conserved across mammalian species and include dramatic increases in circulating GH and IGF-1 levels. Importantly, patients with developmental IGF-1 deficiency due to GH insensitivity (Laron syndrome) do not develop cancer during aging. Rodents with developmental GH/IGF-1 deficiency also exhibit significantly decreased cancer incidence at old age, marked resistance to chemically induced carcinogenesis, and cellular resistance to genotoxic stressors. Early-life treatment of GH/IGF-1-deficient mice and rats with GH reverses the cancer resistance phenotype; however, the underlying molecular mechanisms remain elusive. The present study was designed to test the hypothesis that developmental GH/IGF-1 status impacts cellular DNA repair mechanisms. To achieve that goal, we assessed repair of γ-irradiation-induced DNA damage (single-cell gel electrophoresis/comet assay) and basal and post-irradiation expression of DNA repair-related genes (qPCR) in primary fibroblasts derived from control rats, Lewis dwarf rats (a model of developmental GH/IGF-1 deficiency), and GH-replete dwarf rats (GH administered beginning at 5 weeks of age, for 30 days). We found that developmental GH/IGF-1 deficiency resulted in persisting increases in cellular DNA repair capacity and upregulation of several DNA repair-related genes (e.g., *Gadd45a*, *Bbc3*). Peripubertal GH treatment reversed the radiation resistance phenotype. Fibroblasts of GH/IGF-1-deficient Snell dwarf mice also exhibited improved DNA repair capacity, showing that the persisting influence of peripubertal GH/IGF-1 status is not species-dependent. Collectively, GH/IGF-1 levels during a critical period during early life determine cellular DNA repair capacity in rodents, presumably by transcriptional control of genes involved in DNA repair. Because lifestyle factors (e.g., nutrition and childhood obesity) cause huge variation in peripubertal GH/IGF-1 levels in children,

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further studies are warranted to determine their persisting influence on cellular cancer resistance pathways.

Keywords Growth hormone · Insulin-like growth factor-1 · Lifespan, health span · Longevity · Endocrine · Cellular resilience · Stress resistance

Introduction

Laboratory and epidemiological studies during the past two decades support the developmental origins of health and disease (DOHAD) hypothesis suggesting that early-life events during a sensitive period of development have a fundamental effect on the organism's susceptibility for malignancies in later life(Johnson et al. 2009; Walker and Ho 2012). Growing clinical and experimental evidence suggests that the endocrine milieu present around puberty, when rapid physical growth occurs, induces persisting cellular resilience programs and stress response molecular networks(Dominick et al. 2016; Leiser et al. 2006; Murakami et al. 2003; Page et al. 2009; Leiser and Miller 2010; Panici et al. 2010) that affect the pathogenesis of cancer in adulthood. Accordingly, there is evidence that the origins of breast cancer occur during puberty, a time of rapid breast development (Zhao et al. 2013; Haslam and Schwartz 2011; Olson et al. 2010). Other studies link early-life endocrine influences to the pathogenesis of prostate cancer, bladder cancer, and colon cancer in later stages of life. The endocrine changes that occur during development and puberty are highly conserved across mammalian species and include dramatic increases in GH and the anabolic hormone insulin-like growth factor-1 (IGF-1) (Sonntag et al. 2012; Carter et al. 2002a; D'Costa et al. 1993; Deak and Sonntag 2012; Sonntag et al. 1999, 2000, 2005a). Recent experimental studies have uncovered evidence suggesting that levels of GH and IGF-1 during early life are important in determining susceptibility to cancer later in life (Panici et al. 2010; Ramsey et al. 2002). Importantly, in humans, the peripubertal GH/IGF-1 surge is highly variable. A breakthrough longitudinal study of individuals who carry mutations in the growth hormone receptor gene demonstrates that severe childhood IGF-1 deficiency results in zero cancer incidence later in life (Guevara-Aguirre et al. 2011). Experimental findings obtained in rodent models of developmental GH/IGF-1 deficiency corroborate the clinical data (Panici et al. 2010). Yet, the mechanisms by which early-life GH/IGF-1 levels impact carcinogenesis later in life remain elusive.

To explain the causal link between IGF-1 and carcinogenesis, the growth-promoting and antiapoptotic functions of the IGF-1 pathway were evoked (Pollak et al. 2004; Yakar et al. 2004). It is well known that IGF-1 is a potent growth factor, and proliferation of tumor cells in vitro is stimulated by administration of IGF-1 (Osborne et al. 1976). While the aforementioned studies primarily focused on the possible role of growth-promoting functions of IGF-1, emerging evidence points to potentially highly important, yet under-studied, links among peripubertal GH and IGF-1 levels, cellular resilience programs, and susceptibility to carcinogenesis. Accordingly, there are reports that rodents with developmental GH/IGF-1 deficiency exhibit marked resistance to chemically induced cancers and their cells are resistant to the damaging effects of known mutagens in vitro (Panici et al. 2010; Ramsey et al. 2002; Yakar et al. 2004; Sonntag et al. 2005b; Moore et al. 2008; Olivo-Marston et al. 2009; Ikeno et al. 2003; Bokov et al. 2009; Brown-Borg et al. 2009; Salmon et al. 2005). Studies in rat and murine models of GH and/or IGF-1 deficiency suggest that a critical time window exists around puberty when circulating levels of GH and/or IGF-1 determine both susceptibility to chemically induced carcinogenesis (Ramsey et al. 2002) and incidence of spontaneously occurring malignancies later in life (Panici et al. 2010; Ikeno et al. 2003). These studies suggest that developmental GH/IGF-1 deficiency enhances cellular resistance to genotoxic stresses (Page et al. 2009; Panici et al. 2010). Both in humans and experimental animals, cellular DNA repair pathways determine risk of cancer.

