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Evaluating the Efficacy of Radiofrequency Amplitude-Modulated Electromagnetic Fields as a Novel Treatment for Hepatocellular Carcinoma

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EVALUATING THE EFFICACY OF RADIOFREQUENCY AMPLITUDE-MODULATED ELECTROMAGNETIC FIELDS AS A NOVEL TREATMENT FOR HEPATOCELLULAR CARCINOMA

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ABSTRACT

Hepatocellular carcinoma (HCC) incidence in the US is dramatically increasing. Five-year survival has remained 3-5% for the past several decades, demonstrating urgent need for additional therapies. Intrabuccal administration of amplitude-modulated radiofrequency electromagnetic fields (RF EMF) is a novel, minimally invasive treatment modality. Clinical evidence demonstrates this treatment approach elicits therapeutic responses in cancer patients. *In vitro* we have described a phenotype in HCC cells following RF EMF exposure that included proliferative inhibition, modulation of gene expression, and disruption of the mitotic spindle. This phenotype was specific for HCC cells exposed to HCC-specific RF EMF. We have demonstrated similar efficacy in an *in vivo* model of HCC. Global gene expression data and subsequent validation suggest that modulation frequencies affect intracellular calcium release in cancer cells, resulting in our *in vitro* phenotype and *in vivo* efficacy.

HCC cells were exposed to RF EMF modulated at specific frequencies previously identified in HCC patients. Cell death mechanisms were evaluated using fluorescence microscopy, flow cytometry, immunohistochemistry, and Western blot. MicroRNA arrays compared exposed and control groups of HCC cells, with validation followed by Western blot. NOD SCID mice received HCC subcutaneous cellular xenografts. Following

palpable tumor establishment, mice were exposed to HCC-specific RF EMF, euthanized following excessive tumor burden, and evaluated by immunohistochemistry.

We identified increased levels of miRNAs that target mRNAs used to synthesize proteins important in the PI3K pathway, specifically IP3/DAG signaling and intracellular calcium release. This pathway is frequently disrupted in HCC, making it an excellent candidate for modulation by RF EMF; furthermore, downstream effects include: cell cycle progression, proliferation, inhibition of apoptosis, and cell migration, each of which were implicated in our *in vitro* phenotype. *In vivo*, normal tissue architecture was preserved and xenograft tumors were seen infiltrated with fibrous tissue. Xenograft tumors in RF EMF treated mice also showed significantly decreased growth rate as compared to controls.

These findings uncover a novel mechanism that controls cancer cell growth at specific modulation frequencies, with evidence of modulation of intracellular calcium levels. The optimization of this therapeutic approach may significantly alter the treatment algorithm for patients with HCC.

Keywords: Hepatocellular carcinoma, amplitude-modulated radiofrequency electromagnetic fields, cancer cell growth, cancer treatment

DEDICATION

This work is dedicated to my family. Thank you for all your support and encouragement over the past twenty-seven years.

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LIST OF ABBREVIATIONS

Ca^{++}	Calcium
EMF	electromagnetic fields
ER	endoplasmic reticulum
GSM	Global System for Mobile Communications
HCC	hepatocellular carcinoma
H&E	hematoxylin and eosin
Hz	hertz
HSF	heat shock factor
HSP	heat shock protein
IEI-EMF	idiopathic environmental intolerance attributed to electromagnetic fields
kHz	kilohertz
MEG	magnetoencephalography
MMP	matrix metalloproteinases
MHz	megahertz
μT	microtesla
mT	millitesla
ODC	ornithine decarboxylase
PKC	protein kinase C
RF EMF	radiofrequency electromagnetic fields
ROS	reactive oxygen species

T	tesla
TPA	12-O-Tetradecanoylphorbol-13-acetate
UV	ultraviolet

INTRODUCTION TO RADIOFREQUENCY ELECTROMAGNETIC FIELDS AND DISEASE

Introduction to Radiofrequency Electromagnetic Fields

Radiofrequency electromagnetic fields (RF EMF) lie within the low energy, nonionizing radiation range of the electromagnetic spectrum. The physiologic outcomes following long-term exposure to these fields have yet to be established. Humans receive exposure to RF EMF on a daily basis, and epidemiologic studies evaluating the consequences of uncontrolled environmental or occupational exposure are inconclusive [1-13]. While the overall emphasis of the literature has been to study the potential hazards associated with RF EMF exposure, recent studies demonstrate clinical benefits following controlled exposure to RF EMF [14-21]. The purpose of this dissertation is to evaluate the clinical benefits and biologic effects of exposure to amplitude-modulated RF EMF in hepatocellular carcinoma (HCC).

In the past several decades, these seemingly innocuous fields have been identified as possible risk factors for leukemia and brain tumors such as acoustic neuroma and glioblastoma multiforme [1, 3-6, 8, 9, 11, 13, 22]. Specifically, there has been a focus on risks associated with exposure to power lines as well as long term cellular phone use [1, 3-6, 8, 9, 11, 13, 22]. Though studies have not been able to consistently correlate increased risk with these exposure levels and durations, a stigma has remained. Thus the bulk of the literature evaluating RF EMF effects has emphasized risk rather than benefit. Our work, and that of others, aims to utilize controlled exposure

to electromagnetic fields in the treatment of cancer, specifically malignancies for which more traditional therapeutic options are insufficient [14-17].

Identification of Amplitude-Modulated RF EMF

Amplitude-modulated RF EMF was originally developed as a non-pharmacologic treatment option for physiological insomnia [23]. A 27.12 MHz carrier frequency is amplitude-modulated at specific frequencies that are delivered sequentially, beginning with the lowest one, with each modulation frequency exposure lasting 3 seconds [14]. Following the highest frequency, there is a 1 second pause, then the program is repeated [14].

Following feasibility studies in insomnia, the focus for implementing this therapeutic option shifted to oncology. Modulation frequencies for several common malignancies were previously identified by noninvasively scanning patients diagnosed with cancer with a spectrum of frequencies and identifying frequencies triggering a biofeedback response measured as changes in patient pulse amplitude, blood pressure, and skin electrical resistance [14]. Scanning was done with the same high precision frequency synthesizer device as the one used for therapeutic frequency delivery [14]. A 2009 feasibility study demonstrated complete and partial responses in patients receiving RF EMF; tumor size was monitored by bi-monthly Computed Tomography (CT) scan [14]. The study further showed a favorable safety profile; no patients reported NCI grade 2, 3 or 4 toxicities [14]. The promising findings from this study provided the initial rationale for recent clinical and laboratory investigations.

Amplitude-Modulated RF EMF in the Treatment of Hepatocellular Carcinoma

Recent focus has been an evaluation of the efficacy and mechanistic effects of amplitude-modulated RF EMF as a therapeutic modality for hepatocellular carcinoma. Hepatocellular carcinoma is one of the leading causes of cancer death worldwide [24], with steadily increasing incidence in the U.S. [25]. Patients with progressive HCC are expected to live less than six months. Most patients have severely impaired liver function, limiting therapeutic options [26, 27]. Sorafenib and liver directed therapies have shown limited success, demonstrating the urgent need for additional treatment modalities [28-31].

The inadequacies of current therapies led us to examine the efficacy of RF EMF in the setting of HCC. One hundred ninety-four modulation frequencies ranging from 400 Hz to 21 kHz were identified in patients with HCC and have subsequently been used to treat HCC patients; approximately 85% of the frequencies identified for HCC are unique and were not identified in patients with other primary malignancies [14]. An investigator initiated Phase I/II study conducted in 41 patients with advanced HCC showed long lasting responses in 10% (4/41) of patients, including a patient with biopsy-proven HCC who remained on RF EMF for over five years with documented disease progression at the time of treatment initiation [15]. These findings prompted us to initiate reverse translational experiments to investigate the mechanism of action of RF EMF. We have since demonstrated significant proliferative inhibition *in vitro* following HCC cell line exposure to RF EMF amplitude-modulated at HCC-specific modulation frequencies [32]. Proliferative inhibition was associated with the down regulation of genes contributing to cell motility and chemotaxis. Downregulation of two genes, *XCL2*

and *PLP2*, was validated [32]. Neither decreased gene expression nor growth inhibition were seen in normal hepatocytes (THLE-2 cells), suggesting a tumor specific effect [32]. We also made the exciting discovery that the mitotic spindle was disrupted in HCC cells following RF EMF exposure [32]. Additional *in vitro* data suggest that amplitude-modulated RF EMF may have an impact on novel pathways that may be crucial for long-term anticancer therapeutic effects. Our *in vivo* experiments provide the first animal model to dissect the effects of RF EMF and evaluate safety and efficacy in a xenograft model. Our studies demonstrate that RF EMF may be implemented safely in a clinical setting and elicit novel antitumor responses that may be paradigm-shifting [33].

Conclusions

Section 2 systematically discusses the literature reporting the epidemiologic outcomes and biological impacts following RF EMF exposure. Reported biological effects range from cellular morphology to effects on cellular signaling. The shortcomings of the literature are also discussed.

Sections 3-6 discuss the therapeutic potential of amplitude-modulated RF EMF. Specifically, Sections 3 and 4 provide evidence of modulation-specific effects of RF EMF exposure *in vitro* as well as clinically, with potential therapeutic application in clinical oncology. Sections 5 and 6 describe recent *in vitro* and *in vivo* findings and discuss the future experiments planned to better understand the mechanistic effects of amplitude-modulated RF EMF and expand the treatment modality to additional solid malignancies.

THE IMPACT OF ELECTROMAGNETIC FIELD EXPOSURE ON HEALTH AND HUMAN BIOLOGY

by

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In preparation for submission

Format adapted for dissertation

Environmental Exposure to Electromagnetic Fields

The electromagnetic spectrum is a continuum of energy arranged by frequency and wavelength (Figure 1). Though the general public receives daily environmental exposure to electromagnetic fields (EMF), until recently, it was very seldom a focus of public discussion. Common exposure sources include ultraviolet radiation (UV), power lines, cellular phones, and ionizing radiation used in medical therapy.

Frequent warnings and public service announcements highlight known risks associated with EMF exposure. For example, emphasis has been placed on the increased risk for skin malignancy, which is caused by increased pyrimidine dimer formation following exposure to the UV range of the spectrum. A safety focus has also been placed on the role of X-rays in healthcare, which are used for diagnostic imaging. X-ray penetration depends on tissue properties, leading to the image generated on X-ray film or other media. Prominent warnings are clearly posted in imaging rooms, alerting to the importance of protecting employees and patients, especially pregnant women, from unwanted exposure. Issuing such warnings was uncontested because there are recognized health risks from exposure to EMF at these high energy levels.

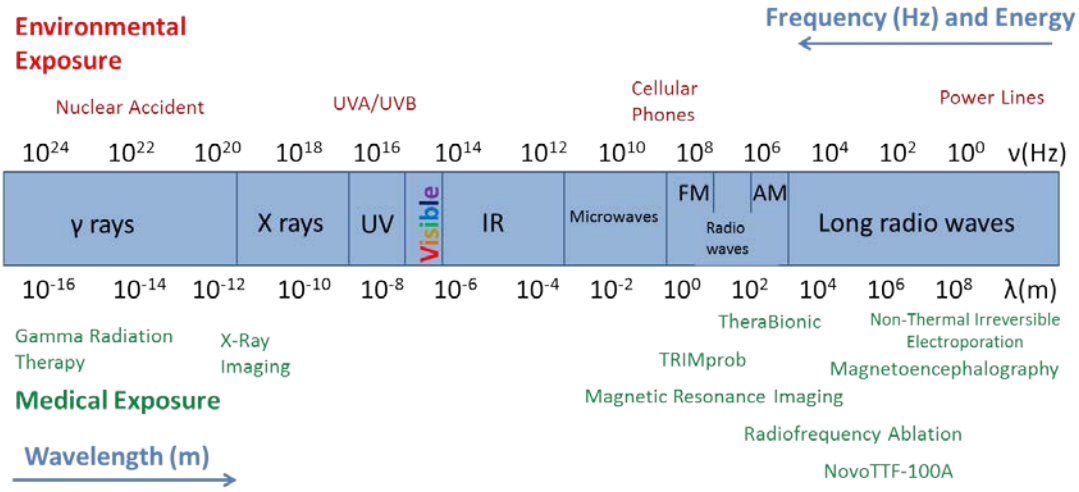

Figure 1. The electromagnetic spectrum and common exposures. The electromagnetic spectrum is depicted in blue. Environmental exposures with known or possible negative consequences are shown in red. Exposures received as part of medical diagnosis or treatment are shown in green.

Until relatively recently, minimal safety emphasis has been placed on the lower energy range of the spectrum because it has been a challenge to identify consequential relevant biologic impact. This energy range is comprised of low frequency fields, i.e. wavelengths greater than that of the visible range of the spectrum (Figure 1). Previously, interest in the lower energy range of the spectrum was limited to an interest in environmental and occupational risk associated with daily exposure to the EMF from power lines. Now there is emerging interest in the effects of environmental and occupational exposure to radiofrequency electromagnetic (RF EMF) fields.

Unlike higher frequency, higher energy waves in the spectrum, exposure to radiofrequency electromagnetic fields does not cause predictable DNA damage, as they are well below the energy threshold required for ionization. DNA strand breakage and free radical formation that results from exposure to high energy gamma radiation is exploited in the radiation therapy used to treat several types of malignancy as well as to destroy proliferating bone marrow cells in preparation for bone marrow transplant. However, the effects of exposure to fields in the lower energy radiofrequency range are far less predictable, and in some cases hotly debated (Havas 2004).

Rapidly changing technology and ever changing man-made sources of radiofrequency electromagnetic field exposure (RF EMF) have led to environmental exposure levels far surpassing those of previous generations. For example, just since the turn of the millennium, the use of cellular phones increased dramatically, with a recent estimate of more than 4.6 billion subscriptions globally (Swerdlow, Feychting et al. 2011). In the past few years the use of smart phones and tablets and the ubiquitous availability of residential and professional Wi-Fi has led to even greater exposure. It has become increasingly important to gain an understanding of possible risks associ-

ated with long-term exposure as well as to exploit possible benefits in a way similar to that done for gamma fields in radiation oncology.

The Controversy Surrounding RF EMF Exposure

Possible Risks Associated with RF EMF Exposure

The body of literature discussing the health risks of exposure to RF EMF is inconclusive. Epidemiologic and laboratory data demonstrate a cause-effect relationship between uncontrolled exposure to high energy radiation emitted from sources of ionizing radiation and incidence of radiation sickness and cancer. However, unlike the outcomes of populations exposed to high levels of ionizing radiation such as those in Chernobyl, Nagasaki, and Hiroshima, there is not undisputed epidemiologic evidence of increased disease risk associated with exposure to lower energy RF EMF (Williams 2003; Kodama, Ozasa et al. 2012; Takahashi, Abbott et al. 2012). Studies evaluating exposure to lower energy RF EMF have been unable to identify a direct cause-effect relationship between RF EMF exposure and malignancy or dementia. Several specific exposures and consequential health risks have been assessed in the literature, with limited consistency among studies.

Leukemia

One research emphasis has been investigating the risk for childhood leukemia as a consequence of living close to power lines, i.e. exposure to 50-60 Hz EMF. This focus is potentially due to children's lower threshold of tolerance to exposure. A study in lymphocytes demonstrated no significant increase in chromosomal damage following exposure to extremely low frequency electromagnetic fields (Hone, Edwards et al. 2003). Similarly, no alteration in apoptosis was identified in multipo-

tent hematopoietic progenitor cells exposed to low frequency magnetic fields (Reipert, Allan et al. 1997). Further, an Australian case-control study evaluating leukemia risk in the offspring of mothers with occupational exposure, found no increased risk for acute lymphoblastoid leukemia (Reid, Glass et al. 2011). While two case-control studies did associate increased risk of acute lymphoblastoid leukemia with living in close proximity to power lines, the significance of the relationship was inconsistent (Kroll, Swanson et al. 2010; Sohrabi, Tarjoman et al. 2010). Finally, a 2003 report from the Children's Health Workshop suggested there was no increased risk for acute lymphoblastic leukemia as a function of living in close proximity to power lines, but the group did admit that more thorough studies are necessary (Brain, Kavet et al. 2003).

