Energy Balance Modulates Mouse Skin Tumor Promotion through Altered IGF-1R and EGFR Crosstalk

Tricia Moore¹, Linda Beltran¹, Steve Carbajal¹, Stephen D. Hursting², and John DiGiovanni¹,²

Abstract

Obesity, an established risk factor for epithelial cancers, remains prevalent in the United States and many other countries. In contrast to positive energy balance states (overweight, obesity), calorie restriction (CR) has been shown to act as a universal inhibitor of tumorigenesis in multiple animal models of human cancer. Unfortunately, the mechanisms underlying the enhancing effects of obesity or the inhibitory effects of CR on cancer etiology remain elusive. Here, we evaluated the impact of dietary energy balance manipulation on epithelial carcinogenesis and identified several potential mechanisms that may account for the differential effects of obesity and CR on cancer. Obesity enhanced tumor promotion during epithelial carcinogenesis, in part, due to altered insulin-like growth factor-1 receptor (IGF-1R)/EGF receptor (EGFR) crosstalk and downstream signaling to effectors such as Akt/mTOR. Obesity-induced changes in cellular signaling subsequently led to altered levels of cell-cycle proteins that favored enhanced epidermal proliferation during tumor promotion. In contrast, CR reduced susceptibility to tumor promotion, attenuated IGF-1R/EGFR crosstalk and downstream signaling, and altered levels of cell-cycle proteins that favored reduced epidermal proliferation during tumor promotion. Collectively, these findings suggest potential targets for the prevention of epithelial cancers, as well as for reversal of obesity-mediated cancer development and progression. Cancer Prev Res; 1–11. ©2012 AACR.

Introduction

Energy balance refers to the relationship between caloric consumption and energy expenditure (1). Epidemiologic and animal studies have established a direct correlation between positive energy balance states (e.g., overweight, obesity) and risk of developing multiple cancers (2–5). In contrast, negative energy balance states, such as calorie restriction (CR), have been shown to consistently inhibit tumorigenesis in animal models regardless of mode of tumor induction (1, 6–8). Previous studies have evaluated the impact of fat consumption and/or CR on 2-stage skin carcinogenesis, especially during tumor promotion, the effect of diet-induced obesity (DIO) per se has not been adequately studied in this model. Several mechanisms have been proposed to explain the inhibitory effects of CR on tumor development (12, 13) including elevated serum corticosterone (6, 14) and alterations in cellular signaling. TPA-induced epidermal AP-1 activation and extracellular signal–regulated kinase (ERK) phosphorylation was reduced in mice subject to 40% CR (15–17). Xie and colleagues (18) reported a reduction in TPA-mediated activation of epidermal phosphoinositide 3-kinase (PI3K) and Ras signaling following 20% CR. Recently, we reported that both CR and DIO modulated steady-state growth factor signaling pathways in mouse epidermis, liver, and prostate (19). Additional studies suggested that these effects may be mediated by diet-induced changes in circulating insulin-like growth factor-1 (IGF-1) levels and altered IGF-1 receptor (IGF-1R) signaling (19, 20).

Diet of varying caloric density were used to induce changes in body mass and body fat content to determine the impact of both positive and negative energy balance on the promotion stage of multistage epithelial carcinogenesis in mouse skin. We provide novel evidence showing that dietary energy balance affects susceptibility to epithelial carcinogenesis, in part, through diet-induced changes in

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IGF-1R and EGF receptor (EGFR) crosstalk and downstream signaling during tumor promotion.

Materials and Methods

Animals

ICR female mice (3–4 weeks of age, Harlan Teklad) were group housed; however, mice maintained on a 30% CR regimen were separated for 1 hour for feeding. Mice were weighed before randomization and then every 2 weeks for the duration of the experiments.

Diets

Four diets ranging in caloric density were used (Research Diets) and have been previously described: 30% CR diet, 15% CR diet, 10 kcal% fat (ad libitum), and 60 kcal% fat (ad libitum; ref. 1). CR was achieved by administering a daily aliquot equivalent to 70% or 85% of the daily energy consumed by the 10 kcal% fat group, as previously detailed (19).