The present study was designed to test the hypothesis that GH/IGF-1 status in a critical period early in life determines cellular DNA repair capacity by altering transcriptional regulation of DNA repair-related genes. To test these hypotheses, we assessed repair of γ-irradiation-induced DNA damage (by single-cell gel electrophoresis/comet assay) and analyzed basal and post-irradiation expression of DNA repair-related genes (by qPCR) in primary fibroblasts derived from control rats, Lewis dwarf rats, and Lewis dwarf rats with peripubertal GH replacement. The Lewis dwarf rat is a useful model of human isolated developmental GH/IGF-1 deficiency, as these animals have normal pituitary function except for a selective genetic GH deficiency, and they mimic many of the pathophysiological alterations present in human GH/IGF-1-deficient children. DNA

repair capacity was also assessed in primary fibroblasts derived from Snell dwarf mice and respective wild-type control mice. Snell dwarf mice lack GH, prolactin (PRL), and thyroid-stimulating hormone (TSH) throughout development and exhibit resistance to spontaneous cancers.

Methods

Experimental animals

Lewis dwarf rats

In the present study, we used male Lewis rats that are heterozygous or homozygous for the spontaneous autosomal recessive *dw-4* mutation, which causes a decrease in GH secretion from the pituitary gland beginning around post-natal day 26 (Carter et al. 2002a, b; Charlton et al. 1988). Lewis dwarf (*dw-4/dw-4*) rats have chronically low levels of GH and IGF-1 and make an excellent animal model of isolated GH/IGF-1 deficiency (Charlton et al. 1988; Bailey-Downs et al. 2012a; Ungvari et al. 2010; Ungvari et al. 2011; Yan et al. 2014). Female heterozygous (*dw-4/–*) Lewis rats were bred with male homozygous Lewis dwarf rats (*dw-4/dw-4*) to generate heterozygous (*dw-4/–*) offspring with a normal phenotype ("control") or homozygous rats (*dw-4/dw-4*) with a dwarf phenotype ("dwarf"). Classification as control or dwarf was based on their body weight as well as serum IGF-1 levels at 33 days of age. Total IGF-1 levels in serum were determined as previously reported (Ungvari et al. 2011). Beginning on day 35, dwarf rats were divided into two experimental groups: (1) dwarf rats given saline ($n = 6$) and (2) GH-replete dwarf rats with GH administered beginning at 5 weeks of age and continued throughout the experimental period of 30 days (termed "GH-replete," $n = 6$). Saline or GH (300 μg recombinant porcine GH, Alpharma, Victoria, Australia) was injected s.c. twice daily. The heterozygous rats were used as controls and given saline injections twice daily from 5 weeks of age to the end of the experimental period. Recently, we reported that at the end of the experimental period, in this experimental cohort, control and dwarf GH-replete rats had significantly higher serum IGF-1 levels (control: ~900–1000 ng/mL; dwarf: ~400 ng/mL; GH-replete: ~800–900 ng/mL) compared to the untreated dwarf rats, indicating that twice daily administration of GH to the dwarf rats effectively normalizes serum IGF-1 (Ungvari et al. 2011). Rats had access to food and water ad libitum and were housed in pairs in the specific pathogen-free barrier facility of the University of Oklahoma Health Sciences Center. Animals were fed standard rodent chow (PicoLab Rodent Diet 20 from Purina Mills [Richmond, IN], containing 20% protein by mass, 24% protein by caloric content). Animals were killed by decapitation 30 days after the treatment period to establish primary fibroblast cultures.

Snell dwarf mice

Snell dwarf ($n = 5$) and littermate control mice ($n = 5$) were produced by a cross between (DW/J × C3H/HeJ)-dw/+ heterozygous parents and maintained in the specific pathogen-free barrier facility of the University of Michigan using husbandry conditions that have been described previously (Vergara et al. 2004). Mice with the dw/dw genotype were identified at approximately 3 weeks of age by their small body size (dwarfs). The mice were housed in microisolator cages with 1/8 in. Bed-O-Cob bedding (The Andersons, Maumee, OH), free access to tap water and Purina 5001 Rodent Chow (St. Louis, MO); in addition, moist or crushed pellets of chow were placed on the floor of cages housing dw/dw animals. Dwarf mice were caged with normal sized females ("warmer" mice) to prevent premature death of the dwarf mice from hypothermia.

Post-developmental liver-specific knockdown of Igf1 in mice

In additional studies, to separate the effects of early- and late-life IGF-1 deficiency, blood that was collected from the tail vein of a mouse model of adult-onset isolated circulating IGF-1 deficiency (*Igf1*$^{f/f}$ + TBG-Cre-AAV8) was used. The generation and husbandry of these mice were recently reported (Tarantini et al. 2016a, b; Toth et al. 2014a, 2015). In brief, male mice homozygous for a floxed exon 4 of the *Igf1* gene (*Igf1*$^{f/f}$) in a C57BL/6 background were purchased from Jackson Laboratories. These mice have the entirety of exon 4 of the *Igf1* gene flanked by loxP sites, which allows for genomic excision of this exon when exposed to Cre recombinase. Transcripts of the altered *Igf1* gene yield a protein upon translation that fails to bind the IGF receptor. To target hepatocytes, adeno-associated viruses (AAVs) were purchased from the University of Pennsylvania Vector Core (Philadelphia, PA). At 4 months of age, approximately

1.3 × 10 (Haslam and Schwartz 2011) viral particles (as assayed by genome content at the University of Pennsylvania) of AAV8.TBG.PI.Cre.rBG or AAV8.TBG.PI.eGFP.WPRE.bGH were administered to *Igf1*$^{f/f}$ mice to knockdown IGF-1 or as a control, respectively. Mice were anesthetized with ketamine/xylazine (100 and 15 mg/kg, respectively) and given retro-orbital injections of virus diluted to the appropriate concentration in 100-μL 0.9% saline. While AAV8 is effective at transducing multiple tissues after i.v. delivery, including the liver, the thyroxine binding globulin (TBG) promoter restricts expression solely to hepatocytes (Toth et al. 2014b, 2015). Blood was collected at 6 months of age. All studies were approved by the Institutional Animal Care and Use Committees of the respective institutions.