While most of the literature has focused on the risk for childhood leukemia as a consequence of environmental exposure to power line EMF, increased risk for leukemia has also been evaluated in adults who live or work in close proximity to power lines. A Norwegian case-control study showed a non-significant increase in leukemia risk in the population with the greatest time-weighted average exposure to power line EMF, with no significant increase in incidence for those with occupational exposure (Tynes and Haldorsen 2003). A case-control study from Brazil evaluated mortality from leukemia and determined that there was a higher mortality rate in the population close to transmission lines (Marcilio, Gouveia et al. 2011). Similarly, a Spanish study found a higher rate of acute myeloid leukemia in the population living in close proximity to thermoelectric power plants and maximum density high-power lines (Rodriguez-Garcia and Ramos 2012). Ultimately, there is not a conclusive study identifying leukemia risk as a function of long-term proximity to power line EMF exposure, as many studies are relying on patient memory and suffer from small num-

bers. A comprehensive evaluation of a dose-response relationship between exposure to EMF emitted from power lines and leukemia incidence will be difficult due to the significant number of confounding variables.

Neurodegenerative Disease

In addition to common occupational exposures such as pesticides and heavy metal, occupational exposure to RF EMF has also been evaluated for increasing employee risk for Alzheimer's disease. A population study in Swedish twins did not demonstrate a significant risk for dementia as a consequence of RF EMF exposure, but the study did suggest that occupational exposure may be linked to earlier onset in those patients who did develop dementia (Andel, Crowe et al. 2010). However, the exposure levels of the participants in this study varied greatly depending on their occupation, with magnetic field exposures ranging from 0.24-4.03 μT (Floderus, Persson et al. 1996; Andel, Crowe et al. 2010). In both a review and a meta-analysis of occupational exposure to EMF and subsequent dementia, the findings were inconsistent and did not clearly identify a cause-effect relationship between EMF exposure and Alzheimer's disease (Santibanez, Bolumar et al. 2007; Garcia, Sisternas et al. 2008). Furthermore, the authors of a review discussing the quality of these epidemiologic studies observed that there were few studies evaluating occupational EMF exposure and Alzheimer's, and that the infrequency of this occupational exposure would necessitate large studies (Santibanez, Bolumar et al. 2007). They further concluded that the only occupational exposure that appears to be linked to Alzheimer's risk is exposure to pesticides (Santibanez, Bolumar et al. 2007).

In contrast to the epidemiologic studies, recent *in vivo* studies suggest that EMF may have a protective effect on the cognition of mice. Arendash *et al.* have

completed two studies demonstrating that RF EMF at frequencies similar to cellular phone exposure (918 MHz) may suppress β-amyloid aggregation in Alzheimer's transgenic mice while also having a cognitive benefit in normal mice (Arendash, Sanchez-Ramos et al. 2010; Arendash, Mori et al. 2012). Similar findings were reported when 50 Hz RF EMF was used as repeated electromagnetic field shocks (REMFS) to prevent cellular senescence through a mechanism of increased expression of heat shock factor-1 (HSF1) (Perez, Zhou et al. 2008). Further, animal models demonstrated increased lifespan and decreased amyloid toxicity following exposure REMFS (Perez, Zhou et al. 2008). These studies suggest that EMF may be used as a noninvasive therapeutic option for Alzheimer's patients.

Similar outcomes were seen in an evaluation of the effects 60 Hz RF EMF on an inducible rat model of Huntington's disease (Tasset, Medina et al. 2012). Rats exposed to EMF had improved neurological scores, reduced oxidative damage, and decreased neuronal loss (Tasset, Medina et al. 2012).

Evaluating this phenomenon epidemiologically, specifically by investigating a link between neurodegenerative disease and cellular phone usage, would be an interesting but difficult study to complete due to risk for recall bias. Current data from epidemiologic and animal studies are not sufficient for drawing a conclusion about a relationship between RF EMF exposure and neurodegenerative disease.

Glioblastoma multiforme

In the age of mobile technology where many "cellular phones" are actually de facto handheld computers, a great debate has emerged regarding RF EMF exposure from these devices and the possibility for increased brain malignancy, specifically glioblastoma multiforme. Glioblastoma multiforme is the most aggressive brain ma-

lignancy with the highest mortality rate (Salacz, Watson et al. 2011). Though there will undoubtedly be additional studies in the future, current prospective studies are limited and retrospective studies have been inconclusive. The largest retrospective study to date, the INTERPHONE Study, was a thirteen country interview-based case-control study (Group 2010). This study demonstrated a decreased risk for glioblastoma and meningioma in cellular phone users in all usage groups except for the highest exposure group (Group 2010). The study also reported that glioblastomas most frequently occurred in the temporal lobe of the same hemisphere as where the phone was held, but these data were not significant, nor are they definitively reliable, as this study was plagued by recall bias and methodology limitations (Group 2010). In a follow-up report of this cohort, the INTERPHONE Group reported no elevated rate in acoustic neuromas in cellular phone users (Group 2011). These studies relied on significant self-reporting ranging from the daily usage to the side of the head exposed. Standardization proves difficult but will be necessary in order to gain a better epidemiologic evaluation of any increased risk that may exist (Han, Kano et al. 2009). Studies that have shown an association between cellular phone use and glioblastoma have shown a very weak association, and consistency in reporting is and will continue to be a challenge.

In vitro and *in vivo* studies have also been unable to definitively link cellular phone RF EMF to glioblastoma risk. Big Blue mice, used as an *in vivo* model of mutagenesis, received cellular phone EMF locally to the head and did not demonstrate increased incidence of mutations. (Takahashi, Inaguma et al. 2002). Further, a study in young rats exposed to 1800 MHz Global System Mobile signal modulation (GSM) RF EMF revealed no changes in heat shock proteins or glial cells, suggesting that RF EMF does not adversely affect the developing central nervous system (Watilliaux,

Edeline et al. 2011). *In vitro*, the exposure of glioblastoma (U87MG) cells to 1.9 GHz pulsed RF EMF did not affect gene expression (Qutob, Chauhan et al. 2006). Moreover, there is no evidence that exposure to cellular phone RF EMF results in cytogenetic effects or changes in p53 activation (Bourthoumieu, Joubert et al. 2010; Bourthoumieu, Magnaudeix et al. 2012). A 2010 review concludes that there is very little evidence supporting the hypothesis that RF EMF exposure results in genotoxicity (Verschaeve, Juutilainen et al. 2010). The current body of literature suggests that cellular phone exposure is unlikely to be directly linked to brain tumor formation.

Without a driving mechanism functioning to increase cancer risk, it will be difficult to conclusively draw an association between cellular phone usage and risk for glioblastoma. The explosion of cellular phone use is still a relatively recent phenomenon, so true long-term risk has yet to be assessed. Still, a recent publication posits that minimal evidence suggesting an association between cellular phone use and cancer makes a direct association less likely, especially when evaluating less than 10-15 years of use (Swerdlow, Feychting et al. 2011). Additionally, though glioblastoma incidence is increasing in the United States, this increase began many years before the explosion of cellular phone use, further suggesting that cellular phone use does substantially contribute to glioblastoma risk (Werner, Phuphanich et al. 1995; Werner, Phuphanich et al. 1995; Hess, Broglio et al. 2004; Dobes, Khurana et al. 2011).

Possible Benefits from RF EMF Exposure

High energy ionizing radiation is frequently used in medicine for both the diagnosis and treatment of disease. X-ray is a very common imaging modality used in settings ranging from fracture identification to pneumonia diagnosis. Targeted gamma radiation is used in the treatment of a wide range of malignancies. Additionally,

radioactive isotopes may be used as tracer elements in diagnostic studies. High intensity RF EMF are commonly used for the treatment of several tumor types, especially hepatocellular carcinoma and liver metastases from other primary tumors (add reference by Minami and Kudo) (Minami and Kudo 2011). The use of low intensity RF EMF in healthcare is much less common, and there are still uncertainties regarding its efficacy. A substantial focus of the studies investigating RF EMF exposure has been the possibility of subsequent negative health effects. However, there are many studies documenting beneficial medical outcomes following RF EMF treatment. RF EMF has been used clinically to accelerate fracture healing and treat insomnia. Additionally, there is now an increasing body of evidence that certain exposures to RF EMF may have efficacy in the diagnosis and treatment of malignancy.

Diagnostics

A significant challenge in oncology is the early detection of malignancy. Screening sensitivity and specificity can be imperfect, especially for some currently used routine screening methods. RF EMF offers a potential modality for the early diagnosis of malignancy. This possibility has been identified in a range of solid tumors (Barbault, Costa et al. 2009). Barbault *et al.* reported a phenomenon of a "tumor-specific frequency signature" that was highly consistent in patients with primary malignancies from the same tissue of origin (Barbault, Costa et al. 2009). Patients without malignancy lacked this frequency signature. It was this characteristic that led the group to identify unique frequency programs specific for various tissue sites of primary malignancy (Barbault, Costa et al. 2009). Similarly, the use of magnetoencephalography (MEG) may be used to differentiate among different neoplastic tissue types in the brain (Pearlman, Frye et al. 2011). MEG has the potential to be used in com-

bination with CT or MRI since these modalities effectively locate lesions but are not able to provide histologic diagnosis; MEG is noninvasive and may provide an alternative to brain biopsy for definitive diagnosis (Pearlman, Frye et al. 2011). An RF EMF application called TRIMprob has also been used to aid in the diagnosis of prostate cancer. By exploiting differences in tissue resonance between neoplastic and normal tissue, a 456 MHz frequency was used to detect prostate cancer in a multicenter study (Da Pozzo, Scattoni et al. 2007). The study found that the tissue-resonance test, TRIMprob, could be a useful screening tool in addition to prostate specific antigen (PSA) and digital rectal exam screening as a way to minimize unnecessary biopsies in patients (Da Pozzo, Scattoni et al. 2007). TRIMprob was also used to screen a population in Italy for rectal cancer and demonstrated specificity and sensitivity, an important finding since this modality is less expensive than colonoscopy (Vannelli, Battaglia et al. 2010).

One significant barrier to cancer screening is deciding what patient population warrants screening. This has been a recent topic of debate with respect to screening mammograms. Many screening modalities carry a risk; this includes radiation exposure for traditional mammograms and sedation or anesthesia as well as colon perforation for screening methods such as colonoscopy. Determining the risk-to-benefit ratio of these methods contributes to the decision of which populations should be screened. Such decisions may prevent early diagnosis in patients not included in screened groups. Screening modalities that carry less risk to patients may allow screening in a broader population.

Screening large populations also carries a significant financial burden. Traditional colonoscopy is associated with an inappropriate incremental cost-effectiveness ratio, according to a 2008 study (Hassan, Di Giulio et al. 2008). Screen-film mam-

mography has virtually been replaced by full-field digital mammography, which is associated with a greater cost than screen-film mammography due to greater compensation for this modality (Henderson, Hubbard et al. 2012). Recent changes in the screening guidelines for breast and cervical cancer have led many to question whether such changes are due in part to the significant costs associated with screening large number of patients (Davisson 2012). Optimization of RF EMF modalities in order to screen patients for cancer is an application that would allow subclinical diagnosis that is minimally invasive and cost-effective. In this scenario, utilizing EMF as a screening modality would indirectly impact the effectiveness and cost of cancer treatment because more malignancies would be detected at earlier stages, especially prior to metastasis. Earlier diagnosis would maximize the effectiveness of the currently available arsenal of pharmacologic agents used in the treatment of malignancy.

Therapeutics

Bone healing. Alternating current electric fields have been used in orthopedic clinics as a noninvasive method for inducing fracture healing. Induction of bone formation following exposure to sinusoidal 10 Hz RF EMF has been documented in embryonic chick tibiae and newborn mouse calvaria (Fitzsimmons, Farley et al. 1986). Enhanced bone repair with RF EMF treatment has also been reported in canines followed for 28 days post-osteomy (Bassett, Pawluk et al. 1974). A review from Brown Medical School suggests that the use of RF EMF to treat refractive fractures and nonunions may have efficacy equivalent to that of a bone graft (Aaron, Ciombor et al. 2004). These fields are thought to act by inducing directed migration and differentiation of bone marrow derived mesenchymal stem cells (Hronik-Tupaj, Rice et al. 2011; Zhao, Watt et al. 2011). Another study suggests that adipose-derived stromal cells

(ASCs), which are considered a useful cell population in regenerative medicine, may be stimulated with 50 Hz RF EMF to promote osteogenesis (Hammerick, James et al. 2010). Currently, RF EMF is used as a therapeutic option clinically in cases ranging from tibial stress fractures to spinal cord injury.

Insomnia. Electromagnetic fields have also been used to treat patients with insomnia. Amplitude-modulated RF EMF has been used in the setting of physiological insomnia to decrease sleep latency and increase the number of complete sleep cycles (Reite, Higgs et al. 1994; Pasche, Erman et al. 1996). Patients received intrabuccal administration of 27.12 MHz with an amplitude modulation band width of 0.1 Hz to 10 kHz (Reite, Higgs et al. 1994; Pasche, Erman et al. 1996). There have been no reports of adverse effects from this treatment, suggesting the RF EMF offers a non-pharmacologic treatment option for insomnia. (Reite, Higgs et al. 1994).

Malignancy. Radiofrequency ablation (RFA) is a therapeutic option with selective use clinically to treat malignancies including breast, colorectal, and hepatocellular carcinoma (Ripley, Gajdos et al. 2012; Wilson, Korourian et al. 2012; Wong, Yeh et al. 2012). This therapeutic approach may be indicated clinically, especially in cases with unresectable micrometastases (Brace, Hinshaw et al. 2011). This modality differs from those discussed below because it is more invasive and destroys tumor tissue through high temperatures (Frequencies ranging from 450-500 kHz) (Ripley, Gajdos et al. 2012; Wilson, Korourian et al. 2012; Wong, Yeh et al. 2012).

There is clinical evidence that certain frequencies within the RF EMF range of the spectrum may also have anti-tumor effects without causing hyperthermia. In patients with breast cancer, hepatocellular carcinoma, ovarian cancer, thyroid cancer and

glioblastoma multiforme clinical outcomes range from stable disease to complete disease response (Barbault, Costa et al. 2009; Costa, de Oliveira et al. 2011; Fonkem and Wong 2012). NovoTTF-100A applies alternating electric fields and was the first EMF device of its kind approved by the United States Food and Drug Administration. Approval followed promising results of a Phase 3 trial for the treatment of recurrent glioblastoma (Fonkem and Wong 2012; Stupp, Wong et al. 2012). This study indicated that NovoTTF-100A had efficacy similar to that of the standard-of-care chemotherapy regimen, but patients receiving NovoTTF-100A suffered from far fewer side effects than those receiving chemotherapy (Fonkem and Wong 2012; Stupp, Wong et al. 2012). The TheraBionic treatment modality, which uses RF EMF amplitude-modulated from a 27.12 MHz carrier frequency, demonstrated therapeutic efficacy in a Phase 1/2 Study in unresectable hepatocellular carcinoma patients (Costa, de Oliveira et al. 2011). This study resulted in a response in 4/41 patients, a response nearly four times that documented for hepatocellular carcinoma patients treated with the multikinase inhibitor sorafenib (Abou-Alfa, Schwartz et al. 2006; Rimassa and Santoro 2009; Costa, de Oliveira et al. 2011). A 2009 feasibility study initially demonstrated efficacy using TheraBionic in the treatment of breast and ovarian cancer (Barbault, Costa et al. 2009). TheraBionic was designed for portability and easy use so that patients do not have to report to the clinic for treatment (Barbault, Costa et al. 2009). Similarly an anticancer effect was observed in Wistar rats carrying subcutaneous leiomyosarcoma xenografts and exposed to a specific program of resonant RF EMF ranging from 10 kHz to 120 kHz (Avdikos, Karkabounas et al. 2007). The anticancer effect was seen both when the cells were pretreated with RF EMF and when the rats were exposed to RF EMF after xenograft growth (Avdikos, Karkabounas et al. 2007). Another approach that is slightly more invasive is Non-Thermal Irreversi-

ble Electroporation (N-TIRE). This modality places electrodes on the target tissue and uses microsecond electric frequency pulses to induce irreversible structural changes in the cell membrane (Arena, Rylander et al. 2009). It has been used in small and large animals for tissue ablation, including tumors, and the authors propose its use in the treatment of pancreatic adenocarcinoma (Arena, Rylander et al. 2009). Finally, in a story that received extensive media attention, the Kanzius machine developed by leukemia patient John Kanzius, uses radiofrequencies to heat metal particles within tumor tissue to destroy the tumor without damaging surrounding normal tissue (Schmidt 2008). Adverse effects associated with traditional cancer therapies often prevent patients from receiving prescribed treatment regimens, so the addition of therapeutic modalities with minimal side effects is an exciting prospect.

