

## Effects of Sevoflurane on Breast Cancer Cell Function *In Vitro*

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**Abstract.** *Some retrospective clinical studies have shown there to be an association between the anaesthetic technique employed during breast cancer surgery and recurrence or metastases. Little is known about the direct effects of volatile anaesthetics on cancer cells. In the present study we investigated the effects of sevoflurane on estrogen receptor-positive (ER<sup>+</sup>) and estrogen receptor-negative (ER<sup>-</sup>) breast cancer cell functions that may contribute to metastatic potential. Materials and Methods: MCF7 ER<sup>+</sup> and MDA-MB-231 ER<sup>-</sup> breast cancer cells were incubated with or without sevoflurane, at concentrations of 1, 2, 3, and 4 mM for 6 h. Cell proliferation migration and invasion assays were then employed to measure for sevoflurane effects. An independent sample t-test analysis was used to compare for differences obtained between the groups. Results: Sevoflurane increased proliferation in MCF7 cells by 50-63% and by 50-67% in MDA-MB-231 cells (p<0.05). Sevoflurane increased migration in both breast cancer cell lines, by 30-58% in MCF7 (p=0.04) and by 30-230% in MDA-MB-231; statistically significant at 2, 3 and 4 mM (p<0.03). Increase in invasion ranged from 100-170% in MCF7, (p=0.02) and 28-72% in the MDA-MB-231 cell line, statistically significant only at the 4-mM concentration. Conclusion: In this in vitro model of breast cancer cell function, sevoflurane increased proliferation, migration and invasion in ER-positive MCF7 cells and increased proliferation, and migration but not invasion in ER-negative cells. However, the observed effect size was small and not dose-dependent.*

Breast cancer is the most common type of cancer and the second leading cause of cancer-related death in women, usually caused by recurrence and metastasis (1). Although treatment is based on surgical removal of the primary tumour, this approach can be associated with inadvertent dissemination of tumour cells

into the lymphatics and bloodstreams (2). Whether this results in clinical metastases depends on the balance between anti-metastatic immune activity and the cancer cells' ability to proliferate, migrate and invade adjacent tissues (3). A number of perioperative factors including the choice of anaesthetic agents or techniques, and the management of acute pain and use of opioids, may potentially influence the later process (4). While one retrospective clinical study in breast cancer (5) has indicated an association between the anaesthetic technique used for primary surgery and the risk for recurrence and metastasis, other retrospective studies in on this specific issue have not (6, 7). A prospective, randomised trial is ongoing in breast cancer patients to determine whether choice of the anaesthetic technique affects cancer outcome, but data may not be available for many years (8). There are little data on the direct effect of anaesthetic agents, particularly volatile agents, on cancer cell biology and available information on non-tumour cells is contradictory (9-12). Sevoflurane is perhaps the most frequently used volatile agent clinically, and its effect on breast cancer cell function has not been evaluated directly. In the present study we investigated the effect of sevoflurane on breast cancer cell functions essential to their metastatic potential: proliferation, migration and invasion, using two different breast adenocarcinoma cancer cells with different metastatic potential.

### Materials and Methods

**Cell cultures.** MDA-MB-231, an oestrogen receptor-negative human breast adenocarcinoma cell line, and MCF7 an oestrogen and progesterone receptor-positive human breast adenocarcinoma cell line were used in the study. Both cell lines were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and cultured according to their specifications. Cells were grown as monolayers in 75 ml standard tissue culture plastic ware (Sarstedt, Dublin, Ireland). For experiments, cells were harvested from 70% sub-confluent cultures by trypsinisation, resuspended in media and added to assay plates as per individual assay's protocol.

**Anaesthetic drug.** Sevoflurane was obtained in a liquid state from Abbot (Abbott Ireland Ltd., Dublin, Ireland). 140 µl of sevoflurane were diluted in 10<sup>3</sup> ml cell culture medium by stirring for 30 min in an airtight, ground-glass flask to produce a relatively stable 10 mM

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**Key Words:** Anaesthesia, sevoflurane, breast cancer.

solution as previously described (10). The prepared solution was further diluted with cell media to 4, 3, 2, and 1 mM immediately before experiments. Decrease of concentration due to evaporation for over 6 h of experiment was compensated by hourly replacing the solution. Corresponding concentrations of water in media were used as controls. The concentration of sevoflurane in experimental dishes was measured at 6 h to ensure for the desired concentration of sevoflurane.

