Project title: Cellular response to low dose radiation: Role of phosphatidylinositol-3 kinase like kinases (PIKK)
Grant No.DE-FG02-05ER64055
Principal Investigator: Adayabalam S. Balajee

The overall research objectives and specific aims of the project remained essentially the same as originally proposed. This final STI report summarizes the key findings during the entire project period (07/15/2005-12/31/2010).

Project Abstract: The working hypothesis for this proposal is that the cellular mechanisms in terms of DNA damage signaling, repair and cell cycle checkpoint regulation are different for low and high doses of low LET radiation and that the mode of action of phosphatidylinositol-3 kinase like kinases (PIKK: ATM, ATR and DNA-PK) determines the dose dependent cellular responses. The hypothesis will be tested at two levels: (I) Evaluation of the role of ATM, ATR and DNA-PK in cellular response to low and high doses of low LET radiation in simple in vitro human cell systems and (II) Determination of radiation responses in complex cell microenvironments such as human EpiDerm tissue constructs. Cellular responses to low and high doses of low LET radiation will be assessed from the view points of DNA damage signaling, DNA double strand break repair and cell cycle checkpoint regulation by analyzing the activities (i.e. post-translational modifications and kinetics of protein-protein interactions) of the key target proteins for PI-3 kinase like kinases both at the intra-cellular and molecular levels. The primary goal of this proposal is to elucidate the differences in cellular defense mechanisms between low and high doses of low LET radiation and to define the radiation doses where the cellular DNA damage signaling and repair mechanisms tend to shift. This information is critically important to address and advance some of the low dose research program objectives of DOE. The results of this proposed study will lead to a better understanding of the mechanisms for the cellular responses to low and high doses of low LET radiation. Further, systematic analysis of the role of PIKK signaling pathways as a function of radiation dose in tissue microenvironment will provide useful mechanistic information for improving the accuracy of radiation risk assessment for low doses. Knowledge of radiation responses in tissue microenvironment is important for the accurate prediction of ionizing radiation risks associated with cancer and tissue degeneration in humans.

Final Scientific and Technical Information (STI) Report:

The main goal of this project is to determine the role of PI-3- kinase like kinases (PIKK) in low dose radiation induced cellular DNA damage response mechanisms in human 2- and 3-dimensional cell systems. Low dose radiation induced cellular response mechanisms involve a complex interplay between DNA repair and cell cycle checkpoint activities that depend on the efficiency of several important signal transduction pathways. Realizing the complex nature, the following biological endpoints were performed to investigate the cellular radiation response mechanisms: (I) DNA damage response including the DNA damage induction/repair and cell cycle checkpoint regulation, (II) mode of activation of PIKK mediated signal transduction pathways (MAPK and Protein kinase C), (III) chromatin remodeling and epigenetic changes, (IV) gene expression and (V) genomic instability and apoptosis.

The findings of our study on the effects of low doses of low LET radiation on different biological endpoints in 2- (primary human fibroblasts, keratinocytes, melanocytes, mesenchymal and neural stem cells) and 3 dimensional (EpiDerm^{TM} tissue with only primary keratinocytes and EpiDermFT^{TM} full thickness with both keratinocytes and fibroblasts; MatTek Corporation, Boston, USA) human cell systems are summarized below:

(I) DNA damage response (DDR) triggered by low (less than 10cGy) and high doses (>10-500cGy) of low LET radiation (γ-rays and Protons) was investigated by immunological probing for diverse DDR proteins in human 2 dimensional and 3-dimensional cell systems as mentioned above. The following DDR proteins which are useful predictors for DSB damage signaling and repair were analyzed: (I) sensors/initiators include ATM ser1981, ATR, 53BP1, gamma-H2AX, MDC1, MRE11, Rad50 and Nbs1; (II) signal transducers include Chk1, Chk2, FANCD2 and SMC1; and (III) effectors include p53, CDC25A and CDC25C. Representative pictures for 53BP1 foci induction and repair after exposure low and high doses of gamma rays in both cell model systems are shown in Fig.1.