Serum analysis and body fat composition

Blood was collected immediately following CO₂ asphyxiation, serum was prepared, and serum IGF-1, insulin, leptin, and adiponectin levels were determined as previously described (19, 21). Corticosterone levels were determined by ELISA (Kamiya Biomedical Co.). Percentage of body fat was determined as previously described (22).

Two-stage skin carcinogenesis

ICR female mice were placed on the 10 kcal% fat control diet at 7 weeks of age and initiated with 25 nmol of 7,12-dimethylbenz[a]anthracene (DMBA; Eastman Kodak Co.). Four weeks following initiation, mice were randomized and placed on the 4 diets (n = 30 per group). Four weeks later, mice received twice weekly topical treatments of 3.4 nmol of TPA (Alexis Biochemicals) for 50 weeks. Tumor incidence (percentage of mice with papillomas) and tumor multiplicity (average number of tumors per mouse) were determined weekly until multiplicity reached a plateau (29 weeks). Carcinoma incidence and average carcinomas per mouse were determined weekly from initial detection until 50 weeks of tumor promotion. Squamous cell carcinomas (SCC) were confirmed by histopathology.

Epidermal hyperproliferation

Female ICR mice were maintained on the diets described above for 15 weeks, after which they were treated twice weekly with either acetone vehicle or 3.4 nmol of TPA (n = 3 per group). Mice were injected with bromodeoxyuridine (BrdUrd; Sigma Aldrich; 100 μg/g body weight) 30 minutes before sacrifice. Whole skin sections were excised, processed, and evaluated for epidermal thickness and labeling index as previously described (20).

Epidermal protein lysates

For short-term in vivo experiments, ICR mice were maintained on the diets for 15 weeks, after which they received a single application of either acetone (vehicle) or 3.4 nmol of TPA. Mice were killed either 6 (acetone, TPA) or 18 hours (TPA) after treatment, epidermis was scraped and protein lysates were prepared as previously described (20).

Cell culture

C50 cells are a nontumorigenic keratinocyte cell line derived from spontaneously immortalized normal mouse keratinocytes. The cells were obtained from Dr. Susan Fischer (UTMDACC, Houston, TX) and were cultured as previously described (23) with no further characterizations. When cells reached approximately 80% confluency, they were serum and growth factor starved for 24 hours. Plates were then stimulated with either 25 ng/mL recombinant human IGF-1 (rhIGF-1; Sigma Aldrich) or 10 ng/mL EGF (BD Biosciences) and harvested at multiple time points for protein or RNA isolation.

Immunoprecipitation and Western blot analysis

Immunoprecipitation and Western blot analyses were conducted using lysates prepared from either epidermis or cultured keratinocytes. For co-immunoprecipitation experiments, lysates were precipitated with IGF-1R (Cell Signaling) or EGFR (Millipore) antibodies using the Dynabead Protein G IP Kit (Invitrogen). Western blot analyses were conducted as previously described (20).

Real-time quantitative reverse transcriptase PCR

For in vivo experiments, ICR mice were maintained on the diets for 15 weeks, after which they received a single application of either acetone vehicle or 3.4 nmol of TPA. Mice were killed either 6 (acetone, TPA) or 18 hours (TPA) after treatment and epidermis was scraped for RNA isolation (n = 3 per group). For in vitro experiments, C50 cells were stimulated with either IGF-1 or EGF, harvested for RNA, and RNA was isolated using the Qiagen RNeasy Protect Mini Kit. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.), and quantitative PCR (qPCR) was carried out, as previously described, using assays on demand specific to TGF-α, HB-EGF, amphiregulin, and EGF (21). RNA was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All measurements were duplicated.

Results

Dietary energy balance effects on skin tumor promotion

A 2-stage skin carcinogenesis experiment was conducted using 4 experimental diet groups to generate lean (30% CR), normal (15% CR), overweight (10 kcal% fat), and obese (DIO, 60 kcal% fat) body phenotypes, as previously described (5, 21, 24). Groups were initiated with DMBA, maintained on 10 kcal% diet for 4 weeks, randomized into the experimental groups and promoted twice weekly with 3.4 nmol of TPA for 50 weeks. Tumor incidence and multiplicity (average number of papillomas per mouse) were calculated weekly until the latter reached a plateau at 29 weeks. CR (15% and 30%) significantly inhibited
papilloma multiplicity when compared with both the overweight control and DIO groups \((P < 0.05; \text{Mann–Whitney } U \text{ test})\); however, no significant differences were observed between the overweight control and DIO groups (Fig. 1A; Supplementary Table S1). Dietary energy balance manipulation had no significant effect on papilloma incidence under these experimental conditions (Fig. 1B; Supplementary Table S1).