One final consideration with RF EMF based therapies is possible synergy with frequently used chemotherapeutics. Little is known regarding the simultaneous use of chemotherapeutic agents and RF EMF therapy. Barbault *et al.* reported the simultaneous use of bevacizumab and cyclophosphamide with RF EMF in a patient with FIGO stage III ovarian cancer and peritoneal carcinomatosis, demonstrating no increased adverse effects when RF EMF was used in combination with chemotherapy (Barbault, Costa et al. 2009). *In vitro*, EMF has been used in combination with Taxol or cisplatin without negatively impacting the cytotoxicity of the chemotherapeutic agent (Watson, Parrish et al. 1998). Further, combining pulsed magnetic fields and mitomycin C has potentiated the effect of the chemotherapy in fibrosarcoma and hepatocellular carcinoma (KMT-17 and KDH-8, respectively) cells (Omote, Hosokawa et al. 1990). *In vivo,* the same effect has been seen in a subcutaneous xenograft murine model of colon carcinoma (CT-26); mice treated with 500 Hz pulsed RF EMF simultaneously with bleomycin, 5FU, or BCNU had decreased tumor size

and increased survival time compared to mice treated only with a chemotherapeutic agent (Plotnikov, Fishman et al. 2004). Additionally, magnetic stimulation in combination with imatinib mesylate was more effective against chronic myeloid leukemia cells than imatinib mesylate alone (Yamaguchi-Sekino, Sekino et al. 2011). These findings suggest that patients would not have any additional adverse effects from receiving both a chemotherapeutic and an RF EMF therapy; moreover, simultaneous treatment with both may have a synergistic effect.

Possible mechanistic effects of EMF exposure

There is a tremendous body of original research reporting a wide range of *in vitro* and *in vivo* biological effects following exposure to RF EMF. In a 2007 review by Glen Gordon, he suggests that biological responses to EMF were crucial even in the beginnings of life on Earth (Gordon 2007). Stephen Hawkings has also concluded that EMF controls all chemical reactions (Hawkings 1996).

A significant portion of studies prior to the 21st century focused on frequencies in the 50-60 Hz range, as this is the range in which the public receives environmental exposure from power lines. Newer studies have emphasized potential negative health effects of long term exposure to EMF from cellular phones, frequencies ranging from 450-2700 MHz with GSM. The literature has evaluated static fields as well as pulsed or modulated fields. Studies have also varied with respect to the reported power or intensity of EMF exposure. Some studies have reported their exposure levels in Tesla (T), based on the magnetic field, rather than reporting the electric field component. The findings from these studies can be broadly grouped into categories: cellular function and metabolism; dysregulation and risk for malignancy; intercellular and systemic effects; cell morphology and differentiation; enzyme effects; pharmacologic effects

(Figure 2). Documented effects of EMF exposure include calcium modulation, immune modulation, matrix metalloproteinase effects, stress response effects, genotoxicity, proliferation effects, melatonin effects, modulation of gene expression, cellular differentiation effects, and others. Overall, the literature has focused on possible negative impacts of EMF exposure, ranging from DNA damage to a possible role as a cancer promoter. Previously, little emphasis has been placed on the possible positive impacts of controlled exposure to EMF; however, this paradigm has begun to shift.

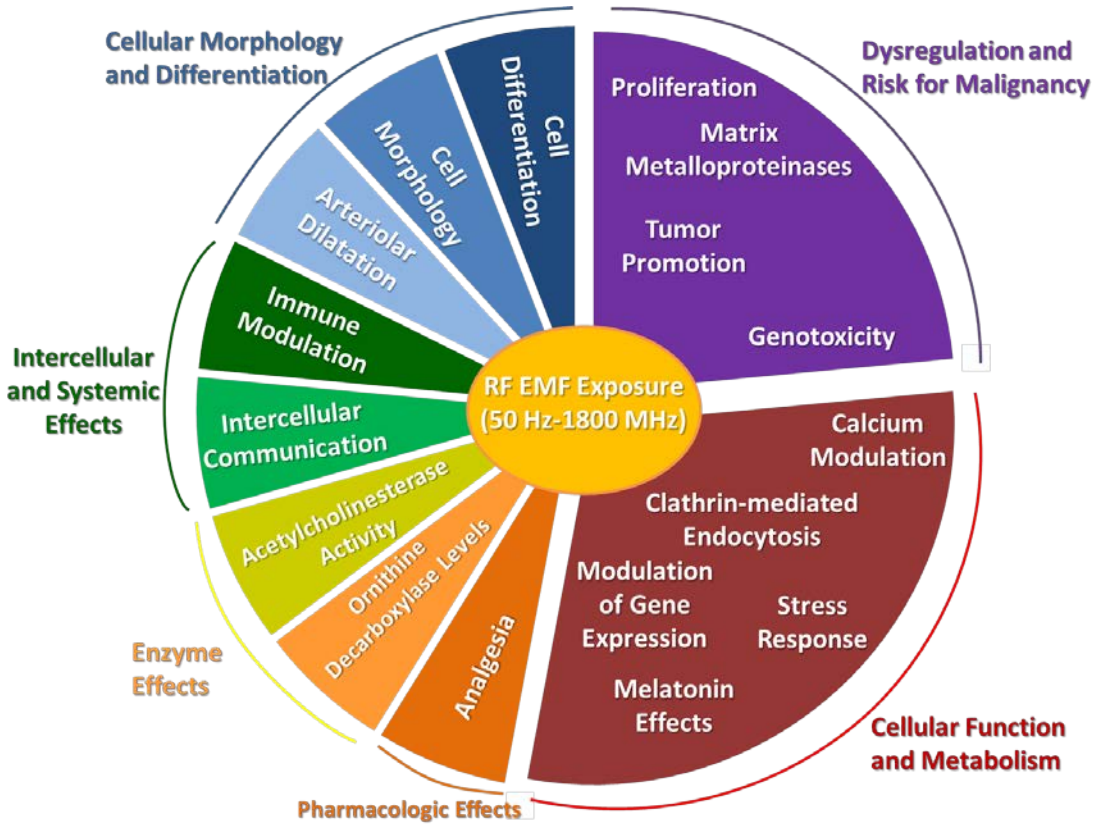

Figure 2. Reported Biological Effects of RF EMF Exposure.

Cellular Function and Metabolism

Calcium Modulation

Calcium (Ca^{2+}) is ubiquitous in the body and crucial for a number of cellular processes ranging from embryonic patterning to transcription factor activation and apoptosis (Berridge, Lipp et al. 2000). Calcium modulation has been included as a possible mechanism of RF EMF exposure from even the earliest studies. This effect is especially appealing because investigators generally agree that the majority of the fields evaluated in these studies are not of the level capable of causing direct effects on cellular structure or chromatin. The first study of RF EMF effects on Ca^{2+} dates back to 1982 when Adey *et al.* illustrated enhanced Ca^{2+} flux in the brains of cats under local anesthesia following exposure to 450 MHz EMF, sinusoidally amplitude modulated at 16 Hz (Adey, Bawin et al. 1982). This study provided the initial rationale for the large volume of studies to follow.

In a study evaluating cytosolic free Ca^{2+} levels following exposure to EMF generated by a Magnetic Resonance Imaging (MRI) unit, there was evidence of increased levels of free Ca^{2+} in human promyelocytic leukemia cells (HL-60) when the fields were varied with time (Carson, Prato et al. 1990). Increased levels of intracellular Ca^{2+} were also seen in lymphocytes and promyelocytic (U937) cells following exposure to static 6 mT magnetic fields (Chionna, Dwikat et al. 2003). However, the same effect was not observed when HL-60 cells were exposed to static magnetic fields (Carson, Prato et al. 1990). When Ca^{2+} was evaluated in Jurkat E6-1 human T-leukemia cells, authors observed no effect on signaling at the level of initial Ca^{2+} stimulation (Lyle, Fuchs et al. 1997). This study contradicted similar studies that did identify Ca^{2+} signaling modulation in Jurkat E6-1 cells, specifically noting an increase in inositol-3-phosphate levels within minutes of exposure initiation (Lindstrom,

Lindstrom et al. 1993; Lindstrom, Berglund et al. 1995; Lindstrom, Lindstrom et al. 1995; Still, Lindstrom et al. 2002). In an evaluation of 50 Hz pulsed RF EMF in human astrocytoma (U-373 MG) cells, the authors found evidence of RF EMF effects on intracellular Ca^{2+} stores or possibly Ca^{2+} transport across the plasma membrane (Pessina, Aldinucci et al. 2001). However, they did not identify any effects on downstream cellular proliferation or apoptosis (Pessina, Aldinucci et al. 2001). Similar outcomes were induced by 1 Hz nanosecond pulsed EMF in ovarian carcinoma (SKOV3) cells; there was a direct increase in intracellular Ca^{2+} following exposure, but in this study increased intracellular Ca^{2+} was seen in combination with increased early apoptosis (Yao, Mi et al. 2008). A study in neuroblastoma x glioma (NG108-15) cells demonstrated that exposure to 50-60 Hz EMF impacted cell differentiation by changing cell membrane surface charges and through modulating hyperpolarizing potassium channels by increasing intracellular Ca^{2+} (Tonini, Baroni et al. 2001). Marino *et al* observed that 60 Hz EMF can alter gap junction intracellular communication (GJIC) in synovial fibroblasts (HIG-82) through changes in Ca^{2+} rather than through direct changes in membrane potential (Marino, Kolomytkin et al. 2003). An evaluation of 50 Hz EMF exposure in neuroblastoma and rat pituitary cells (IMR32 and GH3, respectively) demonstrated increased cellular proliferation and decreased puromycin- and H_2O_2- induced apoptosis following exposure (Grassi, D'Ascenzo et al. 2004). Functional assays showed this was due to increased expression of the Ca^{2+} channel subunit α_1 (Grassi, D'Ascenzo et al. 2004). Further, in a study completed in primary rat cortical neurons, the authors demonstrated that neither the current amplitude nor the current-voltage relationship of barium ion currents through voltage-gated Ca^{2+} channels were impacted by exposure to 900 MHz GSM RF EMF (Platano, Mesirca et al. 2007).

A few studies have examined the downstream effects of EMF on Ca^{2+} signaling, with an emphasis beyond intracellular Ca^{2+} fluctuations. Pilla *et al.* evaluated a model that introduced EMF as the first messenger in the calmodulin-dependent signaling pathways that affect tissue growth, repair, and maintenance (Pilla, Fitzsimmons et al. 2011). In a model of Ca^{2+} efflux, Thompson, *et al.* describing a "windowing" phenomenon, similar to that previously described in chick brains (Blackman, Benane et al. 1980; Blackman, Benane et al. 1980; Blackman, Benane et al. 1982; Blackman, Benane et al. 1988; Blackman, Kinney et al. 1989) in which Ca^{2+} efflux reaches a maximum at discrete EMF windows (Thompson, Yang et al. 2000). In a study completed in mouse hippocampus tissue, Maskey *et al.* demonstrated that exposure to 835 MHz RF EMF altered the expression of calcium binding proteins, possibly causing an indirect effect on intracellular Ca^{2+} levels (Maskey, Kim et al. 2010). Though the specific findings and subsequent hypotheses differ, the literature strongly suggests that some exposure levels to EMF can impact Ca^{2+}; however, the biologic significance of these fluctuations is still unclear (Still, Lindstrom et al. 2002).

Stress Response

Several studies have reported EMF-mediated induction of cellular stress responses through the activation of heat shock proteins (HSPs). Heat shock proteins are activated by various environmental, pathophysiological, and normal metabolic cellular stresses, and some experimental findings suggest that this may also be expanded to include RF EMF exposure (Schlesinger 1990). Di Carlo *et al.* reported that short-term (one-time 30 minutes) exposure to 60 Hz RF EMF or 915 MHz RF EMF protected chick embryos against hypoxia or UV radiation damage through increased levels of HSP70 (DiCarlo, Farrell et al. 1999; Dicarlo, Hargis et al. 1999). In an evalua-

tion of long-term effects of EMF, the same group found evidence that the same exposure frequencies resulted in decreased levels of HSP70 when exposure took place continuously for 4 days for 20, 30, or 60 minutes once or twice daily (Di Carlo, White et al. 2002). Increased HSP70 was also reported when a range of cell lines were exposed to 50 Hz RF EMF, and the authors proposed this was due to increased stability of HSP70 (Alfieri, Bonelli et al. 2006). These findings correspond with those reported in a review by Blank and Goodman in which they discuss the potential for EMF to stress cells and activate a common pathway mediated by HSP70 (Goodman and Blank 1998). A more recent report by Blank and Goodman posited that EMF can interact directly with specific DNA sequences in the promoter of HSP70 (Blank and Goodman 2009). However, when exposure to 1800 MHz RF EMF in various waveforms was evaluated in immune-relevant (mono mac 6 and K562) cell lines, there was no evidence of increased HSP70 (Lantow, Schuderer et al. 2006).

Cells utilize a dynamic set of redox reactions to maintain cellular homeostasis and minimize damage from oxidative processes (Labunskyy and Gladyshev 2012). Dysregulation of this delicate balance damages cellular macromolecules and can lead to age-related pathology such as cancer, diabetes, and neurodegeneration (Labunskyy and Gladyshev 2012). Studies have evaluated cellular redox reactions and ability of cells to respond to reactive oxygen species (ROS) as a consequence of RF EMF exposure. The effects of RF EMF may depend on the antioxidant state of the cells at the time of exposure (Polaniak, Buldak et al. 2010). Poloniak *et al.* demonstrated that in murine squamous cell carcinoma (AT 478) cells RF EMF impacted the antioxidant activities of the cells, with synergistic effects in the presence of the antioxidant vitamin E (Polaniak, Buldak et al. 2010). Further, there is *in vivo* evidence in guinea pigs that following RF EMF exposure, the intensity of the magnetic field as well as the du-

ration of the exposure may affect the cellular oxidative response in a dose dependent manner (Canseven, Coskun et al. 2008).

There is also evidence that RF EMF exposure may prevent cells from successfully responding to oxidative stress. In neuroblastoma (SH-SY5Y) cells exposed to 50 Hz RF EMF, results indicated that RF EMF impacted cellular redox state with increased reduced glutothione and modulation of antioxidant enzymatic expression, suggesting that EMF can decrease cellular ability to tolerate oxidative attacks (Falone, Grossi et al. 2007). Furthermore, in a study carried out in oral mucosa cancer cells (UM-SCC-14-C) to determine the long term effects of galvanic currents induced by dental alloys, cells were treated for 24 hours to direct current electric fields (Wartenberg, Wirtz et al. 2008). Authors described decreased cellular proliferation and increased levels of cyclin dependent kinase (CDK) inhibitors due to ROS levels capable of overwhelming the anti-oxidative mechanism of cells and leading to cellular apoptosis (Wartenberg, Wirtz et al. 2008). When acute myeloid leukemia cells (THP-1) were exposed to sinusoidal 50 Hz RF EMF, there was a reduction in anti-oxidant activity (Patruno, Pesce et al. 2012). This was seen in combination with increased nitrogen intermediates that are involved in the iNOS pathway, specifically peroxynitrite (Patruno, Pesce et al. 2012).

Nictotinamide adenine dinucleotide phosphate (NAD(P)H) functions as a critical cellular molecule in maintaining the redox balance in cells by reducing oxidative molecules, including ROS (Rosenspire, Kindzelskii et al. 2001). In a 2001 study in fibrosarcoma (HT-1080) cells, Rosenspire *et al.* observed that previously reported oscillations of NAD(P)H levels in cells of hematopoietic lineage were also present in polarized and migratory, but not spherical, fibrosarcoma cells (Rosenspire, Kindzelskii et al. 2001). Phased direct currents increased the amplitude of NAD(P)H

oscillations leading to increased levels of ROS and subsequent evidence of DNA damage in as little as five minutes (Rosenspire, Kindzelskii et al. 2001).