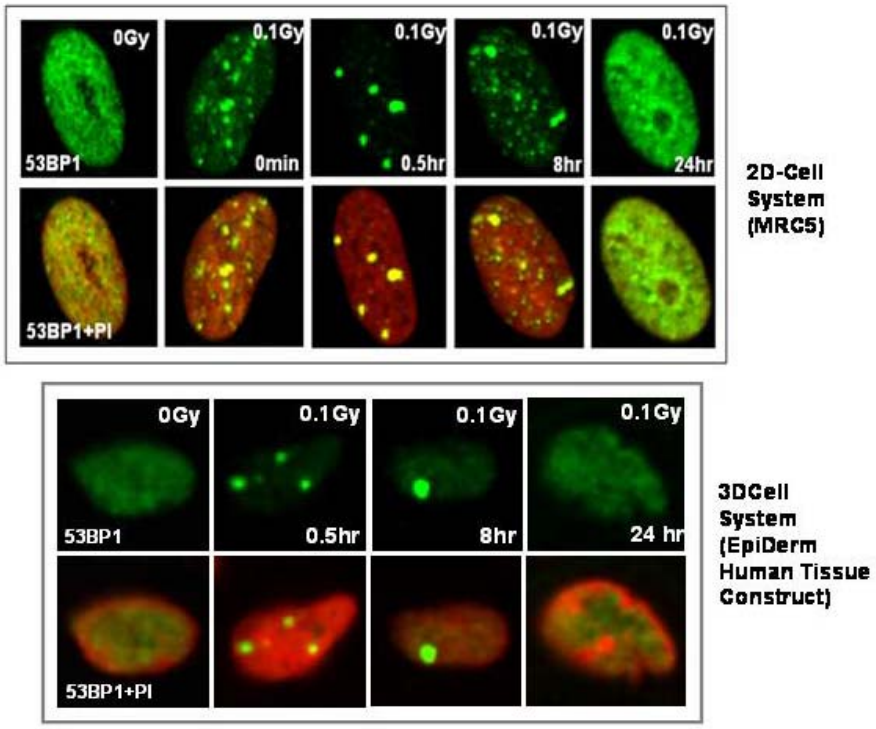

Fig.1. Detection of intra-nuclear assembly of 53BP1 foci at DSB sites in normal human primary fibroblasts (MRC5) and human EpiDerm tissue constructs in response to 10cGy of γ-rays. 53BP1 foci were detected using a primary rabbit antibody and fluorescein conjugated anti-rabbit antibody. Cells were counterstained with propidium iodide (PI).

To determine the role of PIKK in low LET radiation response, human cells deficient in ATM (GM2052F, GM5823E), ATR (GM8398A) and DNA-PK (MO59J) were utilized. Alternately, inhibitors specific for ATM (KU55933), ATM/ATR (CGK733) and DNA-PK (NU7096) were used (10-50μM) to precisely determine the role of PIKK in DDR triggered by low and high doses of low LET radiation. Our results indicate that PIKK is critically required for efficient DSB signaling and repair responses after low doses of gamma rays in both human monolayer cell types (primary fibroblasts, melanocytes, keratinocytes as well as mesenchymal and neural stem cells) and human 3-dimensional EpiDerm tissue constructs. Abolition of PIKK by LY294002 and Wortmannin impaired the low dose radiation induced DNA damage response and resulted in a high level of apoptotic death in both 2-dimensional human fibroblasts and 3-dimensional human skin model system (***Experimental Dermatology, 19, e16-22, 2010)***. Similar results were obtained in both cell systems with protons radiation.

(II) Proportion of cells that showed IR induced DNA damage response (assessed by IR induced foci formation of gamma-H2AX, 53BP1 and phosphorylated ATM) increased as a function of radiation dose (1cGy-5Gy) in both 2D and 3D cell systems. Cells positive for IR induced foci reached a plateau at radiation doses above 2.5 Gy (***Experimental Dermatology, 19, e16-22, 2010***).