Treatments continued for an additional 21 weeks to evaluate the impact of dietary energy balance on tumor progression. As shown in Fig. 1C and D, the number and incidence of SCCs was significantly reduced in the 30% CR group \((P < 0.05, \text{Mann–Whitney } U \text{ test})\) compared with the 15% CR, overweight control and DIO groups; however, dietary manipulation did not affect the overall rate of malignant conversion (see also Supplementary Table S1). A second 2-stage skin carcinogenesis experiment was carried out that yielded nearly identical findings (data not shown).

**Effect of long-term dietary manipulation on body profiles during two-stage skin carcinogenesis**

Body mass, percentage of body fat, and circulatory protein levels (i.e., IGF-1, insulin, leptin, adiponectin)...
were evaluated at the conclusion of the 2-stage skin carcinogenesis study (Supplementary Table S2.1). Body mass and percentage of body fat were significantly different among all groups ($P < 0.05$, Mann–Whitney $U$ test). CR (both 30% and 15%) significantly reduced levels of circulating IGF-1, insulin, and leptin. In contrast, CR increased levels of adiponectin, as compared with both the overweight control and DIO groups; however, only the 30% CR group yielded a statistically significant difference ($P < 0.05$, Mann–Whitney $U$ test). Despite significant differences in both body mass and percentage of body fat, no significant differences in the levels of circulatory proteins were observed between the overweight control and DIO groups at the end of the 50-week experimental period.

**Dietary energy balance modulates TPA-induced epidermal hyperproliferation**

To evaluate potential mechanisms whereby dietary energy balance modulates 2-stage skin carcinogenesis, additional mice were maintained on the 4 diets for 15 weeks, thus generating lean, normal, overweight, and obese body phenotypes. Body mass, percentage of body fat, and levels of circulatory proteins were then determined at this time point (Supplementary Table S2.1). Body mass and percentage of body fat correlated directly with caloric consumption, yielding significant differences in both parameters among all groups ($P < 0.05$, Mann–Whitney $U$ test). Similarly, levels of IGF-1, insulin, leptin, and adiponectin were significantly different across the groups, with the greatest differences occurring between the caloric extremes ($P < 0.05$, Mann–Whitney $U$ test). Specifically, CR reduced whereas DIO increased levels of circulating IGF-1, insulin, and leptin, findings consistent with our previous studies (19). Levels of adiponectin inversely correlated with caloric consumption, with significant differences occurring between each CR group and the overweight control and DIO groups ($P < 0.05$, Mann–Whitney $U$ test). In addition, 30% CR significantly increased circulating corticosterone levels relative to both the overweight control and DIO groups, however, no other statistically significant differences in corticosterone levels were observed.

Given the effect of dietary energy balance on tumor promotion, we evaluated diet-induced changes in the epidermal proliferative response following TPA treatment. Female ICR mice maintained on the 4 diets for 15 weeks were treated twice weekly for 2 weeks with either acetone or 3.4 nmol of TPA. Groups of mice were killed 48 hours following the final treatment and whole skin sections were removed and processed. Epidermal hyperplasia (epidermal thickness) and labeling index [LI; BrdUrd incorporation] were determined as previously described (20). Figure 1E shows representative BrdUrd-stained skin sections, corresponding to both acetone and TPA-treated skins excised from lean, normal, overweight, and obese mice and quantitative analyses are shown in Fig. 1F. In the absence of TPA treatment, 30% CR significantly reduced both epidermal thickness and LI, when compared with all other groups; however, 15% CR only significantly reduced both parameters when compared with the DIO group ($P < 0.05$, Mann–Whitney $U$ test). No differences in either epidermal thickness or LI were observed between the overweight control and DIO groups in the absence of TPA treatment. In contrast, both epidermal thickness and LI were significantly different among all dietary groups following TPA treatment, with the greatest differences again occurring between the 30% CR and DIO groups ($P < 0.05$, Mann–Whitney $U$ test).