**Cell proliferation assay.** Cell proliferation was determined using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Inc., Madison, USA), according to the manufacturer's protocol. Six parallel sets of assays were performed and average data were used for analysis. Cells were added to 96-well plates at a density of 50,000 cells/well. Plates were incubated for 24 h in medium supplemented with FBS to allow for cell attachment, followed by a 24-h incubation in serum-free medium. Sevoflurane was added to the appropriate wells. Cells-only were used as control. Plates with and without sevoflurane were incubated for 6 h. Proliferation was defined as an increase in the number of cells and measured by change in absorbance. Absorbance was measured with a spectrophotometric plate reader using 490 nm filter set.

**Cell migration assay.** Cell migration was determined by a scratch assay. Six parallel sets of assays were performed simultaneously and the average obtained data were used for further analyses. Cells were added to 6-well plates at a density of 500,000 cells/well. The plates were first incubated for 36-48 h in medium supplemented with FBS to allow for cell attachment and confluency. Confluency of cells was checked under microscope and a scratch in cell monolayer was performed using a 10- $\mu$ l pipette tip. Cell plates were visualized using a Nikon Eclipse TS100 phase contrast microscope at  $\times 10$  magnification. Pictures of wells were obtained with a Nikon Coolpix 990 digital camera and Leica  $\times 10$  eyepiece adaptor under standardized settings. Pictures were analyzed using Image Pro Plus version 6.2 (Media Cybernetics) calibrated in a picture of a slide micrometer captured under identical settings. 10 measurements of the distance between the leading edges of the cell growth front were obtained and averaged to determine closure rates. Sevoflurane was added to appropriate wells, cells in media alone were used as control. Plates were sealed with cling-tape and incubated for 6 h. The width of scratch was measured again after 6 h and was compared to the width recorded at beginning.

**Invasion assay.** Cell invasion was investigated by Biocoat Matrigel Invasion Chambers (BD Biosciences, Bedford, USA), according to the manufacturer's protocol. Six parallel sets of assays were performed and average data was used for analysis. 24-well invasion chambers were removed from  $-20^{\circ}\text{C}$  storage and allowed to reach room temperature. The inserts were rehydrated for 2 h by adding 250  $\mu$ l of serum-free medium to each chamber. After that, the medium was replaced with 500  $\mu$ l of a 500,000 cells/ml cell suspension in serum-free medium with or without sevoflurane. 750  $\mu$ l of medium with 20% FBS with or without sevoflurane in corresponding concentrations was added to the outer chamber as chemo-attractant. Plates with and without sevoflurane were incubated for 6 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ . Following incubation, non-invasive cells were removed from the upper chamber using cotton swabs soaked with PBS. Cells that had invaded through the Matrigel membrane were fixed with methanol and stained with Haematoxylin. Inserts were then dehydrated by soaking in solutions with increasing

concentrations of ethanol; the membrane was removed from the insert and mounted on a slide with DPX mounting medium. Cells were visualized at  $\times 10$  magnification; the number of cells in 5 fields per slide was counted and averaged. Invasion was expressed as the ratio of invading cells incubated with drugs compared to the controls.

**Statistical analysis.** Results for migration and invasion were normalized to seek for changes in proliferation. Mean (SD) values were calculated and compared with controls at each concentration, using the independent sample *t*-test for differences between the groups. Percentage differences in values between drug and controls across all concentrations for a given drug were also obtained.  $p < 0.05$  was considered statistically significant.

## Results

Sevoflurane increased proliferation by 50-63% and by 50-67% in MCF7 and MDA-MB-231 cells respectively, but no dose-response effect was observed in either cell type (Figure 1A and 1B). This change was statistically significant ( $p < 0.05$ ) at all concentrations in MCF7 cells and at all, except for the 1 mM sevoflurane concentration, in the MDA-MB-231 cell line.