(III) In contrast to repair proficient human primary fibroblasts (MRC5), persistence of 53BP1 as well as γ-H2AX foci was observed in ATM, ATR and DNA-PK deficient cells upon exposure to low doses of gamma rays (10-100cGy). We next determined whether the persistence of low dose radiation induced DSBs in ATM, ATR and DNA-PK deficient cells leads to genomic instability. For this purpose, some of the well known markers of chromosomal instability such as micronuclei, chromatin bridges and anaphase bridges were analyzed in PIKK proficient (MRC5) and PIKK deficient primary human fibroblast cells. It is interesting to note that more than 55% of the MN observed in ATM deficient cells (GM5823E) were intensely stained with γ-H2AX. ATM deficient cells showed increased incidence of micronuclei even in the absence of ionizing radiation treatment (0.18/cell) and the frequency of micronuclei observed per cell was enhanced after exposure to 0.1Gy (0.3/cell after 24 hr and 0.35 after 48hr) and 1 Gy of γ-rays (0.49/cell after 24hr and 0.68/cell after 48hr). Further, frequency of chromatin and anaphase ridges was also greatly increased as a function of radiation dose (0.1 and 1 Gy) and time (24 and 48hr after treatment) in ATM deficient cells. In contrast to ATM deficient cells, ATM proficient cells (MRC5) did not show any increase in MN after 0.1Gy of γ-rays analyzed at 24hr after exposure. We next determined whether abolition or inhibition of ATM/ATR kinase activity would enhance the frequency of MN in MRC5 cells. As expected, treatment of MRC5 cells with 2 mM caffeine substantially elevated the frequency of MN analyzed at 24hr after both radiation doses (0.02/cell to 0.06 after caffeine treatment for 0.1Gy; 0.07 to 0.19 after caffeine treatment for 1Gy) indicating a critical role for PIKK and ATM in particular in the modulation of cellular responses to low doses of low LET radiation. Although caffeine, an inhibitor of ATM/ATR kinases, greatly potentiated the radiation effects in MRC5 cells, such an effect was not seen in ATM deficient cells. This observation in a way emphasizes the predominant effect of ATM in modulating the cellular responses to low doses of IR.

Similar results were obtained with ATR and DNA-PK deficient human cells. However, low dose radiation effect on MN was more pronounced in ATR deficient cells than DNA-PK deficient cells.

(IV) Comparative analysis of human adult stem cells (mesenchymal stem cells and neural stem cells) and differentiated skin cell types (keratinocytes, melanocytes and fibroblasts) revealed that IR induced DSB repair is slightly attenuated in human adult stem cells. Further, ATM kinase is critical for low dose radiation response in human adult stem cells as ATM ablation dramatically increased IR induced micronuclei in both neural and mesenchymal stem cells (***Manuscript under preparation***). Additionally, ablation of ATM by siRNA in human neural stem/progenitor cells dramatically increased the MN and impaired the ATM associated signaling pathways in response to both low and high doses of γ-rays. Similar observations were made for ATM deficient neurons differentiated from ATM suppressed human neural stem/progenitor (ReNerve) cells. Our study indicates that impaired DDR due to ATM deficiency is a key factor for some of the neuropathological features of AT patients. Further, our study also demonstrates the critical importance of ATM for efficient DDR in post mitotic neurons (Unpublished data).

(V) Cells deficient in ATM, ATR and DNA-PK displayed enhanced genomic instability involving mitotic catastrophe after low doses of gamma rays (1cGy-10cGy). Among the three kinases, ATR appears to be highly critical for genomic stability as ATR deficient primary human fibroblasts displayed the highest level of spontaneous genomic instability (stable chromosome translocations, micronuclei and chromatin bridges) as compared to ATM and DNA-PK deficient human cells (***Manuscript under preparation***).