**Effect of dietary manipulation on growth factor signaling and cell-cycle regulatory proteins during tumor promotion**

Diet-induced changes in growth factor signaling pathways were also assessed. ICR mice were maintained on the 4 diets for 15 weeks, treated with a single application of either acetone or 3.4 nmol of TPA, and killed 6 hours later. Epidermal lysates were prepared for Western blot analyses as previously described (19, 20). As shown in Fig. 2A, phosphorylation of the EGFR, IGF-1R, and downstream signaling targets (i.e., Akt, mTOR, S6 ribosomal, GSK-3β, Foxo3a, Stat3) correlated directly with caloric density in the absence of TPA treatment, corroborating our previous findings indicating that dietary energy balance modulated steady-state growth factor signaling (19). Diet-induced changes in EGF/IGF-1R activation and downstream signaling were further enhanced following TPA treatment, with significant differences in phosphorylation occurring between the 30% CR and DIO groups (Fig. 2A). Specifically, 30% CR significantly reduced activation and/or phosphorylation of the EGFR, IGF-1R, Akt, mTOR, S6 ribosomal, GSK-3β, Foxo3a, and Stat3, as compared with the DIO group ($P < 0.05$, Student $t$ test; Fig. 2A).

We also evaluated diet-induced changes in cell-cycle regulatory proteins. Epidermal protein lysates were prepared as above with the addition of an 18-hour time point. Initially, we analyzed changes in positive cell-cycle regulatory protein levels. CR reduced epidermal levels of cyclin D1, cyclin E, cyclin A, and c-myc compared with DIO; however, these differences were not statistically significant (Fig. 2B). TPA treatment led to increased levels of cyclin D1 (18 hours), cyclin E (6, 18 hours), cyclin A (6, 18 hours) and c-myc (6, 18 hours; ref. 25; see Fig. 2B). The TPA-mediated increases in epidermal cyclin D1, cyclin E, cyclin A, and c-myc were significantly attenuated by 30% CR, compared with DIO, at 18 hours ($P < 0.05$, Student $t$ test). Diet-induced changes in cyclin A and c-myc levels following TPA treatment were also significant at the 6-hour time point ($P < 0.05$, Student $t$ test).

Additional analyses were conducted to determine the impact of dietary energy balance on levels of negative cell-cycle regulatory proteins. Levels of both p27 and p21 were significantly higher in 30% CR mice (vehicle control), as compared with DIO mice (Fig. 2C). Following TPA treatment (6 hours), both p27 and p21 levels were significantly higher in mice maintained on the 30% CR diet, as compared with mice maintained on the DIO diet ($P < 0.05$, Student $t$ test). A similar trend was observed at 18 hours,
although these differences were not significantly different. Preliminary experiments (data not shown) showed increased nuclear localization of both p27 and p21 in the epidermis of CR mice, as compared with mice maintained on the DIO regimen, suggesting that dietary energy balance may also modulate these negative cell-cycle regulatory proteins through additional mechanisms.

**Activation of the IGF-1R stimulates IGF-1R/EGFR crosstalk in cultured mouse keratinocytes**

Both our current and previous data suggested a role for altered IGF-1 levels in modulating the effect of dietary energy balance on epithelial carcinogenesis. A reduction in circulating IGF-1, either due to genetic (LID mouse model) or dietary manipulation (CR) attenuated both IGF-1R and EGFR signaling (refs. 19, 20; Fig. 2), indicating a potential for crosstalk between these 2 receptors. C50 cells were used to examine the role of IGF-1 in modulating both IGF-1R and EGFR signaling and crosstalk, as well as erbB2 activation. Cultured cells were stimulated with IGF-1 (25 ng/mL), harvested at multiple time points (0–120 minutes), and analyzed for IGF-1R activation (measured by IRS-1 phosphorylation) and for EGFR and erbB2 activation (measured by phosphorylation) using Western blot analysis. IGF-1 treatment significantly induced activation of all 3 cell surface receptors (Fig. 3). EGFR phosphorylation was rapid, with maximal activation occurring within 5 minutes and phosphorylation was maintained above the basal level up to 120 minutes. EGFR phosphorylation correlated directly with IGF-1–induced activation of the IGF-1R. Rapid phosphorylation of erbB2 also paralleled both IGF-1R and EGFR activation; however, erbB2 activation was maintained at a...
consistent level across all time points examined. Stimulation of C50 cells with EGF (10 ng/mL) over the same time course led to rapid activation of both the EGFR and erbB2; however, there was no significant effect on IGF-1R activation (Fig. 3).