Isolation of fibroblasts and cell culture techniques

Primary fibroblast cell lines were established from rats as previously described (Ungvari et al. 2011). In brief, skin samples were digested with collagenase (at 37 °C and 5% CO_2 for 30 min), then washed twice with MEM, supplemented with 10% heat- inactivated fetal bovine serum (Hyclone). Cells were plated into 100-mm dishes with MEM media supplemented with 10% heat-inactivated fetal bovine serum plus penicillin/streptomycin/Fungizone (at 5% CO_2 and 3% O_2, at 37 °C). After 18 h, the media was changed to discard unattached cells. The fibroblasts were subsequently cultured, as described previously (Labinskyy et al. 2009).

Primary fibroblast cultures from mice were generated using a previously published protocol (Salmon et al. 2005). In brief, tail skin biopsies ~3–5 mm in length were obtained from the latter half of the intact tail of isoflurane-anesthetized mice after skin sterilization with 70% ethanol. Biopsies were further washed in 70% ethanol, placed in DMEM (high-glucose variant; GIBCO-Invitrogen, Carlsbad, CA), diced to <0.5 mm, and digested overnight with collagenase type II (400 U/mL, 1000 U total per tail, GIBCO-Invitrogen) dissolved in DMEM supplemented with 20% heat-inactivated fetal bovine serum, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; Sigma, St. Louis, MO), and 0.25 μg/mL fungizone (Biowhittaker-Cambrex Life Sciences, Walkersville, MD) at 37 °C in a humidified incubator with 5% CO_2 in air. After collagenase treatment, cells were dislodged from digested tissue by repeated pipetting and passed through sterile nylon netting into sterile 14-mL centrifuge tubes (BD Dickenson, Bedford, MA). Samples were centrifuged for 5 min at 200*g*, and collagenase solution was drawn off the cell pellet. Cells were resuspended in DMEM with 20% heat-inactivated fetal bovine serum, antibiotics, and fungizone. Approximately 2.5×10^5 cells in 3 mL of medium were seeded into tissue culture flasks of 25-cm^2 surface area (Corning Costar, Corning, NY). After 3 days, ~2/3 total volume of medium was removed on day 3 and replaced with fresh DMEM with 20% heat-inactivated fetal bovine serum, antibiotics, and fungizone. Six or seven days after seeding, initial cultures (designated first-passage cells) were either 1) split six- or ninefold by volume to create second-passage cells, with twofold or threefold dilutions at each subsequent passage, or (2) split and seeded at a density of 1 × 105 cells/cm^2 flask surface area at each passage into tissue culture flasks of 75 or 175-cm^2 surface area (Corning Costar). Cells were split by first washing flasks with 1× phosphate-buffered saline solution (PBS: 8.8 g NaCl, 2.25 g Na2HPO$_4$, and 0.26 g NaH_2PO_4/L distilled H_2O, pH 7.3) followed by incubation with ~3 mL of trypsin/100 cm^2 surface area of flask 1× trypsin-EDTA (GIBCO-Invitrogen) for ~5 min at 37 °C in a humidified incubator with 5% CO_2 in air. Trypsin activity was inhibited with an equal volume of DMEM with 20% heat-inactivated fetal bovine serum, antibiotics, and fungizone. Subsequent passages were split at 6-day intervals with ~2/3 total volume of medium removed on day 3 and replaced with fresh DMEM with 20% heat-inactivated fetal bovine serum, antibiotics, and fungizone.

Multiple cell lines were established from each experimental group (n = 5–6 for each group). One cell line per animal was used at the end of the third passage for measurement of cellular DNA repair capacity and expression of DNA repair-related genes.

γ-Irradiation protocol

γ-Irradiation to the cells was administered using a ^{137}Cs gamma irradiator (GammaCell 40, Nordion International). The irradiator was activated for a time calculated to deliver from 3 to 9 Gy, depending on the protocol. Dosimetry was

performed as previously described (Warrington et al. 2011, 2012) to confirm the dose received.

DNA damage analysis by alkaline single-cell gel electrophoresis

To compare susceptibility of cultured fibroblasts to DNA damage induced by γ-irradiation, single-cell gel electrophoresis was performed following our published protocol (Ungvari et al. 2013a; Csiszar et al. 2007, 2008; Ungvari et al. 2007). In brief, 10^5 cells in 100 μL PBS were mixed with 100 μL of 1.5% low-melting agarose and 90 μL of this mixture spotted on CometAssay slides (Trevigen, Gaithersburg, MD) between two layers of 1% low-melting agarose. DNA damage was induced by exposure of the slides to γ-irradiation (4 Gy). The extent of DNA fragmentation was examined by single-cell electrophoresis ("comet assay"), as reported (Ungvari et al. 2013a, 2007; Csiszar et al. 2007, 2008). The comet assay is based on the alkaline lysis of labile DNA at sites of damage. The slides were treated with a lysis solution (NaCl 2.5 M, Na_2EDTA 100 mM, Triton X-100 1% DMSO 10%, Trizma base 10 mM; pH 10; for 1 h at 4 °C in the dark), rinsed with a neutralization buffer (3 × 5 min, 0.4 M Tris, pH 7.5) to remove detergents and salts, then submerged in a high pH buffer (NaOH 300 mM, Na_2EDTA 1 mM, pH > 13) for 20 min to allow for unwinding of the DNA and the expression of alkali-labile damage. Electrophoresis was performed using the same buffer at 25 V (~0.74 V/cm) and 300 mA for 20 min. At the end of the run, the slides were neutralized in Tris-HCl 0.4 M, pH 7.5, submersed in absolute ethanol for 3 min, air-dried for 5–10 min, and DNA was stained with SYBR green (Invitrogen). Fluorescent images of the nuclei were captured using an EVOS FL Cell Imaging System (Invitrogen) at 20× magnification. DNA damage was quantified by measuring the tail DNA content (as a percentage of total DNA) using the Comet Assay-IV software (Perceptive Instruments, Haverhill, Suffolk, UK). For assessment of DNA repair efficiency, the cells were irradiated with 3 Gy, and the extent of DNA damage was assessed by the comet assay at 10, 20, 30, 40, 60, and/or 90 min post-irradiation (Csiszar et al. 2007). The percentage of residual DNA damage was plotted as a function of time. The time constant calculated from this plot and the residual DNA damage at 20 to 60 min post-irradiation were used as indices of DNA repair capacity.