Sevoflurane increased migration in both breast cancer cell lines. In MCF7 cells, this was by 30-58%, and was statistically increased at 2, 3 and 4 mM sevoflurane, but not at 1mM (Figure 2A). Sevoflurane increased migration by 30-230% in MDA-MB-231, which was statistically significant at 2, 3 and 4 mM, but again was not significant at the 1 mM concentration (Figure 2B). Similarly to proliferation data, there was no apparent dose-response relationship in this stimulatory effect of sevoflurane on migration.

Sevoflurane also increased invasion of both breast cancer cell lines (Figure 3A and 3B). The observed increase ranged from 100-170% in MCF7 cells and was statistically significant at all concentrations studied. Increase in invasion ranged between 28-72% in MDA-MB-231 cells, but this change was statistically significant only at the 4 mM concentration. Again, as observed in proliferation and migration studies, no dose-response effect of sevoflurane on cancer cell invasion was evident.

## Discussion

Retrospective data have shown that the choice of anaesthesia for primary breast cancer surgery can influence the risk of cancer recurrence and metastasis (4-6). While a causal link between anaesthetic technique and reduced or increased cancer metastasis requires confirmation in prospective, randomised clinical trials, research should also attempt to evaluate mechanisms by which anaesthetic agents may influence cancer cells or their interaction with the host patient, particularly *via* the immune system at the cellular level. This *in vitro* cell culture study showed that sevoflurane increases proliferation, migration and invasion