Previous studies have identified several potential mechanisms for IGF-1R and EGFR crosstalk (26–30). The ability of IGF-1 to induce heterodimerization between the IGF-1R and the EGFR was evaluated in co-immunoprecipitation experiments carried out using lysates prepared from C50 cells treated with either IGF-1 or EGF. As shown in Fig. 4A, IGF-1 treatment induced a statistically significant increase in IGF-1R/EGFR association from 5 to 60 minutes. In contrast, EGF had no observable effect. Additional experiments were carried out to determine the impact of IGF-1 stimulation on levels of mRNA for the EGFR and EGFR ligands. qPCR was carried out using RNA extracted from C50 cells (stimulated with IGF-1 or EGF and harvested at 0–120 minutes) to determine the relative expression of EGFR, TGF-α, HB-EGF, and amphiregulin. Both IGF-1 and EGF stimulation significantly increased expression of HB-EGF and amphiregulin, with a greater induction occurring with EGF treatment (Fig. 4B). These relative increases in mRNA expression, however, were observed only at later time points (≥ 60 minutes). No increases in TGF-α and EGFR mRNA expression were observed following stimulation of C50 cells with either IGF-1 or EGF (Fig. 4B).

**Dietary energy balance alters IGF-1R/EGFR crosstalk in vivo**

Finally, we evaluated the impact of dietary energy balance manipulation on IGF-1R/EGFR crosstalk during tumor promotion in mouse epidermis *in vivo*. Female ICR mice were maintained on the previously described 30% CR and DIO diets for 15 weeks. These lean and obese mice were then treated with a single application of acetone (vehicle) or 3.4 nmol of TPA and killed 6 or 18 hours later. Epidermal lysates and epidermal RNA were prepared for Western blot and qPCR analyses, respectively. Co-immunoprecipitation experiments were carried out to evaluate diet-induced changes in IGF-1R/EGFR heterodimerization. As shown in Fig. 5A, there was a greater degree of association between the IGF-1R and EGFR in obese mice relative to CR mice in the absence of TPA treatment. Furthermore, TPA (6 hours) induced a significant increase in IGF-1R and EGFR association in both the CR and obese mice although the relative increase in heterodimerization was significantly greater in obese mice (P < 0.05, Student t test).
Dietary manipulation did not modulate mRNA levels of EGFR and EGFR ligands in the absence of TPA treatment; however, DIO significantly increased levels of mRNA for EGFR ligands compared with 30% CR, following treatment with TPA (Fig. 5B). Specifically, DIO significantly increased mRNA levels of TGF-α (18 hours), HB-EGF (6 hours), and amphiregulin (6 hours) following TPA treatment. EGFR mRNA levels were not affected by diet or treatment with TPA. Collectively, the in vitro and in vivo results suggest that dietary energy balance modulates IGF-1R/EGFR cross-talk, at least in part, due to diet-induced changes in mRNA levels of EGFR ligands and changes in IGF-1R and EGFR heterodimerization.

Discussion

In this study, potential mechanism(s) underlying the effects of dietary energy balance manipulation on skin tumor formation during the promotion phase of 2-stage skin carcinogenesis were examined. Consistent with previously published data, both 15% and 30% CR significantly inhibited the promotion of papillomas by TPA compared with both the overweight control and DIO groups but had no significant effect on tumor progression (8–10). Notably, no significant difference in tumor response was seen between the DIO and overweight control groups, although the DIO regimen significantly increased both body mass and percentage of body fat. Previous mammary carcinogenesis studies also reported that despite significant differences in body mass, no significant increase in tumor incidence occurred when dietary fat consumption exceeded 20 Kcal% to 30 Kcal% (31–33). These data support the hypothesis that in some model systems, there is an upper limit to the effects of a positive energy balance state above which further increases in total calories do not further increase tumor response.