(VI) Low dose radiation altered the cell cycle profile in a profound way in a PIKK dependent manner [a dose dependent (1-50cGy) transient G1 arrest and accumulation of G1 cells]. These observations are consistent with distinct differences in the activation and persistence of Chk1 and Chk2 kinases between low and high doses of radiation. In corroboration, activation of ATM and ATR kinases persists much longer (up to 24hr) after

high doses of IR (5-10Gy) compared to shorter periods [up to 2hr after low dose (1-10cGy)].

(VII) Inhibition of ATM/ATR kinase activities abolished the regulation of S and G2/M checkpoints after low doses of IR (10cGy) illustrating that these kinases are important for low dose radiation induced checkpoint regulation. DNA-PK was found to be critical for G2/M checkpoint only after high doses of gamma rays (2.5-10Gy) in primary human fibroblasts.

(VIII) Our findings suggest that ATM kinase is highly critical for an efficient cellular response to low dose radiation both in proliferating and plateau phase cells. Also, transient cell cycle checkpoint regulation imposed by low doses of gamma rays (10-50cGy) is dependent on ATM dependent signaling pathways. At doses higher than 1Gy, ATM appears to be somewhat dispensable for G2/M checkpoint regulation as the loss of ATM to some extent is complemented by DNA-PK. Loss of both ATM and ATR kinase activities is highly detrimental to cell survival after IR exposure. Likewise, inhibition of ATM kinase activity in DNA-PK deficient human glioblastomas abolished the cell cycle checkpoint regulation both at S and G2/M phases.

(IX) Phospho-proteomic approach has identified a novel molecular crosstalk between MAPK and PIKK in response to low and high doses of IR. Effects of low (10cGy) and high doses (1 and 5 Gy) of γ-rays exposure on cell cycle regulation was analyzed in PIKK proficient (MRC5 and NHDF) and deficient (ATM-GM2052F; ATR-GM8398A) cells. After exposure to different doses of γ-rays and the cells were processed for cell cycle analysis at different post-irradiation times (1hr, 8hr and 24hr). PIKK proficient cells displayed an efficient G2/M checkpoint at 1hr and 8hr after radiation treatment and the extent of G2/M arrest was radiation dose dependent. In cells irradiated with 0.1Gy, a transient G2/M delay was evident at 1hr but not with higher doses (1Gy and 5Gy). Further, the proportion of G1 phase cells slightly increased in 0.1Gy irradiated samples at 8hr and 24hr as compared to sham treated control samples. In cells irradiated with 5Gy, a strong

G2/M delay was observed and as much as 67.8% of the cells were found to be in G2/M phase. The proportion of G2/M phase cells gradually declined with increasing post recovery time and at 24hr sampling time only 37.1% of the cells were in G2/M phase. Similar results were obtained with human primary MRC5 fibroblasts. Phosphohistone-H3 staining was used to precisely monitor the progression of cells from G2 to M phase. Activation of MAPK pathway in general and ERK1/ERK2 in particular, has been implicated in G2/M checkpoint after radiation exposure. Thus, an efficient sustained activation of both p38 and ERK1/ERK2 observed in PIKK proficient cells nicely correlates with an efficient sustained activation of both p38 and ERK1/ERK2. Treatment of MRC5 cells, prior to radiation exposure, with a small molecule inhibitor specific for ATM/ATR kinase (GCK733) also abolished the G2/M checkpoint control. Collectively, our findings suggest that the molecular crosstalk between MAPK and PIKK determines the efficiency of IR induced cell cycle checkpoints in human cells (***Manuscript under preparation***).