A recent report evaluated the combination of 60 Hz RF EMF and stress factors in mouse fibroblasts (NIH3T3) cells to determine whether EMF exposure increased cellular transformation (Lee, Jin et al. 2012). Cells were stressed with ionizing radiation, hydrogen peroxide, or overexpression of c-Myc and exposed to EMF for 4 hours (Lee, Jin et al. 2012). There were no indications of increased transformation following EMF exposure alone or in combination with any of the stress factors, suggesting that EMF did not work in synergy with other stress factors to transform the cells (Lee, Jin et al. 2012).

The oxidation of the 20S proteasome has also been examined in human epithelial colorectal adenocarcinoma (Caco 2) cells, demonstrating that continuous 50 Hz, 1 mT RF EMF led to a time-dependent increase in proteasome oxidation with that was attenuated by EGCG, a natural antioxidant (Eleuteri, Amici et al. 2009). A study in preadipocytes (3T3-L1) demonstrated an EMF-induced effect on the oxidative-antioxidative balance by monitoring levels of malondialdehyde following exposure to 180-195 Hz (Zwirska-Korczala, Jochem et al. 2005).

These data suggest an oxidative stress response following some RF EMF exposure programs. Studies that have found evidence of increased ROS production led to a hypothesis by Simko and Mattson that long-term exposure to EMF would cause chronic elevation of ROS and subsequent decrease in melatonin leading to an increased risk for DNA damage and malignancy (Simko and Mattsson 2004). Though there appears to be some evidence of increased cellular stress in response to EMF, the degree to which this occurs and the nature of its cell type specificity has yet to be elucidated.

Modulation of Gene Expression

Several studies have evaluated the impact of RF EMF on gene expression. An impact on gene expression, either through directly impacting transcription or through a mechanism of RNAi, could have downstream effects on subsequent protein levels and consequently on cellular processes. No change in gene expression was noted following human keratinocyte exposure to 900 MHz RF EMF, nor were alterations in gene expression identified in human lymphocytes or neuroblastoma (SH-SY5Y) cells exposed to 50 Hz RF EMF (Luceri, De Filippo et al. 2005; Benfante, Antonini et al. 2008; Roux, Girard et al. 2011). Conversely, decreased levels of proinflammatory chemokines were reported in human keratinocytes following exposure to 50 Hz RF EMF (Vianale, Reale et al. 2008). Additionally, modulation of gene expression was reported in a tissue and tumor -specific manner in cells exposed to amplitude-modulated 27.12 MHz RF EMF (Zimmerman, Pennison et al. 2012). Of note, the negative studies used microarray technology or evaluated the expression level of specific genes. The studies that did identify gene expression used more sensitive assays: global mRNA assessment was completed with RNA-Seq, allowing for the identification of multiple mRNA isoforms; chemokine levels were evaluated using ELISA (Wang, Gerstein et al. 2009). The use of the most sensitive assays will allow a more complete evaluation of the impact of RF EMF on gene expression, as it will allow for the identification of subtle but significant effects.

Melatonin Effects

There have been several studies evaluating the impact of EMF on melatonin levels. Melatonin (N-acetyl-5-methoxytryptamine) is a product of the pineal gland and functions in the maintenance of the natural circadian rhythms of the body

(Hardeland 2012). Additionally, melatonin has a role in the biological response to free radical formation following oxidative stress (Hardeland 2012; Surendran, Geetha et al. 2012). Melatonin has also been reported to have an antitumor effect by mechanisms such as cell cycle inhibition, apoptosis induction, and metastasis prevention, especially in hormone-dependent malignancies (Mediavilla, Sanchez-Barcelo et al. 2010).

Harland and Liburdy reported that exposure to 60 Hz, 1.2 μT magnetic fields blocked the melatonin- and tamoxifen-induced proliferative inhibition in breast cancer (MCF7) cells (Harland and Liburdy 1997). They also proposed that the effect is caused by the magnetic rather than the electric field component of the EMF (Harland and Liburdy 1997). A similar effect was also seen in MCF7 cells exposed to simusoidal 50 Hz, 1.2 μT EMF, suggesting that EMF impacts the antiestrogenic effect of melatonin on breast cancer cells (Girgert, Hanf et al. 2010). These findings have also been reported from other laboratories and have prompted a mechanistic investigation (Reiter 1993; Blackman, Blanchard et al. 1998; Ishido, Nitta et al. 2001). Mechanistic evaluation suggested that 50 Hz, 1.2 μT RF EMF uncoupled signal transduction from the melatonin receptor through adenylyl cyclase in MCF7 cells (Ishido, Nitta et al. 2001).

Since melatonin is thought to play a role in the prevention of metastasis, it is important to evaluate alterations in metastatic potential following exposure to RF EMF. Additionally, there is evidence that melatonin decreases the invasiveness of breast cancer cells through increased expression of beta1 integrin and E-cadherin (Cos, Fernandez et al. 1998). However, in a study completed in three breast cancer cell lines (MCF7, MDA-MB-435, MDA-MB-231) neither melatonin nor pulsed 2 Hz, 0.3 mT RF EMF had an impact on the invasiveness of the cell lines (Leman, Sisken et

al. 2001). This suggests that RF EMF may not impact metastasis or negate any possible anti-metastatic effects of melatonin.

In vivo, studies in rats have shown modulated melatonin levels as a consequence of 900 MHz or 1.5 GHz RF EMF exposure (Imaida, Taki et al. 1998; Imaida, Taki et al. 1998; Imaida, Hagiwara et al. 2000). However, these studies also demonstrated that 900 MHz or 1.5 GHz RF EMF did not affect the liver carcinogenesis process in rats (Imaida, Taki et al. 1998; Imaida, Taki et al. 1998). Elevated melatonin levels following EMF exposure limited the development of preneoplastic liver foci (Imaida, Hagiwara et al. 2000). Finally, without evaluating melatonin, Jimenez-Garcia *et al* also showed that exposure to 120 Hz, 4.5 mT EMF inhibited the growth of chemically induced preneoplastic hepatic lesions (Jimenez-Garcia, Arellanes-Robledo et al. 2010). Though studies suggest there may be an association between melatonin levels and RF EMF exposure, there is still discordance regarding the impact of RF EMF on melatonin levels as well as the mechanism of melatonin and RF EMF interaction.

Clathrin-Mediated Endocytosis

Endocytosis is the process in which cells acquire membrane-bound or extracellular products (McMahon and Boucrot 2011; Ramanan, Agrawal et al. 2011). Clathrin-mediated endocytosis is the most common form of receptor mediated endocytosis and is dependent on clathrin coated pits for capture and engulfment of cargo (McMahon and Boucrot 2011; Ramanan, Agrawal et al. 2011). One report suggests that exposure to GSM modulated 900 MHz RF EMF increased the rate of clathrin-mediated endocytosis in murine melanoma (B16F10) cells (Moisescu, Leveque et al. 2009). Thus RF EMF exposure may impact cell membrane receptor density and indi-

rectly impact signal transduction through a mechanism of increased clathrin-mediated endocytosis.

Dysregulation and Risk for Malignancy

Genotoxicity and Strand Breaks

Exposure to higher energy ionizing radiation results in direct damage to DNA, ranging from pyrimidine dimers following UV radiation to strand breakage following gamma radiation. Several studies have evaluated the potential for DNA damage following exposure to EMF in the radiofrequency range. Ivancsits *et al.* demonstrated that application of intermittent sinusoidal 1 mT RF EMF at 50 Hz led to induction of DNA strand breaks in human diploid fibroblasts; the majority of the breaks were double-strand, and the authors noted an exposure-response relationship with respect to magnetic flux density (Ivancsits, Diem et al. 2002). This outcome was not evident when fibroblasts were continuously exposed to 50 Hz sinusoidal 1 mT RF EMF (Ivancsits, Diem et al. 2002). Another study in human diploid fibroblasts examined micronuclei formation as well as five parameters indicative of chromosomal damage following exposure to 50 Hz intermittent 1 mT RF EMF (Winker, Ivancsits et al. 2005). This study found increased micronuclei formation and increased evidence of chromosome aberrations following RF EMF exposure, leading the authors to suggest that this delivery of RF EMF caused chromosomal damage through indirect mechanisms (Winker, Ivancsits et al. 2005). These reports indicate that evidence of chromosomal damage may be related to whether RF EMF exposure is continuous or intermittent.

However, a study evaluating primary human fibroblasts exposed to switching, or intermittent, magnetic fields ranging from 50-1000 μT found no evidence of DNA

and chromosomal damage, despite the use of a sensitive γH2AX assay used to detect double-strand breaks (Burdak-Rothkamm, Rothkamm et al. 2009). Similarly, a study using the Ames assay to assess genotoxic effects in *Salmonella typhimurium* found no evidence of genotoxicity following 48 hours of exposure to electric, magnetic or electromagnetic fields at 60, 600, or 6000 Hz (Morandi, Pak et al. 1996). The study also examined the effects of EMF in the presence of a mutagen but did not find evidence of increased mutagenesis when the mutagen and RF EMF were used in combination (Morandi, Pak et al. 1996). Though disparate, the literature has demonstrated low energy EMF does not cause the predictable direct effects on DNA and chromosomes that are seen following exposure to higher energy EMF.

Proliferation Effects

The impact on proliferation is an important consideration when evaluating potential neoplastic transformation in cells exposed to EMF. In fact, sustained proliferation may be considered the "most fundamental trait of cancer cells" (Hanahan and Weinberg 2011). Several studies have examined the proliferative effects of EMF. Sollazzo *et al.* has documented increased proliferation of osteoblast-like human cells and in human osteosarcoma (MG-63) cells following exposure to pulsed 2.3 mT EMF with a repetition rate of 75 Hz (Sollazzo, Traina et al. 1997). The proliferative response was directly related to fetal calf serum concentration in the cell medium (Sollazzo, Traina et al. 1997). Similarly, increased proliferation and decreased apoptosis were identified in neuroblastoma and rat pituitary cells (Grassi, D'Ascenzo et al. 2004). Additionally, in an *in vivo* study in rats, increased thyroid stimulation was observed when rats were exposed to 100-300 μT for 5 days per week for one month (Rajkovic, Matavulj et al. 2006). Increased proliferation was also identified when in-

tervertebral disk cells were exposed to pulsed 60 Hz sinusoidal RF EMF, suggesting this modality may have utility in the treatment of degenerative disk disease (Lee, Kwon et al. 2010).

Conversely, several studies in malignant cell lines have demonstrated cytostatic, antiproliferative, and apoptotic effects following exposure to EMF. Proliferative inhibition following exposure to alternating electric or amplitude-modulated RF EMF has been described in hepatocellular carcinoma, glioblastoma, breast cancer, and melanoma cell lines (Kirson, Gurvich et al. 2004; Kirson, Dbaly et al. 2007; Zimmerman, Pennison et al. 2012). These effects were seen in combination with disruption of the mitotic spindle, suggesting abnormal mitoses (Kirson, Gurvich et al. 2004; Kirson, Dbaly et al. 2007; Zimmerman, Pennison et al. 2012). Additionally, Wahab *et al.* has reported an increase in sister chromatid exchange when human peripheral blood lymphocytes were exposed to 50 Hz RF EMF, possibly due to DNA crosslinking at the replication fork as a consequence of RF EMF exposure (Wahab, Podd et al. 2007). Zimmerman *et al.* also noted specificity of the antiproliferative phenotype, which was limited to malignant cell lines and was not seen in the normal epithelial counterparts of the primary malignancy (Zimmerman, Pennison et al. 2012). Similar cell line-specific proliferation effects were also documented when four nonmalignant cell lines were exposed to 60 Hz sinusoidal RF EMF (Sul, Park et al. 2006). Findings in neuroblastoma (SH-SY5Y) cells exposed to 900 MHz RF EMF have demonstrated modulation of the expression of genes controlling cell cycle progression and apoptosis (Buttiglione, Roca et al. 2007). In colon carcinoma (HCT116) cells, pulsed EMF induced apoptosis through mitochondrial-independent pathways, regardless of presence or absence of p53 (Hall, Schoenbach et al. 2007). Nanosecond pulsed EMF has shown efficacy in shrinking B16f10 melanoma *in vivo,* and functional evaluation has

suggested that this is through an apoptotic-like mechanism that bypasses the mitochondria (Nuccitelli, Pliquett et al. 2006; Nuccitelli, Chen et al. 2009; Ford, Ren et al. 2010). A similar outcome was seen for mice inoculated with B16 cells and treated with spatial-temporal regulated Thomas field EMF (Thomas, Kavaliers et al. 1997; Hu, St-Pierre et al. 2010). Additionally, the use of capacitive-resistive electric transfer (CRET) caused proliferative inhibition in hepatocellular carcinoma (HepG2) cells after treatment with 0.57 MHz RF EMF at subthermal levels (Hernandez-Bule, Trillo et al. 2007). Interestingly, in an experiment carried out in human leukemia (K562) cells, Garip and Akan noted that the number of apoptotic cells following cell exposure to 50 Hz RF EMF depended on the oxidative stress level of the cells (Garip and Akan 2010). These findings suggest that EMF may arrest proliferation and induce apoptosis and that this might take place in a cell type and an exposure-specific manner.

Tumor promotion

The possibility of EMF to function as a tumor promoter has been evaluated from several perspectives. In a study by Jin *et al.* results indicated that RF EMF did not function as a tumor promoter when 60 Hz, 0.8-300 μT RF EMF was used to expose mouse fibroblasts (INITC3H/10T1/2) transformed by methylcholanthrene (Jin, Blank et al. 2000). The same results were also seen in three human malignant cell lines (MCF7, HL60, HTB124) (Jin, Blank et al. 2000). Additionally, there was no evidence of tumor promotion in estrogen receptor positive breast cancer (MCF7, T47-D) cells following exposure to 60 Hz RF EMF (Dees, Garrett et al. 1996). Similar findings were reported when a promoter-sensitive anchorage independent mouse epidermal cell line (JB6) was exposed to RF EMF of 60 Hz at 0.10 or 0.96 mT with and

without the addition of the tumor promoting agent tetradecanoylphorbol acetate (TPA) (Snawder 1999).

Conversely, a study in Friend erythroleukemia cells found that 60 Hz RF EMF at 1.0-1000 μT led to a larger population of cells remaining in an undifferentiated proliferative state, similar to the impact of a tumor promoter on the cell line (Chen, Upham et al. 2000). In a study evaluating the effects of 50 Hz sine waves on Kaposi's sarcoma associated herpesvirus (KSHV) in a latently infected primary effusion lymphoma cell line (PEL), EMF was found to increase viral DNA synthesis but only when exposure was carried out in combination with TPA treatment (Pica, Serafino et al. 2006). There also appeared to be a greater ratio of defective virus maturation in EMF exposed cells (Pica, Serafino et al. 2006). Similarly, when human amniotic fluid cells were exposed to 50 Hz, 1 mT RF EMF no translocation of protein kinase C (PKC) from the cytosolic to the membrane fraction was noted, but there was an additive effect of PKC translocation when cells were also treated with TPA in combination with EMF (Richard, Lange et al. 2002). Additionally, when 60 Hz RF EMF exposure was compared to TPA treatment in breast cancer, human leukemia, and rat fibroblast (MCF7, HL-60, 3Y1, respectively) cells, the level of activation of the MAPK/ERK pathway was similar in the RF EMF group to treatment with TPA (Nie and Henderson 2003). Further, the activation of MAPK following RF EMF exposure was inhibited by an inhibitor of protein kinase C alpha, suggesting protein kinase C may have a role in the biological effects of RF EMF (Nie and Henderson 2003). Conflicting results prevent determination of the status of RF EMF as a tumor promoter, but additional mechanistic studies evaluating the MAPK pathway may provide a more definitive conclusion.