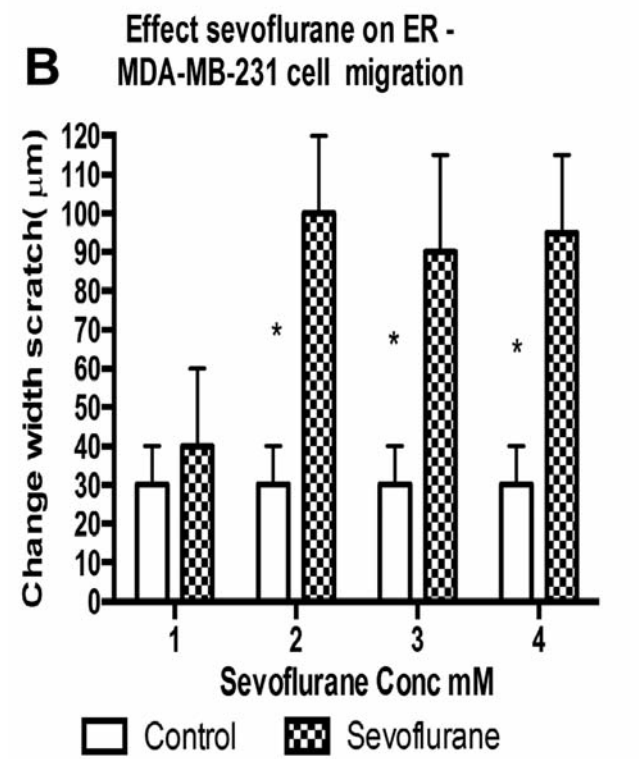
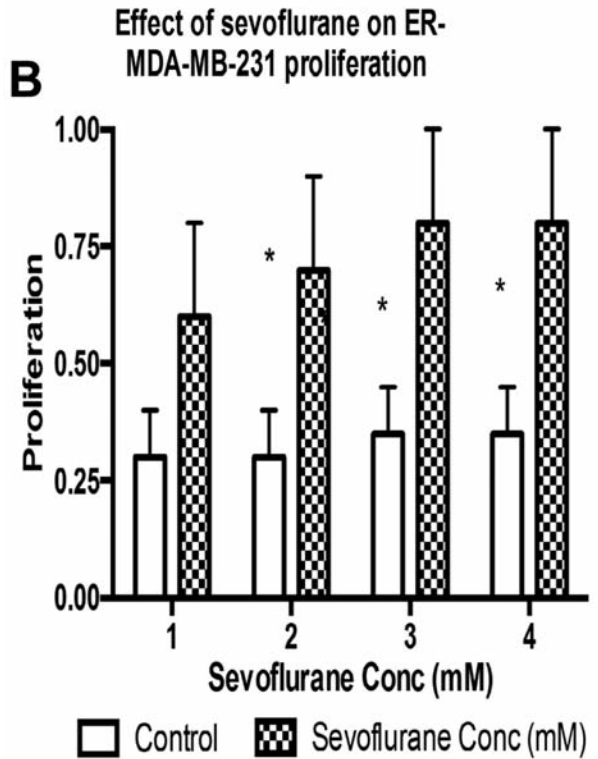
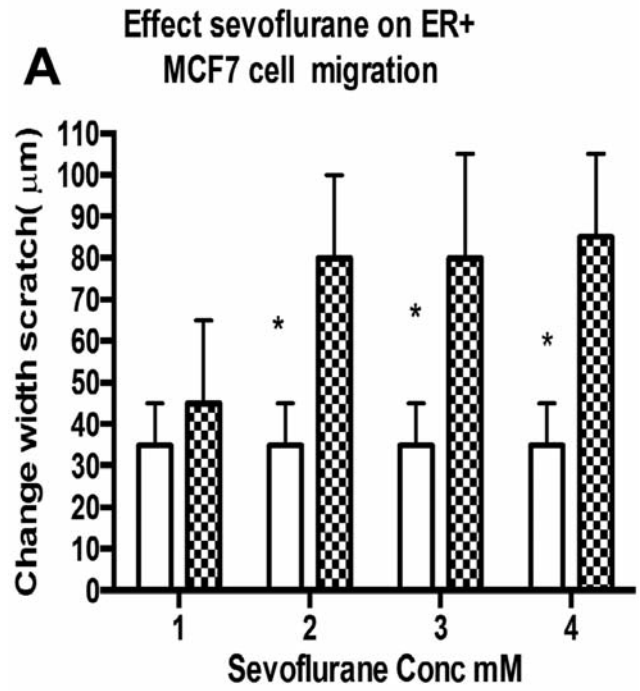
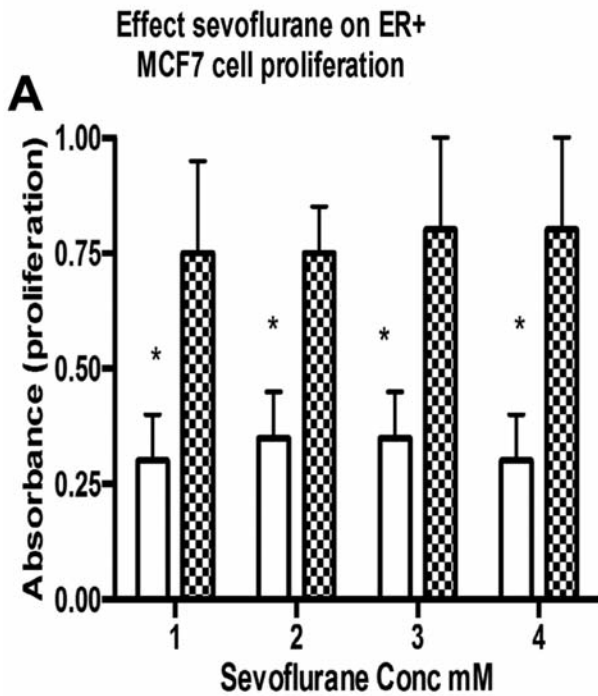


Figure 1. A. Effect of sevoflurane on the proliferation on Estrogen receptor positive (ER<sup>+</sup>) MCF7 breast cancer cells compared to controls. Sevoflurane significantly increased proliferation at all concentrations between 1-4 mM. \* $p < 0.05$ . B. Effect of sevoflurane on the proliferation on Estrogen receptor-negative (ER<sup>-</sup>) MDA-MB-231 breast cancer cells compared to controls. Sevoflurane significantly increased proliferation at 2-4 mM concentrations, but not at 1 mM concentration. \* $p < 0.05$ .

Figure 2. A. Effect of sevoflurane on migration on Estrogen receptor-positive (ER<sup>+</sup>) MCF7 breast cancer cells compared to controls. Sevoflurane significantly increased migration at 2-4 mM concentrations, but not at 1 mM concentration. \* $p < 0.05$ . B. Effect of sevoflurane on migration on Estrogen receptor-negative (ER<sup>-</sup>) MDA-MB-231 breast cancer cells compared with controls. Sevoflurane significantly increased migration at 2-4 mM concentrations, but not at 1 mM concentration. \* $p < 0.05$ .