(X) Using a phosphor-proteomic approach, we earlier demonstrated that the low dose radiation induced activation of both MAPK and PKC pathways depend on the functional status of PIKK. Of interest, a radiation dose dependent (10cGy-5Gy) activation of Hsp27 was earlier noted by us in PIKK proficient MRC5 fibroblasts. In contrast, cells deficient in ATM, ATR and DNA-PK failed to show either the induction or the phosphorylation of Hsp27. To determine whether or not Hsp27 is a crucial factor for the cellular response to low dose radiation, we analyzed the radiation response of human fibroblasts after the stable suppression of Hsp27 by siRNA. The suppression of Hsp27 was found to be 80-90% of the cells transfected with scrambled siRNA. Suppression of Hsp27 greatly diminished IR induced (10cGy-1Gy) activation of phosphorylated H2AX and 53BP1 foci formation. Further, Hsp27 suppressed cells showed 4 fold increase in the induction of micronuclei in comparison to cells transfected with scrambled siRNA. These findings indicate a pivotal role for Hsp27 in initiating the low dose radiation induced cellular response in human cells.

(XI) Most importantly, our ongoing collaborative study identified the involvement of two major tumor suppressor genes that significantly modulate the cellular response to IR: (I) PTEN and (II) HINT1. Loss of PTEN deregulates the expression several key factors

(Rad51, BRCA1 and BRCA2) in homologous recombination repair pathway and disrupts the chromosomal integrity in both mouse and human systems ***(Cell, 128, 157-170, 2007).*** Consistent with a defect in homologous recombination repair pathway, PTEN deficient embryonic stem cells displayed extensive chromosomal aberrations after low doses of gamma rays radiation (10-50cGy). The overall aberration frequency (chromatid breaks, chromosome breaks, dicentrics and Robertsonian centric fusions) after exposure to 0.5Gy of gamma rays was found to be 0.52/metaphase for PTEN proficient cells. In contrast, the aberration frequency was found to be 1.0/metaphase in PTEN deficient cells. Strikingly, chromatid breaks were 3 fold higher in PTEN deficient cells (72/100 metaphases) than in PTEN proficient cells (24/100 metaphases). Interestingly, treatment with caffeine (an inhibitor of ATM and ATR kinases) prior to radiation exposure (0.5Gy of gamma rays) considerably elevated the overall frequency of all types of aberrations in PTEN proficient cells (2.3/metaphase) but only slightly in PTEN deficient cells (1.4/metaphase). Similar results obtained in ATM deficient primary fibroblasts after caffeine and radiation treatments suggest that ATM mediated pathway may be disrupted in PTEN deficient cells. Collectively, these findings illustrate that PTEN is crucial for both DNA repair and cell cycle checkpoint activities after radiation exposure. Further, IR exposure triggered the relocation of phosphorylated PTEN from cytoplasm to nucleus in PTEN proficient MEFs. As PTEN exhibits a dual phosphatase activity for both proteins and lipids, we wish to determine whether the phosphatase activity of PTEN is crucial for modulating the activities of DNA repair and cell cycle checkpoint proteins. For this purpose, cells deficient in PTEN phosphatase domain are being generated. Future studies are warranted to define the precise role of PTEN in IR induced cellular response. Additionally, extensive mitotic irregularities were specifically observed in PTEN deficient cells at these low radiation doses. These findings clearly illustrate the importance of functional PTEN protein for an efficient DNA damage response.

(XII) The persistence of IR induced foci showed a nice correlation with features of genomic instability involving micronuclei, chromosome translocations and chromatin bridges. Using HINT1 deficient mouse model system, we recently demonstrated that the lack of histone acetylation is responsible for persistence of IR induced foci and persistent

foci sites may contain misrejoined chromosome fragments (***Journal of Cell Biology, 183, 253-265, 2008)***.

(XIII) Hint1 modulates the response of ATM kinase activity through Tip60 complex as well as ATM associated signaling pathways and Hint 1 deficient cells show many features of genomic instability. Loss of Hint 1 also affects the histone acetylation events after IR (***Journal of Cell Biology, 183, 253-265, 2008)*** leading to disruption of chromatin remodeling process.