Matrix Metalloproteinase Effects

Matrix metalloproteinases (MMPs) are extracellular or membrane-bound metal-dependent proteases important for breaking down and remodeling the extracellular matrix in physiologic and pathologic processes (Nagase and Woessner 1999; Newby 2005). Results from some studies suggest that there may be a relationship between RF EMF exposure and MMP activity. When acute myeloid leukemia cells (THP-1) were exposed to sinusoidal 50 Hz RF EMF, there was an increase in the nitrogen intermediates that are involved in the iNOS pathway, specifically peroxynitrite (Patruno, Pesce et al. 2012). Peroxynitrite inactivates Tissue Inhibitor of MMP (TIMP)-1, a critical inhibitor of MMP activity, suggesting that EMF may play a role in the composition of the extracellular matrix through modulation of TIMP-1 activity (Patruno, Pesce et al. 2012).

Yet, in a study completed in osteosarcoma cell lines (HOS, MG-63, SAOS-2, NY) to evaluate the possibility of using EMF to enhance the effects of chemotherapeutic drugs, there was no impact on integrin α subunits, integrin β subunits, CD44, or MMP-2/9 (Zhang, Pan et al. 2011). Though the goal of the study was to evaluate the impact of pulsed EMF on chemotherapy efficacy, the study included an evaluation of effects on metastasis-related molecules to verify that pulsed EMF would not promote metastasis (Zhang, Pan et al. 2011). Matrix metalloproteinases were included in the evaluation based on their link to metastasis and aggressive malignancies. Activation of these proteases promotes metastasis by degrading the extracellular matrix thereby providing malignant cells access to distant sites to establish metastases (Xu, Qin et al. 2011; Kim, Kim et al. 2012; Suarez-Roa, Asbun-Bojalil et al. 2012; Zhang, Chen et al. 2012). The authors concluded that with no identified impact on metastatic potential, pulsed RF EMF could be applied in combination with chemotherapy in the treat-

ment of osteosarcoma (Zhang, Pan et al. 2011). Matrix metalloproteinases have not been extensively evaluated with respect to responses to RF EMF exposure, though findings of a relationship between TIMP-1 and EMF warrant additional studies.

Intercellular and Systemic Effects

Immune Modulation

Electromagnetic fields have also been explored as potential modulators of the immune or inflammatory response. There have been reports of people who suffer from "allergies" or hypersensitivity to EMF exposure, with symptoms ranging from itch and heat sensation to pain and edema (Gangi and Johansson 2000). Criteria for the classification and diagnosis of this condition, Idiopathic Environmental Intolerance attributed to EMF (IEI-EMF), have not been standardized (Baliatsas, Van Kamp et al. 2012). In a review by Gangi and Johansson, they proposed a model in which mast cell activation and subsequent degranulation is altered by EMF exposure, leading to release of histamine and other substances (Gangi and Johansson 2000). Such release may lead to the symptoms reported from individuals with IEI-EMF, though release of histamine following EMF exposure may take place without hypersensitivity in the general population. In a 2004 review of possible EMF mechanisms, one consideration was that EMF led to direct activation of macrophages and consequential increase in phagocytosis; this would in turn increase the free radical concentration in cells (Simko and Mattsson 2004). A study following this review reported increased phagocytosis and IL-1beta levels in mouse macrophages exposed to 50 Hz RF EMF (Frahm, Lantow et al. 2006). A 2009 review concluded that the immune system is very likely impacted by environmental exposure to EMF far below international safety limits (Johansson 2009).

Conversely, a recent original report suggested that EMF does not impact the innate immune system. Cytokine and other inflammation-related genes were evaluated in a human monocytic cell line (THP-1), primary monocytes, and macrophages following exposure to a complex multiform waveform or a 50 Hz sine wave with no effects on cytokine or gene expression (Bouwens, de Kleijn et al. 2012). Similar results followed in additional experiments in non-immune cells (Bouwens, de Kleijn et al. 2012). Overall, an immune modulatory effect from EMF exposure cannot be definitively accepted or disproved; however, this hypothesis is appealing because environmental exposure is systemic, and the immune system is responsible for responding to foreign invaders, a group that might not be limited to discrete pathogens.

Intercellular communication

Gap junctions are composed of hexameric proteins called connexins and are critical for intercellular communication and coordinated biological processes, including embryonic formation and cardiac muscle contraction (Evans and Martin 2002). Decreased numbers of gap junction-like structures have been identified in extravillous trophoblast (HTR-8/SVNeo) cells following exposure to 1817 MHz RF EMF (Cervellati, Franceschetti et al. 2009). Similar findings were reported for cells of osteoblastic lineage and synovial fibroblasts exposed to pulsed 15 Hz EMF and to 60 Hz EMF (Lohmann, Schwartz et al. 2003; Marino, Kolomytkin et al. 2003; Sul, Park et al. 2006). Specifically, there is evidence that EMF exposure impacts the level of connexin 43 (Lohmann, Schwartz et al. 2003; Zeng, Chiang et al. 2003; Aaron, Boyan et al. 2004). These findings suggest that RF EMF may have an impact on intercellular communication in some cell lineages.

Cell Morphology and Differentiation

Cellular Differentiation and Tissue Generation

Cellular differentiation is a critical aspect of organismal development and is also critical for successful tissue repair. For example, fracture repair is dependent on the differentiation of osteoblasts. RF EMF is used clinically to promote fracture healing, as discussed above, and a recently described study demonstrated that 50 Hz sinusoidal RF EMF promoted the differentiation and mineralization of rat osteoblasts without increasing cellular proliferation (Zhou, Ming et al. 2011). Further, the increased differentiation was associated with increased expression of differentiation factors that were elevated in an intensity dependent manner (Zhou, Ming et al. 2011). Similarly, increased differentiation was reported in human leukemia (K562) cells following repeated exposure to 50 Hz RF EMF (Ayse, Zafer et al. 2010). However, one-time exposures did not increase differentiation of K562 cells, suggesting that a single application may not be sufficient to generate a significant biological response (Ayse, Zafer et al. 2010). Premature maturation and differentiation was also seen in newborn rat cerebellar granule neurons following exposure to 50 Hz RF EMF (Lisi, Ciotti et al. 2005). Differentiation of human mesenchymal stem cells (hMSCs) was also enhanced by exposure to 15 Hz RF EMF, demonstrating the potential for EMF to promote chondrogenesis of hMSCs (Mayer-Wagner, Passberger et al. 2011). In one study the increased differentiation was associated with elevated levels of transforming growth factor beta (TGF-β), which plays a role in regulating cartilage and bone development (Aaron, Wang et al. 2002). These studies demonstrate that multiple cell types may respond to RF EMF with increased rates of differentiation.

Additional studies have emphasized the potential role of EMF in tissue repair and engineering. Exposure to EMF enhanced the proliferation of both chondrocytes

and epidermal stem cells grown on collagen scaffolds, suggesting that EMF may have clinical efficacy in clinical tissue and skin engineering (Aaron, Wang et al. 2002; Chang, Loo et al. 2010; Bai, Zhang et al. 2012). Though there are few studies evaluating the impact of EMF in tissue engineering, the published studies suggest an efficacy similar to that for fracture healing.

Cell morphology

When promyelocytic (U937) cells and lymphocytes were exposed to static magnetic fields of 6 mT, cells underwent changes in cell shape developing prolific lamellar microvilli and becoming long and irregularly shaped, respectively (Chionna, Dwikat et al. 2003). Both cell types had reduced smoothness of the cell surface and irregular distribution of surface glycoproteins, features commonly seen in apoptotic cells (Chionna, Dwikat et al. 2003). Blackman *et al.* also reported that rat pheochromocytoma (PC12) cells were impacted following exposure to 50 Hz RF EMF (Blackman, Benane et al. 1993). PC12 cells are frequently used as a model of neuronal differentiation and produce neurites following treatment with nerve growth factor, but this action was inhibited by treatment with EMF (Blackman, Benane et al. 1993). These findings suggest the exposure to RF EMF may affect cell morphology, which may consequently impact cell metabolism.

Arteriolar dilatation

Endothelin-1 is a potent vasoconstrictor released by endothelial cells and critical for maintaining homeostasis of the vasculature (Yanagisawa, Kurihara et al. 1988). Production of endothelin-1 was decreased in cells following exposure to a 0.2 T static magnetic field, which has since be shown to mediate endothelin-1 production

in part through modulation of a nitric-oxide related pathway (Jiang, Morimoto et al. 1996; Pacini, Vannelli et al. 1999; Morimoto, Takahashi et al. 2005).

Enzyme Effects

Ornithine decarboxylase levels

Ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis, has been documented at elevated levels in hepatoma cells (Reuber H35) following exposure to 450 MHz RF EMF sinusoidally modulated at 16 Hz (Byus, Kartun et al. 1988). The authors suggested that ODC may serve as a sensitive biomarker of biologic effects following EMF exposure (Byus, Kartun et al. 1988).

Acetylcholinesterase levels

Acetylcholinesterase is responsible for hydrolyzing the neurotransmitter acetylcholine. RF EMF has been linked to decreased levels of acetylcholinesterase in the cerebellum synaptosomal membranes following exposure to 50 Hz sinusoidal waves with inhibitory activity seen at 60, 200, 350, and 475 Hz (Ravera, Bianco et al. 2010). Acetylcholinesterase activity was not affected when membranes were solubilized, indicating that the membrane is important for mediating the modulation of enzymatic activity in this response to RF EMF exposure (Ravera, Bianco et al. 2010).

Pharmacologic Effects

Analgesia

In an *in vivo* study in Wistar rats, investigators found that rats exposed to 45 EMFs selected based on the chemical shift (in parts per million) of the H^1- NMR spectrum of morphine experienced an analgesic effect that was similar to treatment

with the drug (Verginadis, Simos et al. 2012). The fundamental frequency of the spectrophotometer was 200 MHz, and the resonant frequencies were selected by multiplying the fundamental frequency (200 MHz) by the chemical shift (Verginadis, Simos et al. 2012). Treatment with naloxone, a drug used to treat morphine overdose, inhibited the analgesic effects (Verginadis, Simos et al. 2012).

There is a breadth of literature examining the biological effects of EMF exposure, though many of these effects have limited documentation. Frequently, study outcomes are not reproducible by other investigators, so it is difficult to determine which effects are biological in nature and which effects may be artifacts or bias are caused by experimental methodology. Additional studies examining some of these effects will be required in order to classify them as reproducible biological consequences of RF EMF exposure.

Shortcomings of Mechanistic Literature

Similar to the epidemiologic studies evaluating RF EMF exposure, the *in vitro* and *in vivo* studies are also inconsistent. Though there are some possible mechanistic effects of EMF that appear more frequently in the literature, there is not a definitive mechanism linked to the reported effects of RF EMF exposure. Based on the volume of literature, there is a wealth of biological effects that could be the result of a multitude of possible mechanisms, and it unlikely there will be a single mechanistic explanation for the documented outcomes resulting from RF EMF exposure.

There are many explanations for disparate findings, and in some cases, inability to replicate published findings. In many cases, there are slight but possibly significant differences in the exposure conditions. Many times the Methods reported are incomplete for exact experimental replication. Additionally, there is inconsistency in

how the exposures are reported, with some studies emphasizing the magnetic rather than the electric field component. This adds to the challenge when trying to compare similar studies. In a review of EMF methodology, McCann *et al.* critically evaluates the methodology shortcomings in the literature, emphasizing key considerations for studies evaluating exposure risk (McCann 1998). This review also notes the importance of reporting both positive and negative data when completing exposure studies (McCann 1998). Additionally, even if the frequency(ies) are identical, there may be differences in the delivery. For example, studies using a constant exposure may yield different results from studies in which the frequencies were pulsed (Carson, Prato et al. 1990; Blackman, Benane et al. 1995). There may also be different effects from using modulated frequencies, rather than continuous waves, as some studies have shown modulation specific effects (Juutilainen, Hoyto et al. 2011). There have also been studies that have demonstrated different outcomes depending on factors such as the temperature and pH of the cell medium (Blackman, Benane et al. 1991; Radeva and Berg 2004). Finally, there is also the consideration of exogenous fields beyond the intended EMF exposure. It is difficult to account for and cancel the appreciable influence of the Earth's fields as well as the fields intrinsic to cell culture incubators. There are devices designed to cancel these extraneous fields, but the use of such devices is seldom reported in the literature. Ultimately, there are a significant number of confounding factors that must be considered when setting up and reporting EMF exposure experimental conditions.

Another crucial consideration is the cell lines in which the fields are evaluated. The literature includes reports that use cells ranging from human breast cancer cell lines to mouse fibroblasts. Given the inconsistencies in the literature and the broad range of cells used, it is possible that the differences seen may be in part a function of

the metabolism of individual cell types. For example, Zimmerman *et al.* recently reported an antiproliferative effect that was specific to malignant cell lines receiving exposure to a discrete program of amplitude-modulated RF EMF (Zimmerman, Pennison et al. 2012). This supports what was previously reported regarding frequency specificity of antiproliferative effects (Kirson, Gurvich et al. 2004). Similar findings were reported even earlier in prostate, endometrial, and ovarian cancer cell lines, where the authors identified enhanced proliferation, but only for some of the cell lines (Watson, Parrish et al. 1998). This study showed greater response in cells from tissues that were regulated by sex hormones (Watson, Parrish et al. 1998). Literature also suggests that some cells may be in general more susceptible to RF EMF. For example, Czyz *et al.* demonstrated that in mouse pluripotent embryonic stem cells, the effects of 50 Hz EMF depended on the p53 expression level of the cells (Czyz, Nikolova et al. 2004). Muehsam and Pilla further propose that the initial state of the cell is seminal when evaluating the biologic effect of EMF exposure, suggesting that this may explain the difference in response between repairing and resting bone exposed to EMF (Muehsam and Pilla 1999). Without a substantial body of literature that evaluates cells with similar metabolic properties receiving EMF under the same set of exposure conditions, it will be difficult to draw conclusions with respect to the biologic effects of EMF exposure.

Conclusions

Though there is a significant volume of literature evaluating the medical and biological impacts of EMF exposure, there is no clear understanding of the potential risks and/or benefits from exposure. The general consensus in the field more than a decade ago was that additional research was necessary to better understand the medi-

cal and physiologic impacts of EMF exposure (Lacy-Hulbert, Metcalfe et al. 1998; Repacholi 1998). Thus far there is little evidence for a direct cause-effect relationship between EMF and disease risk (Kheifets, Renew et al. 2010). For example, a meta-analysis of over 24,000 cases of breast cancer with 60,000 controls demonstrated no association between low levels of EMF and breast cancer risk (Chen, Ma et al. 2010). These data are similar to the findings of the INTERPHONE Study, which demonstrated that exposure to cellular phone RF EMF did not increase risk for glioblastoma multiforme or acoustic neuroma (Group 2010; Group 2011). Moreover, a review of animal models used to evaluate a link between EMF exposure and cancer risk demonstrated that most of these studies were negative (McCann, Kavet et al. 1997). Similarly, there is little evidence supporting the role of RF EMF in promoting metastasis (Zhang, Pan et al. 2011). Further mechanistic studies are certainly necessary, but one conclusion that may be drawn from the current body of literature is that there is likely not one single mechanistic effect that can be associated with exposure to RF EMF and applied to all cell or tissue types. Additional studies should continue to evaluate mechanistic effects of EMF exposure so that exposure guidelines may be appropriately adjusted and possible healthcare benefits may be exploited.

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TREATMENT OF ADVANCED HEPATOCELLULAR CARCINOMA WITH VERY LOW LEVEL OF AMPLITUDE-MODULATED ELECTROMAGNETIC FIELDS

by

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Background: Therapeutic options for patients with advanced hepatocellular carcinoma (HCC) are limited. There is emerging evidence that the growth of cancer cells may be altered by very low levels of electromagnetic fields modulated at specific frequencies.

Methods: A single-group, open-label, phase I/II study was performed to assess the safety and effectiveness of the intrabuccal administration of very low levels of electromagnetic fields amplitude-modulated at HCC-specific frequencies in 41 patients with advanced hepatocellular carcinoma and limited therapeutic options. Three daily 60 minute outpatient treatments were administered until disease progression or death. Imaging studies were performed every 8 weeks. The primary efficacy end point was progression-free survival ≥ 6 months. Secondary efficacy end points were progression-free survival and overall survival.