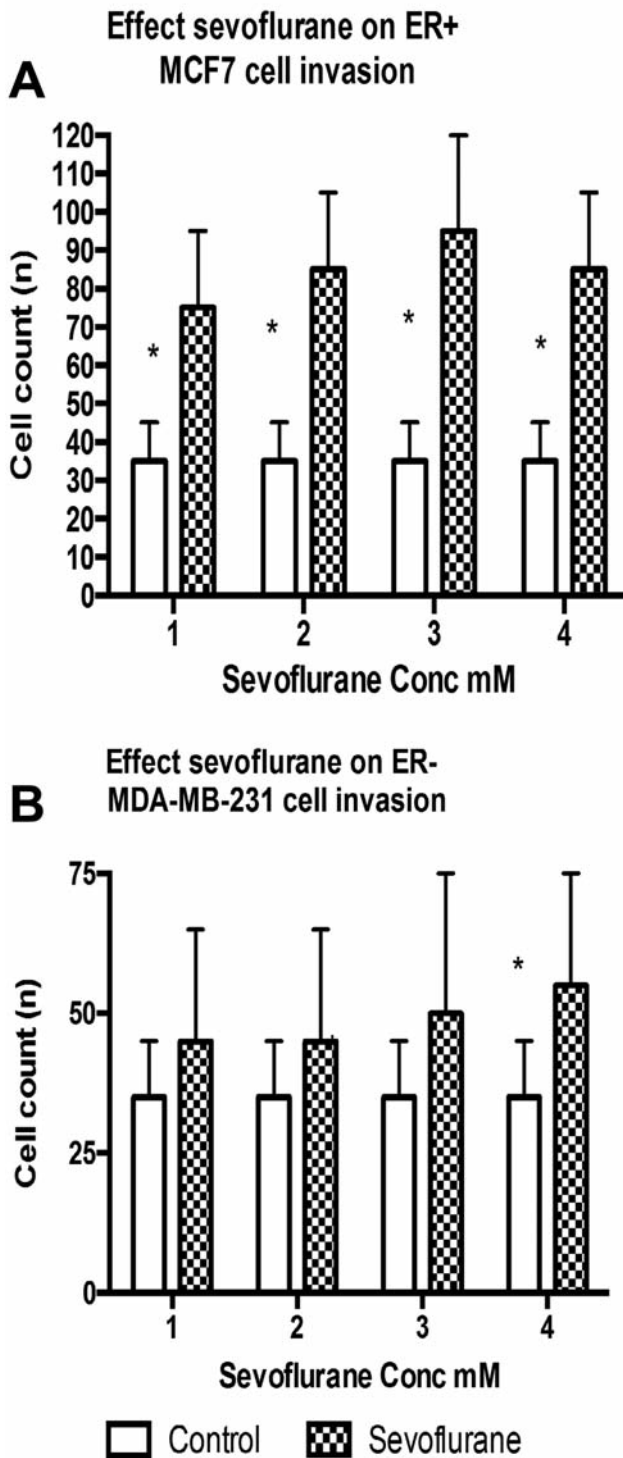


Figure 3. A. Effect of sevoflurane on invasion on Estrogen receptor-positive (ER<sup>+</sup>) MCF7 breast cancer cells compared with controls. Sevoflurane significantly increased invasion at all concentrations between 1-4 mM. \**p*<0.05. B. Effect of sevoflurane on invasion on Estrogen receptor-negative (ER<sup>-</sup>) MDA-MB-231 breast cancer cells compared to controls. Sevoflurane significantly increased invasion only at 4 mM concentration. \**p*<0.05.

functions in ER<sup>+</sup> breast cancer cells and only proliferation and migration in ER<sup>-</sup> breast cancer cells. A small amount of data is available on the effect of volatile anaesthetics on cancer cells. Recently, an halogenated volatile agent, isoflurane, was shown to facilitate renal cancer cell migration *via* the Hypoxia-inducible factor cell signalling pathway progression in an *in vitro* model (13). This supports our present data in suggesting that frequently used volatile anaesthetics can exert pro-tumorigenic effects on human cancer cell lines. In contrast, cell culture studies on lung cancer cells have indicated that sevoflurane actually inhibits migration and invasion by inactivating the p38 MAPK signalling pathway (14). This discrepancy in the effect shown for sevoflurane between our breast cancer cell data and lung cancer cell data raises the question of whether the effect of anaesthetic agents on cancer varies with cancer type. This seems a plausible explanation, given the widely recognised fact that different tumour types behave differently in the clinical environment.