Targeted qPCR array to analyze mRNA expression of genes involved in regulation of DNA repair processes

A quantitative real-time RT-PCR technique was used to analyze mRNA expression of genes known to be involved in regulation of DNA repair pathways in fibroblasts derived from control rats, Lewis dwarf rats, and GH-replete dwarf rats as reported (Tarantini et al. 2016b; Csiszar et al. 2013; Tucsek et al. 2013, 2014; Toth et al. 2013). The fibroblasts were harvested for analysis before or 2, 4, or 6 h after exposure to γ-irradiaton (3 Gy). In brief, total RNA was isolated with a Mini RNA Isolation Kit (Zymo Research, Orange, CA) and was reverse transcribed using Superscript III RT (Invitrogen) as described previously (Bailey-Downs et al. 2012b). mRNA expression of DNA repair genes was analyzed using validated TaqMan probes (Applied Biosystems) and a Strategen MX3000 platform, as previously reported (Tucsek et al. 2014). Quantification was performed using the ΔΔCt method as described (Tarantini et al. 2016b; Csiszar et al. 2013; Tucsek et al. 2013; Toth et al. 2013; Tucsek et al. 2014).

Statistical analysis

Data were normalized to the respective control mean values and are expressed as means ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All statistical comparisons were performed using Prism 5.0 for Windows (Graphpad Software, La Jolla, CA) and were considered significant at $p < 0.05$.

Results

Early-life GH/IGF-1 status of donor animals does not affect the severity of γ-irradiation-induced initial DNA damage in cultured primary fibroblasts

To assess DNA damage induced by γ-irradiation, a random sample of 200 cells was analyzed from each slide. Figures 1a and 2a show examples of images of the comet assay using cells derived from Lewis dwarf rats and Snell dwarf mice, respectively. In each cell line without γ-irradiation, all the DNA was confined to the nuclei, as indicated by the percentage of DNA in the tail less than 5%. In cells with γ-irradiation-induced DNA strand breaks, a

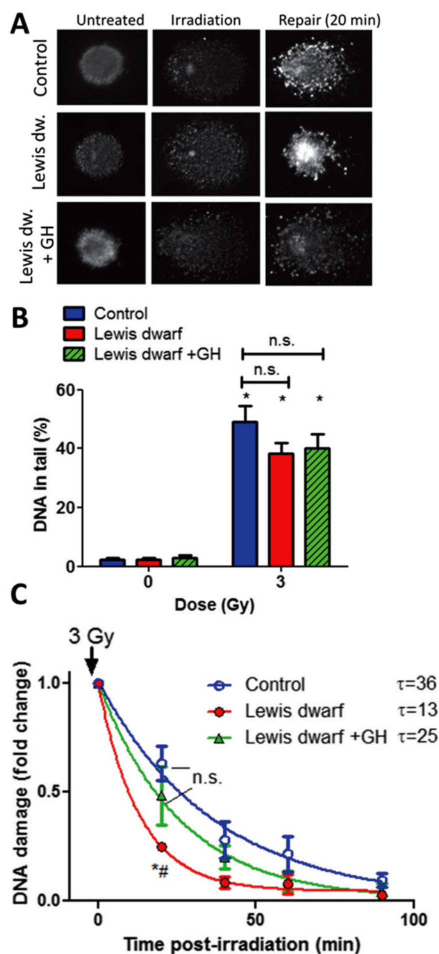

Fig. 1 Early-life GH/IGF-1 status of donor rats elicits persisting alterations in DNA repair capacity in cultured primary fibroblasts. Fibroblasts were γ-irradiated, and DNA damage was assessed by single-cell electrophoresis ("comet assay") at different time points post-irradiation. Damaged DNA migrates during electrophoresis from the nucleus toward the anode, forming the shape of a "comet" with a head (cell nucleus with intact DNA) and a tail (relaxed and broken DNA). **a** Representative images of damaged DNA in irradiated (3 Gy) fibroblasts derived from control rats, Lewis dwarf rats, and Lewis dwarf rats with early-life GH treatment. Median values of tail DNA content in each sample were determined. Note the marked decline in tail DNA content at 20 min post-irradiation due to DNA repair processes. **b** γ-Irradiated fibroblasts exhibit comparable DNA damage. Shown are γ-irradiation (3 Gy)-induced increases in tail DNA content in fibroblasts derived from control rats, Lewis dwarf rats, and Lewis dwarf rats with peripubertal GH treatment. *$p < 0.05$ vs respective non-irradiated controls (one-way ANOVA, Tukey's multiple comparisons). **c** Fibroblasts derived from control rats Lewis dwarf rats exhibit increased DNA repair capacity, which is prevented by peripubertal GH treatment. For assessment of DNA repair efficiency, the percentage of residual DNA damage was plotted as a function of time post-irradiation. Tail DNA content at each time point is shown. The residual DNA damage at 20 min post-irradiation was the lowest in fibroblasts from untreated Lewis dwarf rats, indicating more efficient DNA repair processes in these cells, which was reversed by peripubertal GH treatment. *$p < 0.001$ Lewis dwarf vs control; # Lewis dwarf vs Lewis dwarf GH (one-way ANOVA, Tukey's multiple comparisons); data are mean ± SEM (n = 5–6 for each time-point). The time constant calculated from these plots (τ) is shown next to the legends