(XIV) We have recently shown that the loss of major tumor suppressor gene p15/Ink4b triggered by low (γ-rays) and high LET (Fe ions) radiations results in tumorigenesis and the severity of tumorigenesis process depends on radiation quality and dose (***Carcinogenesis, 30, 1889-1896, 2010***). Interestingly, the tumorigenesis process induced by high LET radiation (Fe ions) seems to be accompanied by the formation of Robertsonian type centric fusions in Ink4b /Arf deficient murine astrocytes. Future in depth studies would clarify whether formation of chromosome fusion event (Robertsonian type) is an initiator for tumorigenic process. Additionally, epigenetic events triggered by chromosome fusion events would help in deducing the molecular events that initiate and promote IR induced tumorigenesis process.

Some of the results mentioned above have been published in peer reviewed journals. Results pertaining to the analysis of MAPK, Hsp27 and PTEN as well as the effects of PIKK on cell cycle checkpoint regulation are being compiled for publication. Few additional experiments are underway for the completion of our study on comparative analysis of DNA damage response in human adult stem cells and differentiated cell types.

Abstracts presented in scientific meetings:

1. **A.S. Balajee**, K. Hopkins and H.B. Lieberman. (2005) Role of Rad9 in base excision repair. 52nd Annual Meeting of Radiation Research Society, Denver, Colorado. October 16-19. pp325.
2. K. Hopkins, **A.S. Balajee** and H.B. Lieberman (2005) Deletion of mouse Rad9 leads to high spontaneous frequencies of sister chromatid exchange. 52nd Annual Meeting of Radiation Research Society, Denver, Colorado. October 16-19. pp309.
3. **A.S. Balajee,** R. Baskar and C.R. Geard (2005) Histone H2AX is dispensable for base excision repair activity. 52nd Annual Meeting of Radiation Research Society, Denver, Colorado. October 16-19.pp286.
4. Y. Su, J. A. Meador and **A.S. Balajee** (2006). Ionizing radiation induced DNA damage signaling and repair responses in human 3-dimensional skin model system. 53rd Annual Radiation Research Meeting, Philadelphia. Nov 4-8.
5. Y. Su, J. A. Meador and **A.S. Balajee**. (2007) Molecular crosstalk between PIKK and MAPK in cellular response to ionizing radiation. 13th International Congress on Radiation Research, San Francisco, July 8-12.
6. J.A. Meador, Y. Su and **A.S. Balajee** (2007). Zebularine selectively kills human glioblastoma cells deficient in DNA dependent protein kinase (DNA-PK). Annual Meeting of American Association for Cancer Research, Los Angeles April 14-18. #1970
7. W. H. Shen, **A. S. Balajee**, J. Wang and Y. Yin (2007). PTEN maintains chromosomal integrity through physical interaction with centromere and control of DNA repair. Annual Meeting of American Association for Cancer Research, Los Angeles April 14-18. #5688
8. H. Li, **A.S. Balajee**, T. Su, T. K. Hei and I. B. Weinstein (2007). The novel tumor suppressor gene Hint 1 may play a role in DNA damage response by modulating the activity of Tip60. Annual Meeting of American Association for Cancer Research, Los Angeles April 14-18. #1084
9. J.A. Meador, Y. Su and A.S. Balajee (2008) Modulatory role of Phospatidylinositol-3 Kinase like Kinases (PIKK) in cellular response to low LET radiation. DOE Low

Dose Research Program Investigator's Workshop VII, Jan 21-23, Bethesda, MD, USA.