While we observed that proliferation of ER<sup>+</sup> cells was significantly increased with exposure to sevoflurane, earlier data on non-cancer, non-tumour cells indicated that sevoflurane produced an anti-proliferative effect (15). This difference is probably attributable to different behavioural patterns of cancer cells compared with non-cancer cells when exposed to sevoflurane.

While both ER<sup>+</sup> and ER<sup>-</sup> breast cancer cell lines exhibited similar stimulatory effects, there was a difference between them in terms on cell invasion, where a minimal effect of sevoflurane was observed in ER<sup>-</sup> cells. It is clinically recognised that ER<sup>-</sup> breast cancers behave differently and are associated with poorer prognosis, than ER<sup>+</sup> cells. In this case, the apparent lack of an effect of sevoflurane on the invasion of ER<sup>-</sup> cells would theoretically reduce their malignant potential, but this may be merely an isolated observation.

The fact that no dose response of sevoflurane on breast cancer cell function was observed in either cell type is also interesting. This may suggest that the effect of sevoflurane on these functions is not primarily mediated by receptors but rather by a diffuse effect throughout breast cancer cellular function. Given that the effect on migration and invasion differs between ER<sup>-</sup> and ER<sup>+</sup> cell lines, this raises the possibility that estrogen receptors may play a role in mediating the effect of sevoflurane on invasion or migration. The direct effect of sevoflurane has been more extensively studied on neuron cells exposed to ischemia and reperfusion injury. In these studies, it was established that the neuroprotective effect of sevoflurane is delivered through various mechanisms including GABA receptors, NMDA receptors, inhibition of intracellular calcium response and effect of sevoflurane on glutamate uptake and antioxidant formation (16). Sevoflurane was shown to have an immunomodulatory effect in sepsis-

injured alveolar epithelial cells (17), but this lies far from the context of the present study, where we found that sevoflurane is stimulatory to functions of breast cancer cells which facilitate cancer metastasis and progression.

While cell culture studies provide valuable data on the effect of anaesthetic agents on cancer cell functions *in vitro*, more relevant data would be obtained from breast cancer animal models. Indeed such a mouse model has recently been described and studies on the effect of opioids and other perioperative drugs on cancer outcome using this animal model are anticipated (18). In another study, using serum from patients with breast cancer, who were randomized to either paravertebral block/propofol or opioid/sevoflurane anaesthesia, we have shown that the latter increased proliferation and migration in MDA-MB-231 breast cancer cells (19). While in another breast cancer cell culture study performed by our group, we showed that the *NET1* gene is associated with the mechanism of the effect of morphine on breast cancer cell function (20).

In conclusion, this study examined the *in vitro* effects of sevoflurane anaesthesia on ER<sup>+</sup> and ER<sup>-</sup> breast cancer cells and has presented evidence of a pro-tumourigenic effect of sevoflurane on proliferation and migration for both cell types and only on invasion for ER<sup>+</sup> cells. The extent of the effects that sevoflurane produces in the specific *in vitro* model is relatively small.

### Role of the Investigators

Patricija Ecimovic contributed to the design of the study, conducted most of the original experimental work, assisted analysing the data and drafted the manuscript. Blathnaid McHugh assisted with the conduct of the experimental work supervised by Dr. Ecimovic; David Murray contributed to the design of the study and supervised the conduct of the experimental work; Peter Doran contributed to the design of the study, assisted in analyzing the data and drafted the manuscript. Donal Buggy developed the ideas and concept for the study, funded it, analyzed the data and drafted the manuscript.

### Conflicts of Interest

No investigator has any conflict of interest to declare in this study.

### Acknowledgments

This work was funded in part by National Institute for Academic Anaesthesia (NIAA) UK and the Sisk Healthcare Foundation, Ireland.

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*Received August 28, 2013*

*Revised September 9, 2013*

*Accepted September 11, 2013*