bright fluorescent tail along the electric field was observed because small DNA fragments migrated out of the nuclei (Figs. 1a and 2a). Figures 1b and 2b show the DNA content in the tail (indicating the severity of DNA damage) as a function of the dose of γ-irradiation in each experimental group. The severity of γ-irradiation-induced DNA damage in Lewis dwarf fibroblasts (Fig. 1b) and Snell dwarf fibroblasts (Fig. 2b) did not differ significantly from that in their respective controls.

Early-life GH/IGF-1 status of donor animals elicits persisting alterations in DNA repair capacity in cultured primary fibroblasts

For assessment of DNA repair efficiency, the percentage of residual DNA damage in cultured fibroblasts was plotted as a function of time post-irradiation. We found that the percentage of residual DNA damage post-irradiation was lower in fibroblasts derived from Lewis dwarf rats than in control cells (Fig. 1c), indicating that early-life GH/IGF-1 deficiency associates with persisting increases in cellular DNA repair capacity. The percentage of residual DNA damage post-irradiation did not differ significantly in fibroblasts derived from Lewis dwarf rats with early-life GH treatment and in control cells (Fig. 1c), indicating that GH treatment of donor Lewis dwarf rats decreased cellular DNA repair capacity. These results highlight the importance of early-life GH/IGF-1 status in regulation of cellular DNA repair capacity. This concept is also supported by findings obtained in Snell dwarf fibroblasts showing decreased residual DNA damage post-irradiation in this mouse model of developmental GH/IGF-1 deficiency (Fig. 2c). To separate the effects of early- and late-life IGF-1 deficiency on cellular DNA repair in mice, a comet assay was performed using blood derived from a mouse model of adult-onset isolated circulating IGF-1 deficiency (*Igf1*$^{f/f}$ + TBG-Cre-AAV8). The IGF-1 levels in this model are decreased by ~70% as reported (Tarantini et al. 2016a, b; Toth et al. 2015, 2014a). We found that adult-onset IGF-1 deficiency did not have any obvious effect on cellular DNA repair (Fig. 2d).

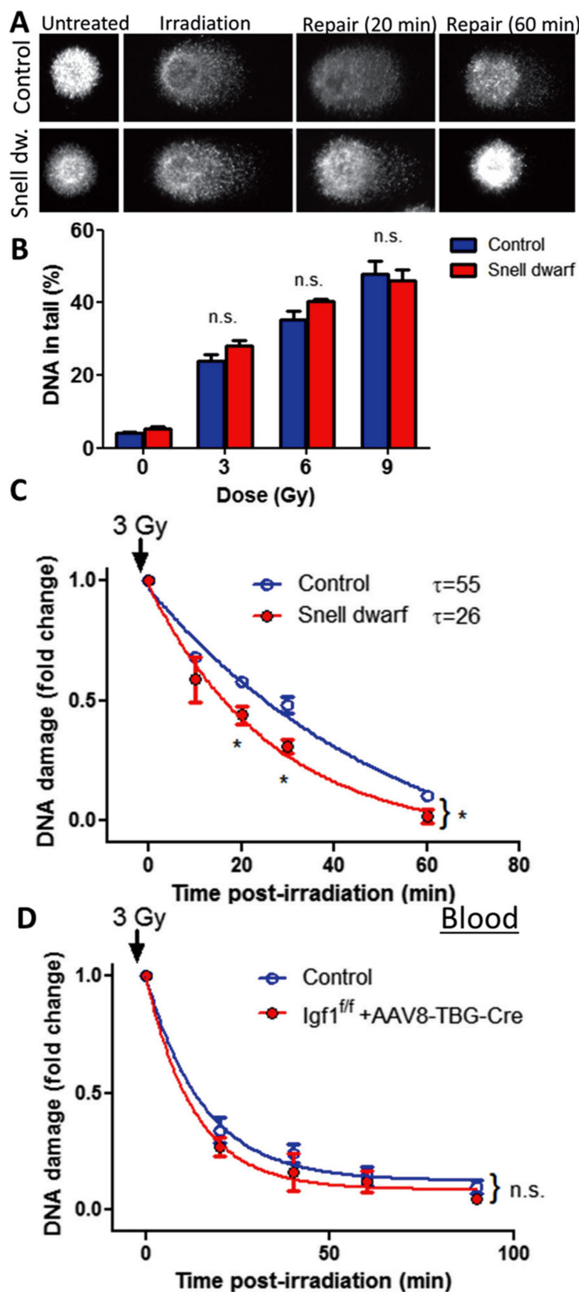

Fig. 2 Developmental GH/IGF-1 deficiency in Snell dwarf mice associates with increased DNA repair capacity in cultured primary fibroblasts. **a** Representative images of damaged DNA in irradiated (3 Gy) fibroblasts derived from control mice and GH/IGF-1-deficient Snell dwarf mice. Note the greater decline in tail DNA content at 20 and 60 min post-irradiation in cells derived from Snell dwarf mice due to improved DNA repair processes. **b** γ-Irradiated fibroblasts exhibit comparable DNA damage. Shown are increases in tail DNA content induced by increasing doses of γ-irradiation (3 to 9 Gy) in fibroblasts derived from control mice and Snell dwarf mice (n.s.: not significant vs control; *t* test). **c** Fibroblasts derived from Snell dwarf mice exhibit increased DNA repair capacity as compared to controls. For assessment of DNA repair efficiency, the percentage of residual DNA damage was plotted as a function of time post-irradiation. Tail DNA content at each time point is shown. The time constant calculated from this plot was shorter, and the residual DNA damage post-irradiation was lower in fibroblasts from Snell dwarf mice as compared to cells derived from control mice, indicating a more efficient DNA repair capacity in these cells. $^{*}p < 0.001$ Snell dwarf vs control (*t* test). Data are mean ± SEM ($n = 5$ for each time-point). **d** White blood cells derived from mice with adult-onset IGF-1 deficiency ($Igf1^{f/f}$ + TBG-Cre-AAV8) and control mice exhibit comparable DNA repair capacity. Data are mean ± SEM ($n = 5$ for each time-point)