10. J. A. Meador, Y. Su, R. Morris, R. Du and **A. S. Balajee** (2009). Analysis of ionizing radiation induced DNA damage and repair in multipotent adult stem cells and differentiated human skin cell types. 55th Annual Meeting of Radiation Research Society, Atlanta, Georgia, USA, October 10-12.
11. J.A. Meador, Y. Su and A.S. Balajee. (2009) Analysis of ionizing radiation induced DNA damage induction and repair in human multipotent adult stem cells and differentiated cell types. DOE Low Dose Research Program Investigator's Workshop VIII, April 6-8, Bethesda, MD, USA.
12. C. Leloup, H. Hang, **A.S. Balajee**, K. Hopkins and H.B. Lieberman. (2010) Role of *MRad9b* in DNA repair. 56th Annual Meeting of Radiation Research Society, Maui, Hawaii, September 26-29. PS2.47.
13. C.V. Camacho, B. Mukherjee, B. McEllin, N. Tomimatsu, L-H. Ding, C. Nirodi, D. Saha, M. Story, **A.S. Balajee**, R. Bachoo and S. Burma. (2010). Loss of p15/Ink4b accompanies tumorigenesis triggered by complex DNA double-strand breaks. 56th Annual Meeting of Radiation Research Society, Maui, Hawaii, September 26-29. PS6.46.
14. J.A. Meador, D. Delia and **A.S. Balajee** (2010) Role of ATM kinase in ionizing radiation induced DNA damage response in human neural stem/progenitor cells and differentiated cell types. DOE Low dose research program investigator's workshop IX, April 12-14, Bethesda, MD, USA.

Research publications in peer reviewed journals:

1. S. Sudha, D.J. Stumpo, **A.S. Balajee**, C.B. Block, P.M. Lansdorp, R.M. Brosh and P.J. Blackshear (2007). RecQL, a member of the RecQ family of DNA helicases, suppresses chromosomal instability. **Mol. Cell. Biol**. 27, 1784-1794.
2. W. Shen #, **A.S. Balajee** #, J. Wang, H. Wu, C. Eng, P.P. Pandolfi and Y. Yuxin (2007). Role of nuclear PTEN in the maintenance of chromosomal integrity. **Cell** 128, 157-170 (#Shared first author).
3. R. Baskar, **A.S. Balajee** and C.R. Geard (2007). Effects of low and high LET radiations on

bystander human lung fibroblast cell survival. **Int. J. Radiat. Biol**.83, 551-559.

4. Baskar R, **Balajee AS**, Geard CR, Hande MP (2008). Isoform-specific activation of protein kinase c in irradiated human fibroblasts and their bystander cells. **Int. J. Biochem Cell Biol.** 2008; 40:125-134.
5. E.T. Sakamoto-Hojo and **A.S. Balajee*** (2008) Targeting Poly (ADP) ribose polymerase 1 and PARP-1 interacting proteins for cancer treatment. **Anti cancer agents in Med. Chem.** 8, 402-416.
6. J.A. Meador, M. Zhao, Y. Su, G. Narayan, C.R. Geard and **A.S. Balajee***. (2008) Histone H2AX is a critical factor for cellular protection against DNA alkylating agents. **Oncogene** 27, 5662-5671.
7. H. Li, **A.S. Balajee**, T. Su, B. Cen, T.K. Hei and I.B. Weinstein (2008) The HINT1 tumor suppressor regulates both gamma-H2AX and ATM in response to DNA damage. **J. Cell Biol**. 183, 253-265.
8. H. Bu, B. Shen, Y. Su, C.R. Geard and **A.S. Balajee*** (2009) Protein kinase C epsilon is involved in ionizing radiation induced bystander response in human cells. **Int .J. Biochem. Cell Biol**., 41, 2413-2421.
9. Y. Su, J.A. Meador, C.R. Geard and **A.S. Balajee*** (2010) Analysis of ionizing radiation induced DNA damage and repair in human 3-dimensional skin model system. **Exp. Dermatol**. 19, 16-22.
10. C. Camacho, B. Mukherjee, B. et al. (2010) Loss of p15/Ink4b accompanies tumorigenesis triggered by complex DNA double strand breaks. **Carcinogenesis**, 31, 1889-1896..
11. Y. Su, J.A. Meador, G. Calf, L. Proietti DeSantis, Y. Zhao, V.A. Bohr and **A.S. Balajee*** (2010). Human RecQL4 helicase plays critical roles in prostate carcinogenesis .**Cancer Res.** 70, 9207-9217.

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