Results: Treatment was well tolerated and there were no NCI grade 2, 3 or 4 toxicities. Fourteen patients (34.1%) had stable disease for more than 6 months. Median progression-free survival was 4.4 months (95% CI 2.1-5.3) and median overall survival was 6.7 months (95% CI 3.0-10.2). There were three partial and one near complete responses.

Conclusion: Treatment with intrabuccally-administered amplitude-modulated electromagnetic fields is safe, well tolerated, and shows evidence of antitumor effects in patients with advanced hepatocellular carcinoma.

Keywords: Hepatocellular carcinoma; phase II study; radiofrequency electromagnetic fields; tumor-specific modulation frequencies; 27.12 MHz

Treatment of inoperable or metastatic solid tumors is a major challenge in oncology, which is limited by the small number of therapeutic agents that are both well tolerated and capable of long-term control of tumor growth. Hepatocellular carcinoma (HCC) is the second most common cause of cancer death in men and the sixth in women worldwide (Jemal *et al.*, 2011). HCC is the most common tumor in certain parts of the world, particularly in East Asia, Africa, and certain countries of South America. This tumor is less frequent in Europe and in the United States but has become the fastest rising cancer in the United States (Jemal *et al.*, 2011). In the United States alone, it is estimated that 24,120 new cases were diagnosed and there were 17,430 deaths from HCC in 2010 (Jemal *et al.*, 2010), a 27% increase in the number of new cases since 2004 (Jemal *et al.*, 2004). The prognosis of patients suffering from advanced HCC is poor with an average survival of fewer than six months (Jemal *et al.*, 2011; Kassianides and Kew, 1987).

Therapies for HCC are limited. Resections of the primary tumor or liver transplantation are the preferred therapeutic approaches in patients who are surgical candidates (Bruix and Sherman, 2005). Although these interventions result in long-term survival for some patients, only a minority benefit from them because of limitations due to tumor size, patient's overall condition, and presence of hepatic cirrhosis (Cance *et al.*, 2000). Only a small number of randomized trials show a survival benefit in the treatment of HCC. Chemoembolization has been shown to confer a survival benefit in selected patients with unresectable HCC (Llovet *et al.*, 2002). Data from two phase III randomized placebo controlled studies demonstrate improved survival in patients with advanced HCC receiving the multikinase inhibitor sorafenib (Llovet *et al.*, 2008b; Cheng *et al.*, 2009). Additional therapies for this disease are sorely needed, especially for the large number of patients with advanced

disease who cannot tolerate chemotherapy or intrahepatic interventions because of impaired liver function (Thomas and Zhu, 2005).

The intrabuccal administration of low and safe levels of electromagnetic fields, which are amplitude-modulated at disease-specific frequencies (RF AM EMF) (Fig. 1) was originally developed for the treatment of insomnia (Pasche *et al.*, 1990). The highest levels of electromagnetic fields encountered during treatment are found at the interface between the tongue and the mouth probe and are compliant with international safety limits (ICNIRP, 1998). Tumor-specific modulation frequencies have been identified for several common forms of cancer and one report suggests that this novel therapeutic approach is well tolerated and may be effective in patients with a diagnosis of cancer (Barbault *et al.*, 2009). However, the safety and potential efficacy of this treatment approach in the treatment of advanced hepatocellular carcinoma are unknown. We designed this single-group, open label, phase I/II study to assess the feasibility of this treatment in patients with advanced HCC and limited therapeutic options.

PATIENTS AND METHODS

Patients

The study was aimed at offering treatment to patients with Child-Pugh A or B advanced HCC and limited therapeutic options. Patients were classified as having advanced disease if they were not eligible for surgical resection or had disease progression after surgical or locoregional therapies or had disease progression after chemotherapy or sorafenib therapy. Patients with measurable, inoperable HCC were eligible for enrollment. Prior local or systemic treatments were allowed as long as they were discontinued at least 4 weeks prior to enrollment. Inclusion criteria

included Eastern Cooperative Oncology Group (ECOG) performance status of 0, 1, or 2 and biopsy-confirmed HCC. Also allowed were patients with no pathological confirmation of HCC with a level of alpha-fetoprotein higher than 400 ng/mL and characteristic imaging findings as assessed by multislice computer tomography scan (CT) or intravenous contrast ultrasound (US). As per the University of São Paulo Department of Transplantation and Liver Surgery guidelines, liver biopsies are avoided in patients eligible for transplant or with severely impaired liver function. Exclusion criteria included confirmed or suspected brain metastasis, Child-Pugh C, prior liver transplant, and pregnancy.

Study design

This was an investigator-initiated, single center, uncontrolled phase I/II trial in patients with advanced HCC. The trial was approved by the local human investigation committee and conducted in accordance with the Helsinki declaration. Written informed consent was obtained from each patient. The protocol was registered: clinicaltrial.gov identifier # NCT00534664.

Administration of amplitude-modulated electromagnetic fields

The generator of amplitude-modulated electromagnetic fields consists of a battery-driven radiofrequency (RF) electromagnetic field generator connected to a 1.5 meter long 50 Ohm coaxial cable, to the other end of which a stainless steel spoon-shaped mouthpiece is connected via an impedance transformer (Fig. 1A). The RF source of the device corresponds to a class C amplifier operating at 27.12 MHz. The carrier frequency is amplitude-modulated (Fig. 1B) with a modulation depth of 85 ± 5% whereas the modulation frequency is generated by a digital direct synthesizer

(DDS) with a resolution of 10^{-7}. The treatment sequence is controlled by a microcontroller (Atmel AT89S8252, Fribourg, Switzerland), i.e. duration of session, sequence of modulation frequencies and duration of each sequence can be programmed via PC over a RS232 interface. The RF output is adjusted to 100 mW into a 50 Ohm load which results in an emitting power identical to that of the device used for the treatment of insomnia (Pasche *et al.*, 1990;Reite *et al.*, 1994). The U.S. FDA has determined that such a device is not a significant risk device and it has been used in several studies conducted in the U.S. (Reite *et al.*, 1994;Pasche *et al.*, 1996;Kelly *et al.*, 1997). A long-term follow-up survey of 807 patients who have received this therapy in the U.S., Europe and Asia showed that the rate of adverse reactions was low and was not associated with increases in the incidence of malignancy or coronary heart disease (Amato and Pasche, 1993). The maximum specific absorption rate (SAR) of the applied RF averaged over any 10 g of tissue has been estimated to be less than 2 W/kg, and the maximum temperature increase is significantly lower than 1 °C anywhere in the body due to RF absorption. The induced RF field values within the primary and metastatic tumors are significantly lower. In contrast, the RF fields induced during radiofrequency ablation of tumors cause hyperthermia and result in SAR in the range of 2.4×10^5 W/kg (Chang, 2003), i.e. more than 100,000 times higher than those delivered by the device used in this study.

We have previously reported the discovery of HCC-specific modulation frequencies in 46 patients with hepatocellular carcinoma using a patient-based biofeedback approach and shown the feasibility of using amplitude-modulated electromagnetic fields for the treatment of patients with cancer (Barbault *et al.*, 2009). The treatment program used in this study consisted of three daily outpatient

treatments of one hour duration, which contained HCC-specific modulation frequencies ranging between 100 Hz and 21 kHz administered sequentially, each for three seconds (Fig. 1C and Suppl. Table 1).

The treatment method consists of the administration of amplitude-modulated electromagnetic fields by means of an electrically conducting mouthpiece, which is in direct contact with the oral mucosa (Figure 1D). The patients were instructed on the use of the device and received the first treatment at the medical center's outpatient clinic. A device was provided to each patient for the duration of the study. The patients were advised to self-administer treatment three times a day. Treatment was administered until tumor progression was objectively documented. At that time, treatment was discontinued. Treatment compliance was assessed at every return visit by recording the number of treatments delivered in the preceding two months.

Efficacy endpoints and disease assessment

The primary end point of this trial was the proportion of patients progression-free at 6 months. Secondary end points were progression-free survival (PFS) (first day of treatment until progression of disease or death) and overall survival (OS) (first day of receiving treatment to death). Objective response was assessed using the Response Evaluation Criteria in Solid Tumors group classification (RECIST) for patients with disease assessed by either helical multiphasic CT (Therasse *et al.*, 2000). Whenever contrast-enhanced US radiologic assessment was used, it was performed and reviewed by the same radiologist specialized in HCC (M. C. C.) as this imaging modality is investigator dependent. Tumor measurements were performed at baseline and every 8 weeks. Only patients with at least one repeat tumor measurement during therapy were considered for response analysis. Throughout the study, lesions measured at baseline

Fig. 1

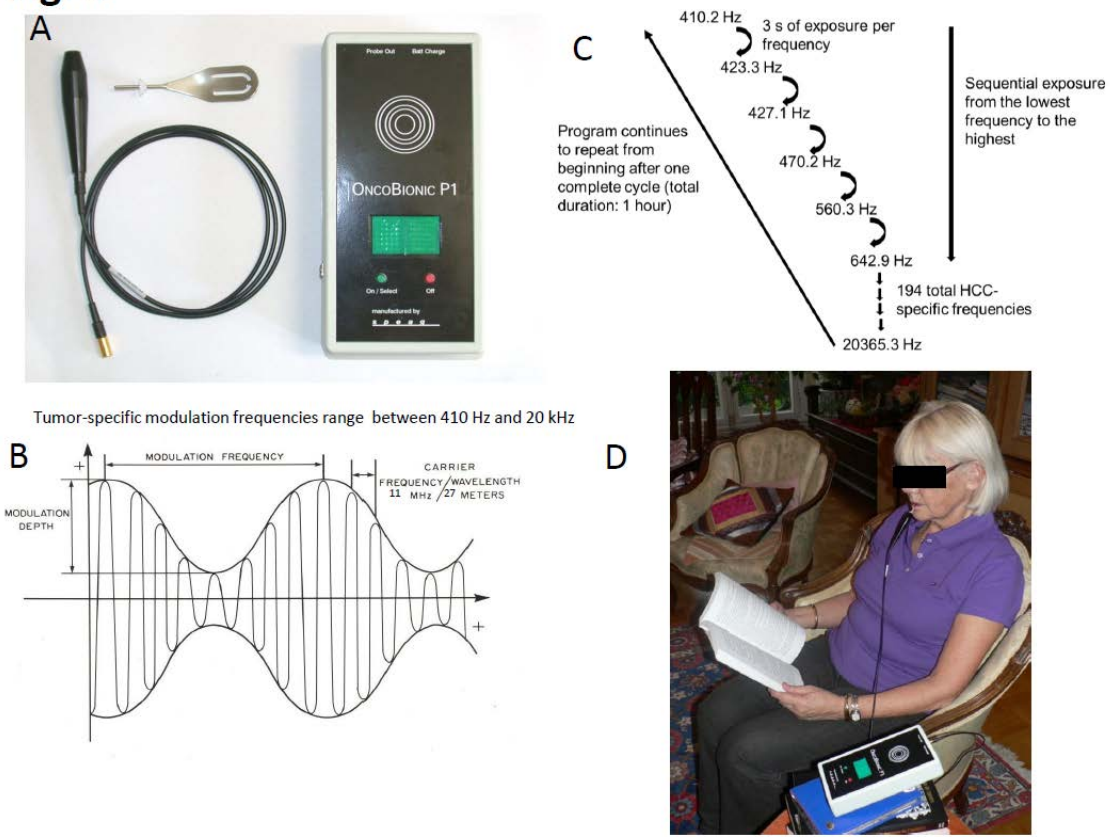

Fig. 1. Delivery of HCC-specific modulation frequencies (A) The generator of amplitude-modulated electromagnetic fields is a battery-driven radiofrequency (RF) electromagnetic field (EMF) generator connected to a spoon-shaped mouthpiece. **(B)** Schematic description of amplitude-modulated (AM) electromagnetic fields. The carrier frequency (27.12 MHz) is sinusoidally modulated at specific frequencies. **(C)** Patient receiving treatment with RF AM EMF. **(D)** HCC treatment program consisting of sequential emission of 194 modulation frequencies for 60 min.

were evaluated using the same technique (CT or contrast-enhanced US). Overall tumor response was scored as a complete response (CR), partial response (PR) or stable disease (SD) if the response was confirmed at least 4 weeks later. Alpha fetoprotein (AFP) levels were measured every 8 weeks in all patients throughout the study but changes in AFP were not an end point for assessment of response. Pain was assessed according to the NCI-CTCAE v.3.0 (http://ctep.cancer.gov/protocol Development/electronic_applications /docs/ctcaev3.pdf).

Statistical Analyses and efficacy assessment

All eligible patients who began treatment were considered assessable for the primary and secondary end points. A Simon two-stage Phase II minimax design was used (Simon, 1989) to evaluate the rate of progression-free survival at 6 months. The interim analysis was performed once enrollment into the first stage was completed. In the first stage, 23 patients were observed. If 2 or fewer patients had progression-free survival $\geq$ 6 months, the trial would be terminated early for lack of efficacy. If the progression-free survival of 3 or more of the first 23 patients was equal or greater than 6 months, then an additional 18 patients would be enrolled to a maximum of 41 patients. If eight or more of the 41 had PFS of at least 6 months we would conclude that the treatment was efficacious. This design had a Type I error rate of 5% and a Type II error rate of 10% for the null hypothesis of a six month PFS rate of 10% vs the alternative of 27.5%. Kaplan-Meier estimates of survival, PFS, and duration of response were calculated with standard errors based on Greenwood's formula. These calculations were performed using Proc Lifetest in SAS 9.2 (SAS Institute Inc., Cary, NC).

RESULTS

Patient recruitment and follow up

From October 2005 to July 2007, 267 patients were assessed for eligibility (Fig. 2). Forty three patients with advanced HCC and Child-Pugh A or B were enrolled in this study. The date of last patient follow up is June 9, 2011. Twenty patients (46.5%) had histological confirmation of HCC. Twenty three patients (53.5%) were diagnosed based on elevated levels of alpha-fetoprotein and characteristic imaging findings such as vascular invasion and characteristic differences in tumor blood flow. One patient was excluded because liver biopsy established the diagnosis of metastatic breast cancer. Another patient was excluded because of severely impaired liver function (Child-Pugh C11). These two patients who did not meet the inclusion criteria were registered as screening failures. Hence, a total of 41 patients were eligible to receive experimental therapy (Fig. 2).