Early-life GH/IGF-1 status of donor animals elicits persisting alterations in cellular expression of DNA repair-related genes

Expression of genes involved in regulation of DNA repair pathways was analyzed in fibroblasts derived from control rats, Lewis dwarf rats, and GH-replete dwarf rats. We found that early-life GH/IGF-1 deficiency in donor animals was associated with significant alterations (as determined by one-way ANOVA) in cellular expression of *Gadd45a*, *Xrcc5*, *Ercc6*, and *Ddit3* (Fig. 3). Importantly, short-term early-life GH treatment of donor animals prevented these gene expression changes (Fig. 3). We analyzed the time course of changes in expression of known γ-radiation-induced common stress response genes. We found that many of these genes (including *Gadd45b*, *Bbc3*, *Mdm2*) were upregulated (as determined by *t* test) in Lewis dwarf fibroblasts post-irradiation. The radiation-induced expression of *Gadd45b*, *Bbc3*, and *Mdm2* (but not baseline expression of these targets) was significantly greater in Lewis dwarf fibroblasts as compared to that in cells derived from control animals or Lewis dwarf rats with early-life GH treatment (one-way ANOVA; Fig. 3).

Discussion

This is the first study to demonstrate that GH/IGF-1 status during a critical developmental time-window elicits persisting alterations in cellular DNA repair pathways. Proficient cellular DNA repair systems substantially reduce cancer incidence, and thus, upregulation of DNA repair pathways by peripubertal GH/IGF-1 deficiency could well have relevance for decreased organismal susceptibility to carcinogenesis later in life. A critical role