Genetic reduction of circulating IGF-1 levels (i.e., LID mouse model) attenuated TPA-induced epidermal hyper-proliferation, which correlated with reduced responsiveness to skin tumor promotion by TPA (20). In comparison to the overweight control and DIO groups, both the 30% and 15% CR groups had significantly reduced epidermal thickness and LI in the absence of TPA. Following TPA treatment, we
observed a significantly progressive effect of dietary energy balance across all the diet groups on the epidermal proliferative response, with 30% CR exhibiting the lowest response and DIO exhibiting the highest. Several other studies have shown that energy balance impacts cellular proliferation in both normal and tumorigenic tissue (e.g., mammary, colon, liver, epidermis, and bladder; refs. 34–38). Collectively, our results suggest that manipulating dietary energy balance impacts TPA-mediated epidermal hyperproliferation, thus modulating epithelial carcinogenesis in mouse skin.

Previous studies have indicated an important role for IGF-1R and EGFR activation in TPA-induced epidermal hyperproliferation and tumor promotion in mouse skin (reviewed in ref. 39). Inhibition of TPA-induced EGFR activation in vivo, using either RG13022 (tyrosine kinase inhibitor) or GW2974 (dual specific erbB2/EGFR inhibitor) significantly reduced TPA-induced epidermal hyperproliferation as well as skin tumor promotion (GW2974; refs. 40, 41). In addition, epidermal IGF-1 overexpression increased epidermal proliferation, both in the presence and absence of TPA, and increased sensitivity to 2-stage skin carcinogenesis (42, 43). Furthermore, dietary energy balance induced changes in steady-state activation of these critical growth factor receptors in epidermis and other epithelial tissues of FVB/N and C57BL/6 mice (19). As shown in Fig. 2A, 30% CR reduced activation and/or phosphorylation of the IGF-1R, EGFR, and downstream signaling following treatment with TPA compared with the DIO group. Xie and colleagues reported a similar reduction in TPA-mediated epidermal Akt activation in CR mice (18).

The impact of dietary manipulation on levels of positive and negative cell-cycle regulatory proteins was also evaluated. Notably, in mice treated with TPA, the levels of positive cell-cycle regulatory proteins (cyclin D1, cyclin E, cyclin A, c-myc) correlated directly with caloric consumption, whereas the levels of negative cell-cycle regulatory proteins (i.e., p21, p27) inversely correlated with caloric consumption. As reported earlier, elevated IGF-1R activation or increased Akt activity, respectively, leads to increased levels of positive cell-cycle regulatory proteins (i.e., p21, p27) inversely correlated with caloric consumption. Data represent mean ± SEM of 3 independent experiments. a–c, values with the same lettering indicate statistically significant differences (P < 0.05, Student t test). B, qPCR analysis of EGFR and the EGFR ligands TGF-α, HB-EGF, amphiregulin, and EGF. Data represent mean ± SEM. * , significantly different from 30% CR group at the corresponding time point (P < 0.05, Mann–Whitney U test).
Energy Balance Modulates IGF-1R/EGFR Crosstalk

In conclusion, we have shown that CR reduced, whereas DIO increased, signaling through the IGF-1R and EGFR following TPA treatment. Both in vitro and in vivo studies (20) suggest that levels of circulating IGF-1, which are modulated by dietary energy balance, regulate activation of the IGF-1R which in turn regulates crosstalk with the EGFR (and erbB2). These diet-induced changes in IGF-1R and EGFR activation subsequently altered downstream signaling to Akt and mTOR (and other signaling pathways), thus modulating levels and activity of cell-cycle regulatory proteins. These changes in cell-cycle regulatory proteins correlated directly with diet-induced changes in TPA-induced epidermal proliferation. Supplementary Figure S1 summarizes the effects of dietary energy balance on signaling pathways and cell-cycle proteins observed in the present study in relation to altered epidermal proliferation and tumor promotion. Taken together, these effects of dietary energy balance on IGF-1R/EGFR signaling and crosstalk, cell-cycle regulation, and epidermal proliferation provide a plausible mechanism for the inhibitory effects of negative energy balance and the enhancing effects of positive energy balance on susceptibility to skin tumor promotion during 2-stage skin carcinogenesis. These studies provide new information about potential molecular targets for prevention of epithelial cancers and for reversing the effects of obesity on cancer development.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: T. Moore, S.D. Hursting, J. DiGiovanni
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