Two patients were lost to follow-up as they did not come back for their scheduled appointments. Repeated efforts were made to reach the patients and their families. The date of death of only one patient is known, and no information on response to treatment is available for either patient. Four patients withdrew consent while receiving therapy after 8.0, 9.3, 20.3, and 21.0 months, respectively (Fig. 2). One patient elected to receive chemotherapy, one patient had poor treatment compliance as defined by administration of less than 50% of planned treatments at two consecutive return visits, one patient elected to enroll in another experimental protocol, and one patient requested to be considered for liver transplantation as part of an extended indication, which does not fulfill the Milan criteria (Mazzaferro *et al.*, 1996). This latter patient experienced disease progression and was ultimately not eligible for liver transplantation. Of the 35 patients who discontinued experimental

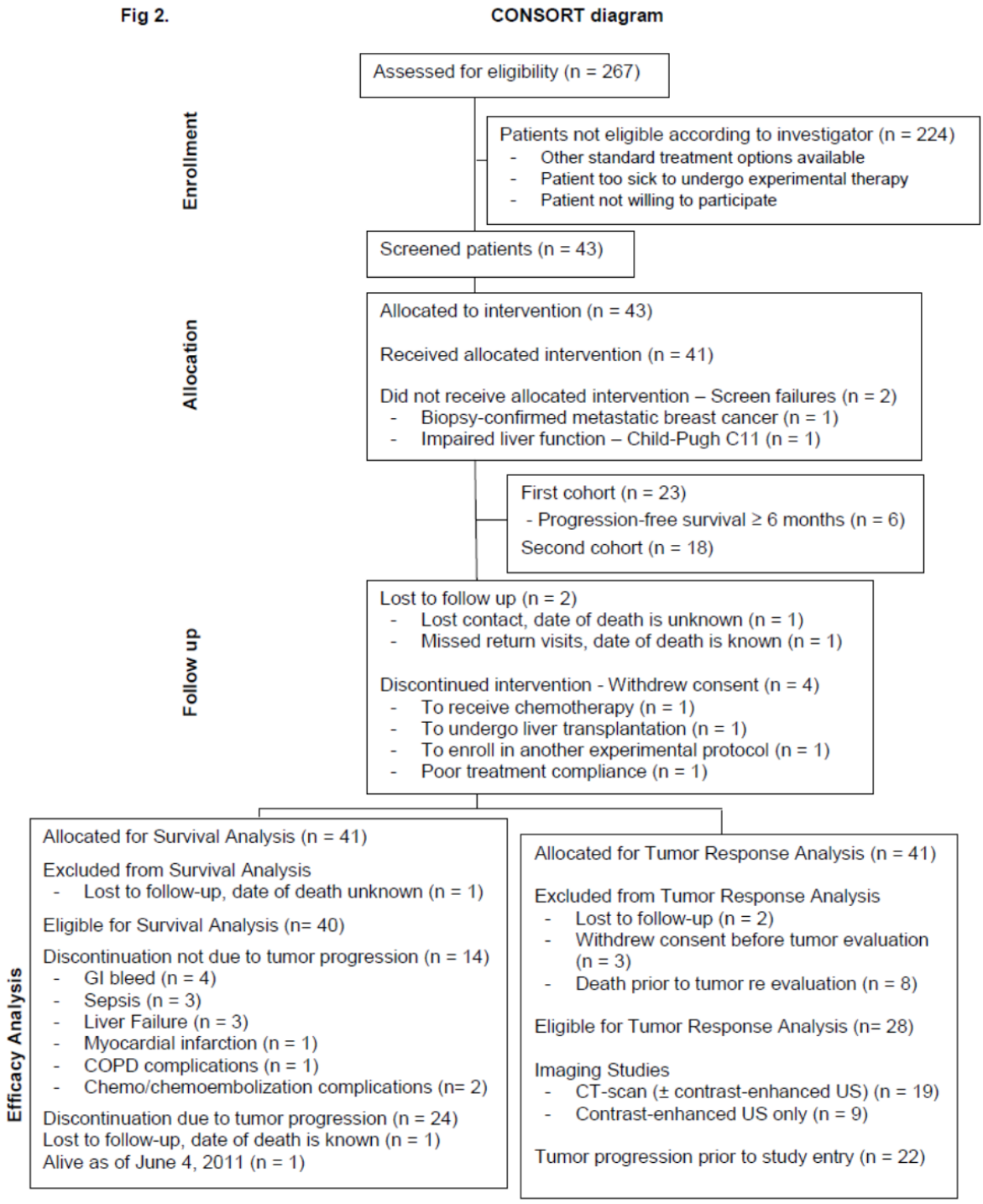

Fig. 2. CONSORT diagram

therapy, four died of gastrointestinal bleeding, three of sepsis, three of hepatic failure, two of chronic obstructive pulmonary disease (COPD), two of chemotherapy and chemoembolization related complications, and one of myocardial infarction (Fig. 2). The remaining 24 patients discontinued because of disease progression assessed by imaging or significant clinical deterioration as assessed by the investigator (Fig. 2). Estimated sixty-day mortality was 27.8%; seven of 10 deaths were directly related to progression of disease. They were caused by liver failure in association with significant hepatic tumor involvement, without other cause of death, other than tumor involvement. Two deaths were secondary to gastrointestinal bleeding. One death was due to liver failure.

Thirty one patients (75.6%) had radiological evidence of disease progression at the time of enrollment as defined by comparison of baseline imaging studies with imaging studies obtained within the previous six months. Thirty four (82.9%) patients had received therapy prior to enrollment, six (14.6%) of them systemic chemotherapy or sorafenib (Table 1). Seven (17.1%) patients had not received therapy prior to enrollment for the following reasons: 1) Severely impaired liver function in five cases, 2) Two patients refused to receive chemotherapy for metastatic disease. As shown in Table 2, the majority of patients had severely impaired liver function as demonstrated by the fact 22 (53.7%) patients had Child-Pugh B disease and 35 (85.4%) BLCL stage C disease.

Table 1: Treatments received by patients with advanced HCC prior to enrollment (n = 41)

No prior treatment	7
Chemoembolization	25
I^{131}-Lipiodol	1
Octreotide	1
Percutaneous alcohol injection therapy	1
Surgery	9
Systemic chemotherapy or sorafenib	5

Two patients had surgery and chemoembolization, two patients surgery and systemic chemotherapy, one patient surgery and chemoembolization and systemic chemotherapy, one patient surgery and percutaneous alcohol injection, one patient surgery and sorafenib, one patient chemoembolization and systemic chemotherapy, one patient surgery and octreotide.

Treatment efficacy

Six of the first 23 patients (26.1%) had progression-free survival ≥ 6 months, which led us to continue enrolling patients up to the pre-planned total of 41 patients (Fig. 2). In total, fourteen patients (34.1%) had stable disease for more than 6 months, which met our preplanned primary efficacy end point. Median progression-free survival was 4.4 months (95% CI 2.1-5.3) and median overall survival was 6.7 months (95% CI 3.0-10.2) (Fig. 3A-B). One patient, previously enrolled in the SHARP study (Llovet *et al.*, 2008b) and with evidence of disease progression at the time of enrollment, remains on therapy with a near complete response for 58 months (Fig. 3C). Estimated survival at 12, 24 and 36 months is 27.9% (SE = 7.1%), 15.2% (SE = 5.7%), and 10.1% (SE = 4.8%).

A total of 28 patients were evaluable for tumor response (Fig. 2). Four (9.8%) patients had a partial response assessed with CT with or without contrast-enhanced ultrasound (Table 3). All partial responses were independently reviewed by two authors (M.S.R and D.M.). Three patients had biopsy-confirmed HCC and three had radiological evidence of disease progression at the time of enrollment

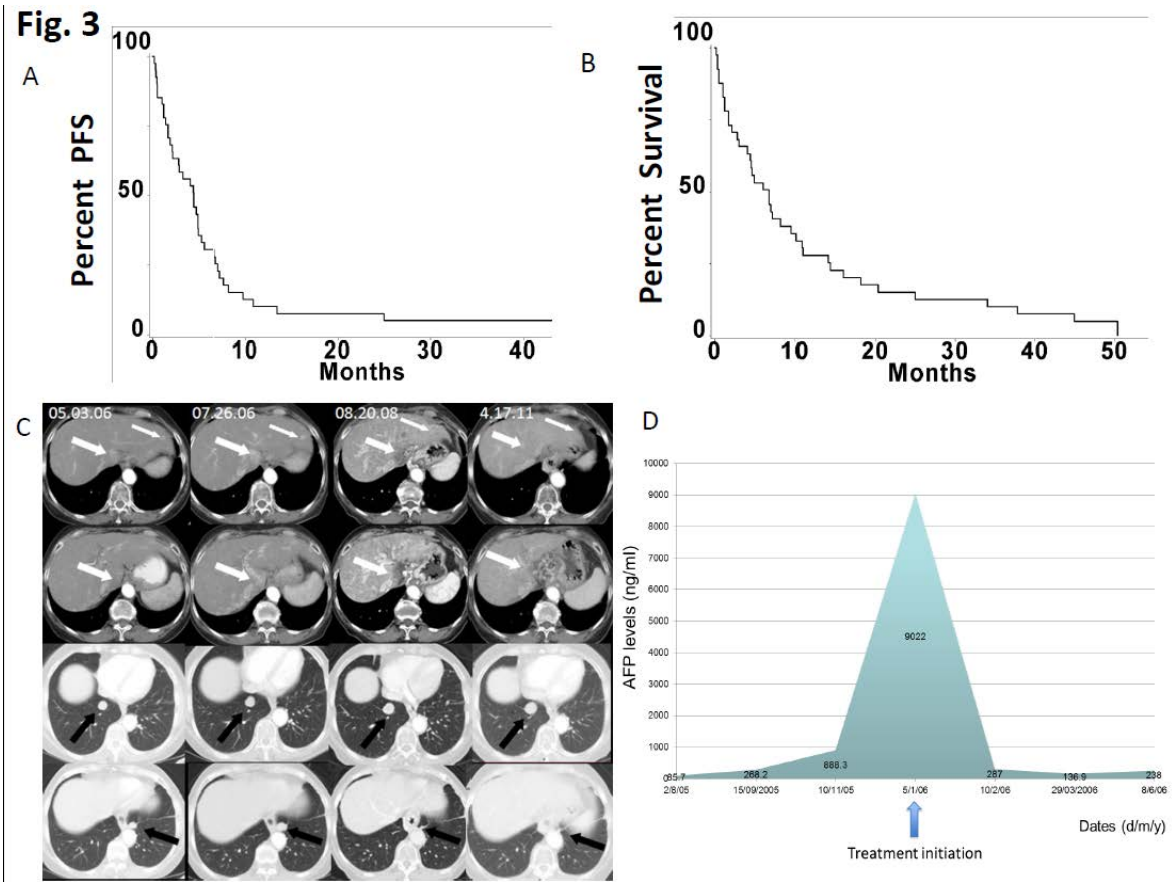

Fig. 3. Progression-free and overall survival. (A) Median progression-free survival was 4.4 months (95% CI 2.1-5.3). **(B)** Median overall survival was 6.7 months (95% CI 3.0-10.2). **(C)** Long-term partial response in a patient with biopsy-proven hepatocellular carcinoma. 76 year-old woman with hepatitis C and Child-Pugh A5, BCLC C, biopsy-proven hepatocellular carcinoma with bilateral pulmonary metastases, who had evidence of disease progression (+36% by RECIST criteria) between 05.03.06 (first column) and 07.26.06 (second column) while enrolled in the SHARP study (Llovet *et al.*, 2008b). Treatment with amplitude-modulated electromagnetic fields was initiated on 08.09.06. Subsequent restaging multiphasic contrast enhanced CTs with images from corresponding levels (across rows) are demonstrated in the third and fourth columns over the course of 57 months. Note that the hypervascularity of the focal hepatic lesions (arrows in first two rows) became relatively hypoenhancing on arterial phase (08.20.08). The patient developed main portal vein thrombosis with cavernous transformation as a complication of her cirrhosis. However, the intrahepatic lesion size is stable regardless of enhancement pattern. Note also that the left lung base lesion resolved (4th row), and the right lung base lesion remained stable (3rd row) over the duration of treatment. **(D)** Alpha-fetoprotein (AFP) response in a 67 year old patient with Child-Pugh A5, BCLC C HCC and Hepatitis C (Hepatitis B negative).

Table 2: Patients' baseline characteristics

Age, years	No	%
Median age	64	
Range	18-85	
≥65	19	46.3
<65	22	53.6
Sex		
Female	6	14.6
Male	35	85.4
ECOG performance status		
0	5	12.2
1	28	68.3
2	8	19.5
Child-Pugh status		
A5	15	36.6
A6	2	4.9
B7	6	14.6
B8	5	12.2
B9	11	26.8
No cirrhosis	2	4.9
BCLC status		
B	6	14.6
C	35	85.4
AFP>ULN		
Yes	28	68.3
No	13	16.7
Etiology		
ETOH	2	4.9
Hepatitis B	6	14.6
Hepatitis B+C	1	2.4
Hepatitis C	22	53.7
ETOH + Hepatitis C	1	2.4
NOS	9	22.0
Portal thrombosis	10	24.3
Extra hepatic disease		

Yes	16	39.0
Location		
Lung	6	14.6
Bone	3	7.3
Lymph nodes	4	9.8
Peritoneal carcinomatosis	1	2.4
Adrenal gland	1	2.4

(Table 4). Two patients had Child-Pugh A, one Child-Pugh B disease, and one had no cirrhosis. One of these patients without biopsy-proven disease subsequently withdrew consent after 4.9 months to undergo liver transplantation. The patient died of progression of disease 9.4 months later prior to undergoing liver transplantation. One patient with Child-Pugh B disease had a partial response lasting 11.7 months and died of gastrointestinal bleeding. One patient died of disease progression at 44.6 months. Overall, there were six long-term survivors with an overall survival greater than 24 months and four long-term survivors with an overall survival greater than three years. Importantly, five of the six (83%) long-term survivors had radiological evidence of disease progression at the time of study enrollment (Table 4). Two of three patients with the longest survival (44.6 and +58 months) had radiological evidence of disease progression at the time of enrollment, BLCL stage C disease, as well as portal vein thrombosis, three predictors of short survival (Llovet *et al.*, 2003). Serial alpha-fetoprotein (AFP) measurements, which predict radiological response and survival in patients with HCC (Chan *et al.*, 2009; Riaz *et al.*, 2009), were available for 23 patients. AFP decreased by 20% or more in four (9.8%) patients following initiation of therapy (Table 5). Fig. 3D shows the time course of a 37 fold decrease in AFP in a patient who had a long-lasting (11.7 months) partial response as assessed by CT.

Table 3: Independently reviewed best response (N = 41)

Best Response	No	%
Partial response*	4	9.8
Stable disease**	16	39.0
Progressive disease	8	19.5
Not available for response assessment	13	31.7

* Duration of the partial responses were +58.0 months, 46.9 months, 14.5 months and 5.3 months (patient withdrew consent to undergo liver transplant)
** To be classified as a stable disease, patients needed to have stable disease for ≥ 12 weeks

Eleven patients reported pain prior to treatment initiation, three patients reported grade 3, five grade 2, and three grade 1. Five patients reported complete disappearance of pain and two patients reported decreased pain shortly after treatment initiation. Two patients reported no changes and two patients reported increased pain. There were no treatment-related grade 2, 3 or 4 toxicities. The only treatment-related adverse events were grade 1 mucositis (1 patient) and grade 1 somnolence (1 patient) over a total of 266.8 treatment months.

DISCUSSION

Treatment with amplitude-modulated electromagnetic fields did not show any significant toxicity despite long-term treatment. The lack of toxicity experienced by the 41 patients presented in this report as well as the 28 patients from our previous report (Barbault *et al.*, 2009) can be readily explained by the very low and safe levels of induced radiofrequency electromagnetic fields, which are more than 100,000 times lower than those delivered during radiofrequency ablation procedures (Chang, 2003). Hence, the putative mechanism of action of this novel therapeutic approach does not depend on temperature changes within the tumor.