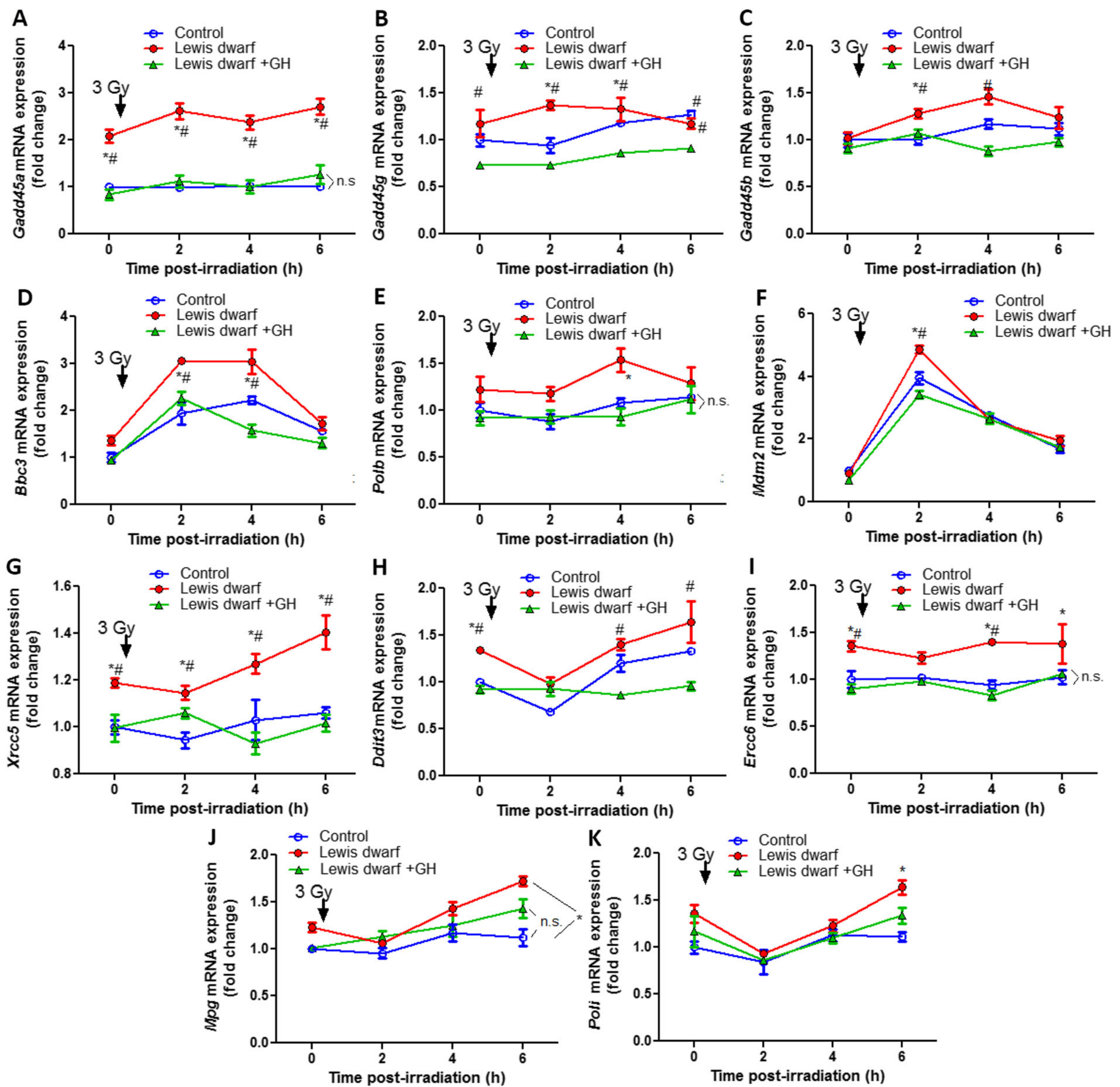

Fig. 3 qPCR data showing expression of genes involved in regulation of DNA repair pathways, including γ-radiation-induced common stress response genes, in fibroblasts derived from control rats, Lewis dwarf rats, and GH-replete dwarf rats. Expression of target genes was compared under basal conditions and at 2, 4, and 6 h post-irradiation. Data are mean ± SEM ($n = 3$–6 for each data point). *$p < 0.05$ Lewis dwarf vs control, #$p < 0.05$ Lewis dwarf vs Lewis dwarf + GH

for pubertal IGF-1 levels in determining cancer risk is supported by both clinical studies (Guevara-Aguirre et al. 2011) and experimental findings (Panici et al. 2010; Ramsey et al. 2002). In Lewis dwarf rats, the reductions in IGF-1 around puberty profoundly influence late-life pathologies (Sonntag et al. 2005b, 2013), including a decrease in cancer risk at old age (Guevara-Aguirre et al. 2011; Pollak et al. 2004). Importantly, in these animals, restoration of IGF-1 levels by GH treatment early in life dramatically increases susceptibility to cancer (Ramsey et al. 2002). Similar evidence was obtained in IGF-1-deficient Ames dwarf mice supporting the concept that a critical time window exists during postnatal development when circulating IGF-1 levels regulates cellular mechanisms that maintain genomic integrity and thus determine resistance to cancer later in life (Panici et al. 2010). In addition, IGF-1 is also known to exert growth-promoting and antiapoptotic effects

(Pollak et al. 2004; Yakar et al. 2004), which may contribute to the delayed growth of tumors and metastases associated with IGF-1 deficiency (Sonntag et al. 2012; Pollak et al. 2004; Yakar et al. 2004; Wu et al. 2003; Burgers et al. 2011; Renehan et al. 2004).

Our findings have important clinical significance. Circulating levels of IGF-1 are highly variable during puberty (range: from ~100 to 800 ng/mL) (Sorensen et al. 2012; Bidlingmaier et al. 2014). The significant variability in peripubertal IGF-1 levels, in addition to genetics, can be largely attributed to differences in nutritional status and increasing prevalence of childhood obesity. In the USA among children, 17% are estimated to be obese. Several studies have demonstrated that childhood obesity is associated with increased basal IGF-1 levels (Ong et al. 2002; Garnett et al. 2004), increased GH-induced IGF-1 generation, and increased IGF-1 bioavailability via reductions in IGF binding proteins (Ballerini et al. 2004; Bouhours-Nouet et al. 2007; Burt Solorzano and McCartney 2010). Because childhood obesity increases cancer predisposition, future studies are warranted to determine the effects of this obesity epidemic on cellular DNA repair pathways. There are also important ethnic differences; for example, African American prepubertal children have higher circulating IGF-1 levels as compared to their Latino and Caucasian peers (Alderete et al. 2011). It remains to be determined how these differences affect cellular DNA repair pathways.

The mechanisms by which developmental/peripubertal GH/IGF-1 status regulates cellular DNA repair processes are likely multifaceted. Our findings support the concept that peripubertal IGF-1 levels affect the expression of multiple DNA repair genes later in life. As shown in Fig. 3a, we found that in fibroblasts of rats, lacking the peripubertal IGF-1 surge expression of *Gadd45a* is significantly upregulated. This is a potentially important finding, because *Gadd45a* is an important component of the p53 pathway that contributes to the maintenance of genomic stability (Hollander et al. 1999, 2001; Jung et al. 2007). Mice lacking the *Gadd45a* gene are more prone to tumors induced by ionizing radiation and genotoxin exposure (Hollander et al. 2001), and their fibroblasts have defective NER (Hollander et al. 1999) and BER (Jung et al. 2007). Furthermore, Gadd45a was also shown to have a key role in active DNA de-methylation (Barreto et al. 2007). It has been proposed that Gadd45a may regulate promoter methylation and thereby activation of various tumor suppressor genes such as *Mlh1*. Importantly, early-life IGF-1 deficiency is also associated with upregulation of *Gadd45a* in tail-derived fibroblasts of the Snell dwarf mouse model as well (Ungvari, Miller and Csiszar, 2016, unpublished data). Another potentially interesting target regulated by early-life IGF-1 status is *Xrcc5*. The Ku80 protein, which is encoded by the *Xrcc5* gene, is known to bind to DNA double-strand break ends and is required for the non-homologous end joining pathway of DNA repair. Peripubertal IGF-1 levels also elicit persisting changes in the expression of *Ercc6* encoding the DNA excision repair protein ERCC-6/CSB. Further studies are evidently warranted to evaluate potential causal links among these gene expression changes, alterations in DNA repair efficiency, mutagenicity, and susceptibility for carcinogenesis. Importantly, our data show that peripubertal IGF-1 status determines the upregulation of the known p53-dependent tumor suppressor gene *Mdm2* and the p53 upregulated modulator of apoptosis *Bbc3* upon γ-irradiation. p53 (encoded by the *Tp53* gene) is a critically important tumor suppressor, which protects the genome from mutations and genetic alterations. Tp53 is one of the most commonly mutated genes in human cancers, and genetic deficiency of p53 was shown to promote the development of a variety of different cancers (Harvey et al. 1993). p53 functions as a transcription factor, which is activated in response to DNA damage, initiating the transcription of a number of genes. Importantly, *Gadd45a* is also a target of p53. Although there is ample evidence in the literature suggesting that a link exists between IGF-1 signaling and p53-regulated pathways, it is currently unknown how peripubertal IGF-1 deficiency affects this aspect of the regulation of cancer susceptibility. In addition to Lewis dwarf rats and Snell dwarf mice (Carter et al. 2002a; Flurkey et al. 2001), there are many mouse models in which early-life disruption of GH/IGF-1 signaling is associated with significant lifespan extension and/or reduced cancer burden later in life (e.g., Ames (Brown-Borg et al. 1996) dwarf mice, GHRHR-deficient lit/lit mice (Flurkey et al. 2001), GHR knockout mice (Berryman et al. 2008), and mice lacking both GH and GHR (Gesing et al. 2016)). It will be informative to determine how DNA repair pathways are altered in the tissues of these models in vivo. Initial evidence shows that two proteins involved in DNA repair (MGMT, NDRG1) are upregulated in Snell dwarf mice, growth hormone receptor gene disrupted mice (GHRKO), and in mice deficient in the pregnancy-associated protein-A (PAPPA-KO) (Dominick et al. 2016).

The molecular mechanisms by which peripubertal GH/IGF-1 status regulates expression of genes involved in cellular resilience and DNA repair processes remain

obscure. There is growing evidence linking silencing of tumor suppressor genes by DNA methylation to carcinogenesis. Previous results (Murakami et al. 2003; Salmon et al. 2005; Ungvari et al. 2011; Harper et al. 2007; Hsieh and Papaconstantinou 2009) showing that rodent fibroblasts retain their unique stress resistance signatures against DNA damaging agents in culture through many rounds of mitosis are consistent with the presence of epigenetic control mechanisms regulating DNA repair pathways that are induced in vivo by neurohormonal factors and maintained in extended culture. Thus, future studies should elucidate cellular epigenetic changes as a result of differing IGF-1 histories (Dominick et al. 2016). Studies correlating IGF-1-dependent alterations in DNA methylation status and expression profiles of DNA repair genes should be quite revealing. In addition to the putative role of DNA methylation, translational control of proteins involved in DNA repair may also be important (Dominick et al. 2016). We have recently showed that peripubertal IGF-1 deficiency also influences the cellular aging phenotype in mice by altering miRNA-mediated post-transcriptional gene regulation (Tarantini et al. 2016a).

Limitations of the study

There are important limitations of our study, including the limited endpoints tested. We have explored how DNA repair and gene expression are coordinated only in response to γ-irradiation. Future studies should test responses to multiple types of DNA damage and how specific DNA repair pathways are affected by early-life IGF-1 deficiency (Leiser et al. 2006; Murakami et al. 2003; Salmon et al. 2005; Ungvari et al. 2013b). Importantly, previous studies by the Miller laboratory show that UV-induced DNA damage is attenuated in fibroblasts from Snell dwarf mice (Salmon et al. 2008). It will also be interesting to determine whether transcription patterns reflect the relative cellular sensitivities to DNA-damaging stressors across multiple species. It is also a limitation of our studies that we do not have data on DNA repair in cells of GH replete Snell dwarf mice. Importantly, an earlier study found that Snell dwarf mice treated with GH starting at 4 weeks of age had no effect on lifespan (Vergara et al. 2004). Because in these studies, GH treatment did not reverse the dwarf phenotype, one may speculate that starting the GH treatment at 4 weeks may be too late to interfere with cellular stress resistance pathways. We found that in white blood cells, adult onset IGF-1 deficiency did not affect DNA repair efficiency. It would be also informative to assess DNA repair efficiency in white blood cells derived from Snell dwarf mice in future studies. Finally, because there is emerging evidence that there are important sex differences in the longevity phenotypes in models of early-life disruption of GH/IGF-1 axis (e.g., in mice heterozygous for the deletion of the IGF-1 receptor (Holzenberger et al. 2003) and in mice with early-life knockdown of circulating IGF-1 (Sonntag et al. 2016, submitted)), future studies should determine how sex influences epigenetic regulation of DNA repair pathways by peripubertal GH/IGF status.

Future studies should also determine whether levels of IGF-1 or GH early in life are more important in regulation of cellular resilience and DNA repair pathways. On the one hand, mice deficient in the pregnancy-associated protein-A (PAPPA-KO) exhibit a longevity phenotype and upregulation of DNA repair proteins without any change in GH (Dominick et al. 2016). On the other hand, liver-specific GHRKO mice have no lifespan increase despite significant decline in circulating IGF-1 (List et al. 2014).

Previous studies suggest that paracrine IGF-1 signaling may compensate for circulating IGF-1 deficiency in certain tissues (e.g., in the brain (Sun et al. 2005; Sun and Bartke 2007)). Thus, it would be of great interest to determine whether the effects of early-life IGF-1 status on cellular resilience and DNA repair pathways show organ/tissue specificity. It would also be interesting to know whether local GF-1 production in individual tissues/organs can be modified by circulating IGF-1/GH status during development.

In conclusion, GH/IGF-1 levels during a critical period during early life determine cellular DNA repair capacity in rodents likely by post-transcriptional control of genes involved in DNA repair. Because lifestyle factors (e.g., nutrition, childhood obesity) cause huge variation in peribubertal GH/IGF-1 levels in children, further studies are warranted to determine their persisting influence on cellular cancer resistance pathways.

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Author contribution AP, AC, and ZU designed research; MNVA, AP, KV, VP, EN, TG, and RAM performed experiments; AP, MNVA, AC, RAM, WES, and ZU analyzed and interpreted data; AP, AC, and ZU wrote the paper; MNVA, WES, RAM revised the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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