Table 4: Characteristics of patients with either partial response (PR) and/or long-term survival in excess of 24 months

Age at Enrollment and Sex	Race	Cause/ Cirrhosis (Child-Pugh)	Previous Treatment/ Resection	AFP↑ / Pathology Confirmation	Extra hepatic metastasis/ Portal Thrombosis	BCLC Okuda CLIP MELD	Progression Prior to Study Entry / Response	Treatment duration / Overall Survival (months)	Cause of death	Treatment received after completion of experimental therapy
62 M	Caucasian	Hep C Yes (A5)	Yes No	Yes Yes	No No	B 1 0 6	Yes N/A	2.0 32.0	Tumor progressed	Systemic chemotherapy
67 F	Caucasian	Hep C Yes (B9)	Yes No	Yes Yes	No No	C 2 2 11	Yes PR	11.7 11.7	GI bleed	None
30 M	Black	NOS No	Yes Yes	No Yes	No No	B N/A N/A N/A	No PR	13.5 37.6	Tumor progressed	Chemo-embolization and systemic chemotherapy
61 M	Caucasian	Hep C Yes (A5)	Yes No	No No	No No	C 1 1 6	Yes SD	26.8 26.8	COPD	None
56 M	Caucasian	Hep B/C Yes (A5)	No No	Yes No	No No	B 1 0 10	Yes SD	4.9 50.3	Tumor progressed	Chemo-embolization
63 M	Caucasian	Hep C Yes (A5)	Yes No	Yes No	No No	C 1 1 4	Yes PR	4.9 14.3	Tumor progressed	None

76 F	Caucasian	Hep C Yes (A5)	No No	No No	No Yes	C 1 1 6	Yes SD	44.6 44.6	Tumor progressed	None
76 F	Caucasian	Hep C Yes (A5)	No Yes	No Yes	Yes Yes	C 1 1 6	Yes PR	+58.0 +58.0	On therapy	Still receiving experimental treatment

Table 5: **Changes in alpha-fetoprotein (AFP) levels**

Patient Age Gender	AFP 6 months (ng/ml)	Baseline AFP (ng/ml)	8 week AFP (ng/ml)	AFP variation (%)	Treatment Duration (months)	End Treatment Status	Virus Status
65 M	4.31	9.76	5.95	-39.0	3.0	Progression-death	HepC
67 F	888.3	9022.0	238.0	-97.3	11.7	GI Bleed-death	HepC
64 M	4.7	4.5	2.6	-42.2	8.8	AMI-death	HepB
18 M	6.7	35.7	16.4	-55.7	7.8	Revoked Consent-death	NOS

AFP - alpha-fetoprotein
AFP 6 months – AFP measured within six months prior to enrollment
GI - gastrointestinal
AMI - acute myocardial infarction
NOS – not otherwise specified
HepB – hepatitis B virus
HepC – hepatitis C virus
Baseline AFP – AFP at treatment initiation
8 week AFP – AFP at 8 weeks during treatment

These data are comparable to recent phase II studies evaluating the effectiveness of standard chemotherapy as well as novel targeted therapies in HCC (Abou-Alfa *et al.*, 2006; Boige *et al.*, 2007; Chuah *et al.*, 2007; Cohn *et al.*, 2008; Dollinger *et al.*, 2008; Siegel *et al.*, 2008). In a large phase II study assessing the effects of sorafenib in patients with HCC and Child-Pugh A and B who had not received prior systemic treatment, Abou-Alfa et al. observed partial responses using the WHO criteria in 2.2% of patients (Abou-Alfa *et al.*, 2006). Investigator-assessed median time to progression was 4.2 months, and median overall survival was 9.2 months. Of note, all 137 patients from that study had evidence of disease progression after 14.8 months (Abou-Alfa *et al.*, 2006) while, at the same time point, four (9.8%) of the patients enrolled in this study did not have evidence of disease progression. These findings suggest that RF AM EMF may increase the time to radiologic progression in advanced HCC.

The majority of patients enrolled in this study had either failed standard treatment options or had severely impaired liver function that limited their ability to tolerate any form of systemic or intrahepatic therapy. Indeed, 16 patients (39.0%) had Child-Pugh B8 or B9 disease. Among these patients the median progression-free survival was 4.4 months (95% CI 1.6 to 7.6 months), which is identical to that of the entire group. Five of these 16 patients (31.3%) received therapy for more than 7.5 months, which indicates that this therapy is well tolerated even in patients with severely impaired liver function.

Prior treatment with standard chemotherapy or sorafenib does not seem to impact the effectiveness of amplitude-modulated electromagnetic fields in the treatment of HCC. Indeed, three of the four patients who had a partial response while receiving amplitude-

modulated electromagnetic fields had received prior systemic therapies (chemotherapy and sorafenib) and one had received intrahepatic therapy with ^{131}I-lipiodol.

Tumor shrinkage as assessed by radiological imaging as well as changes in AFP levels were documented in patients with advanced hepatocellular carcinoma receiving RF EMF modulated at HCC-specific frequencies administered by an intrabuccal probe. Antitumor activity in patients with advanced HCC was exemplified by partial responses observed in four patients (9.8%) and decreases in AFP levels greater than 20% in four patients. A total of 18 patients (43.9%) either had objective response or stable disease ≥ 6 months.

Importantly, this therapeutic approach has long-lasting therapeutic effects in several patients with metastatic cancer. Two of these patients, one with recurrent thyroid cancer metastatic to the lungs (Fig. 4) enrolled in our feasibility study (Barbault *et al.*, 2009) and the patient shown in Fig. 3C, are still receiving treatment without any evidence of disease progression and without side effects almost 5 years after being enrolled in these studies. These findings suggest that, in some patients, this therapeutic approach may achieve permanent control of advanced cancer with virtually no toxicity.

Our phase I/II study has several limitations. First, only 19 of the 41 patients had biopsy-proven HCC, the others were diagnosed by clinical criteria, an approach similar to that used in a recently reported phase II trial evaluating the clinical and biological effects of bevacizumab in unresectable HCC (Siegel *et al.*, 2008). Importantly, analysis restricted to these 19 patients shows rates of progression-free survival at 6 months, median progression-free survival and overall survival that are similar to those without

biopsy-proven HCC (Suppl. Fig. 1C and 1D). Furthermore, three of the four partial responses were observed in patients with biopsy-proven HCC.

Fig. 4

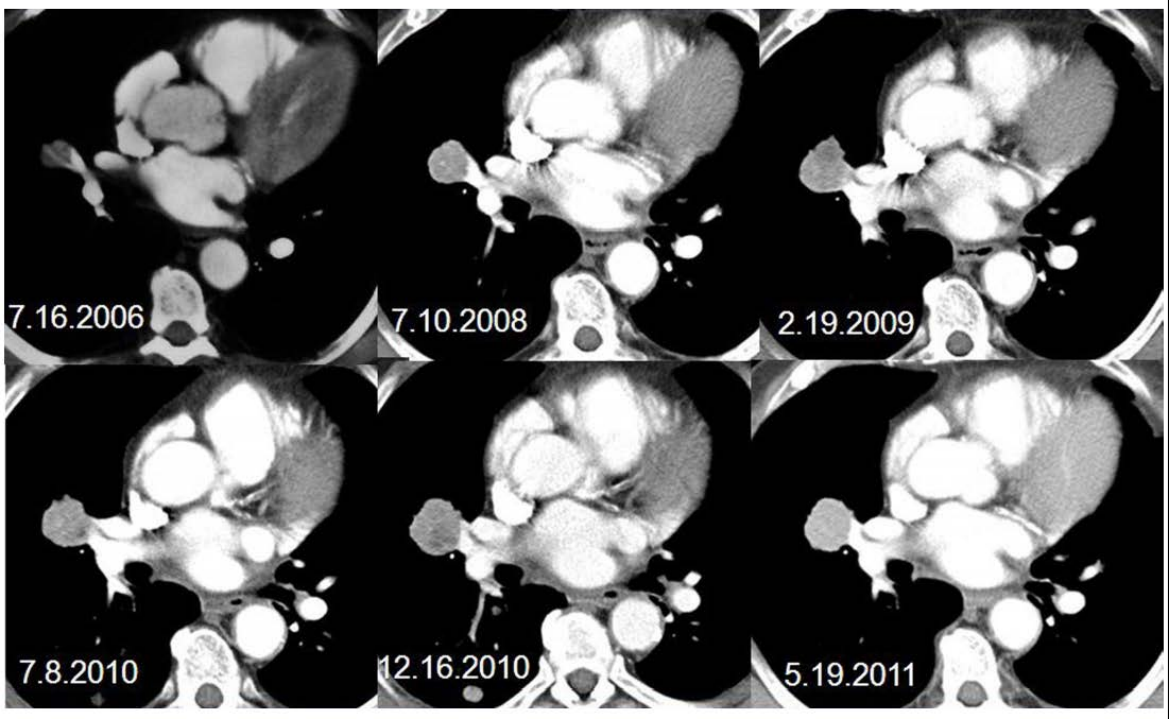

Fig. 4. 70 year old man with recurrent thyroid cancer metastatic to the lungs: stable disease at 57.5 months. Long-term stable disease in a 70 year-old man with recurrent biopsy-proven thyroid carcinoma metastatic to the lungs enrolled in the previously published feasibility study (Barbault *et al.*, 2009). Treatment with amplitude-modulated electromagnetic fields was initiated on August 20, 2006. As of June 9, 2011 the patient is asymptomatic and still receiving treatment with no evidence of disease progression. Images through the target metastatic lesion in the right hilum demonstrate minimal size change over the four years, given differences in CT acquisition techniques over that time interval.

Hence these findings strongly suggest that treatment with amplitude-modulated electromagnetic fields yields similar results in patients with and without biopsy-confirmed HCC. Another potential limitation of our study consists in the use of contrast-enhanced ultrasound for the monitoring of some patients with HCC. It should be pointed out that recent studies indicate that the use of this imaging technique is comparable to that of CT-scan with respect to the measurement of HCC tumors (Choi, 2007; Maruyama *et al.*, 2008).

Anti-tumor response is considered the primary end point for phase II studies to proceed to further investigations. Studies applying Cox proportional hazards analysis indicate that this end point is consistently associated with survival in trials of locoregional therapies for HCC (Llovet *et al.*, 2002) and a recent consensus article suggests that randomized studies are necessary to capture the true efficacy of novel therapies in hepatocellular carcinoma (Llovet *et al.*, 2008a). In summary, the encouraging findings from this study warrant a randomized study to determine the impact of amplitude-modulated electromagnetic fields on overall survival and time to symptomatic progression.

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Supplementary Information

Subset analyses

As of June 9, 2011, the median progression-free survival and overall survival for the 16 patients with Child-Pugh A disease are 4.3 (95% CI 1.8-4.9) and 6.4 (95% CI 3.0-24.9) months, respectively, with one patient alive still receiving treatment (6.2%) (Suppl. Fig. 1A and 1B). The corresponding median progression-free survival and overall survival for the 20 patients with Child-Pugh B disease are 2.8 (95% 0.5-6.6) and 3.6 (95% CI 0.5-9.5) months, respectively with no patient receiving treatment and no patient alive (Suppl. Fig. 1A and 1B). The progression-free survival and overall survival of the two patients without cirrhosis but with biopsy-proven HCC are 15.0 and 37.1 months for one of them and 7.8 and 15.3 months for the other.

Analysis restricted to the 19 patients with biopsy-confirmed HCC shows progression-free survival of 29.0% (SE = 10.8%) at 6 months with a median of 4.7 months (95% CI 1.8-6.7 months) and survival at six months of 62.4% (SE = 11.3%) with a median of 6.8 months (95% CI 3.0 to 14.1 months) (Suppl. Fig. 1C and 1D). Subset analysis of the 11 patients fulfilling similar diagnostic criteria as those applied in phase III studies such as the SHARP and Asia-Pacific sorafenib studies (Llovet *et al.*, 2008;Cheng *et al.*, 2009), i.e. biopsy-proven disease and assessment of disease with CT, shows an objective response rate (RR) by RECIST of 18.2% (2/11), and median PFS and OS of 4.9 months (95% CI .6 to 10.8 months) and 10.8 months (95% CI 2.1 to 34.0 months) (Suppl. Fig. 2A and 2B). These preliminary results based on a small number of patients appear to be either comparable or superior to the Asia-Pacific phase III study in which RR was 3.3%, median PFS 2.8 months and median OS 6.5 months (Cheng *et*

al., 2009), and the SHARP study in which RR was 2.3%, median PFS 5.5 months and median OS 10.7 months (Llovet *et al.*, 2008).

Twenty five (61.0%) patients had received chemoembolization (Table 1) prior to enrollment, and their median progression-free survival and overall survival were 4.8 and 6.5 months, respectively, which are almost identical to the median progression-free survival and overall survival of all patients: 4.8 months and 6.9 months, respectively.

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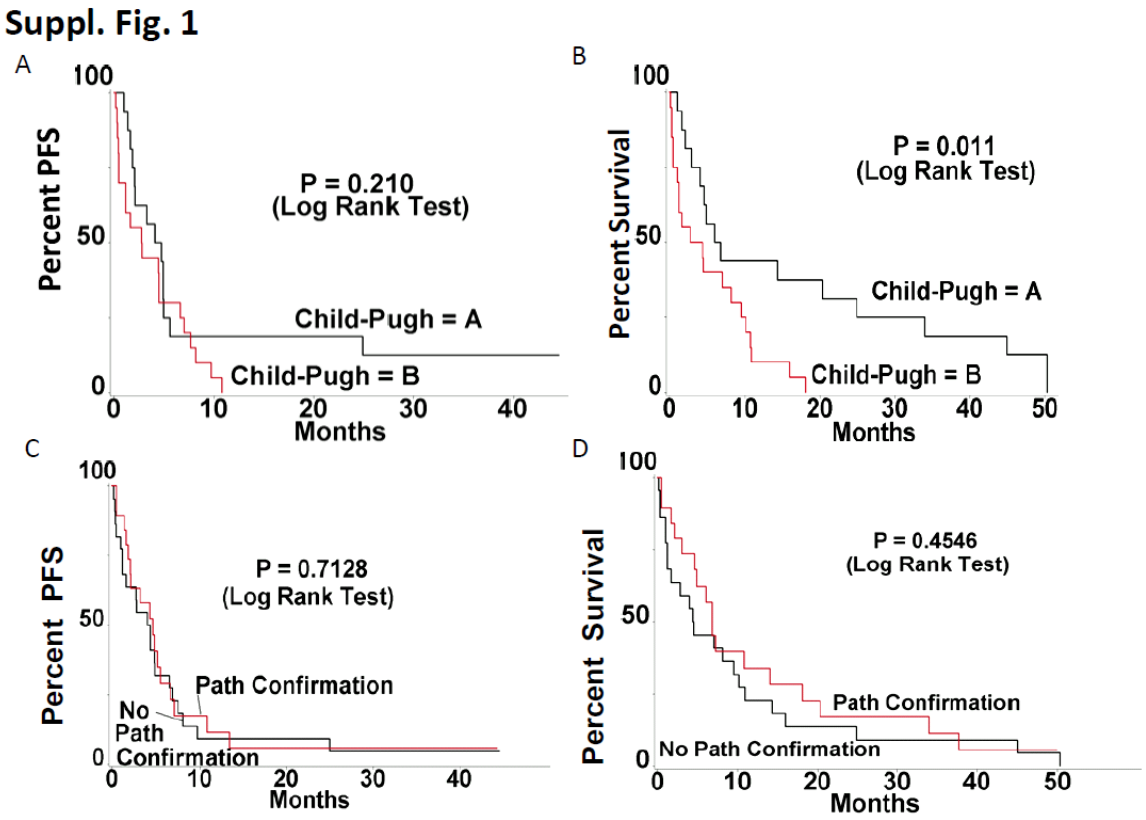
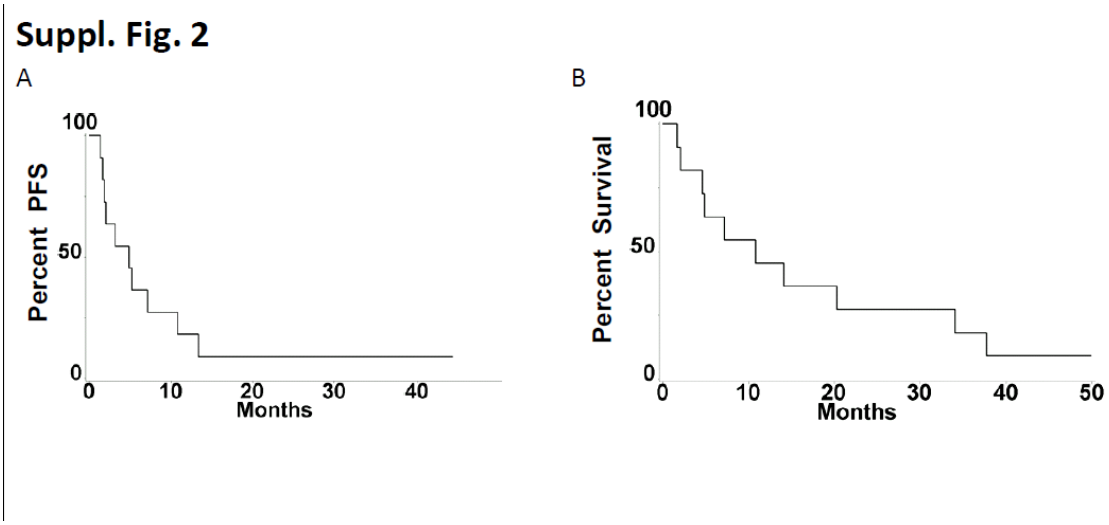


Suppl. Fig. 1. Progression-free and overall survival based on pathology confirmation of diagnosis. (A), Median progression-free survival for the 16 patients with Child-Pugh A disease was 4.3 months (95% CI 1.8-4.9). **(B),** Median overall survival for the 16 patients with Child-Pugh A disease was 6.4 months (95% CI 3.0-24.9). **(C),** Median progression-free survival was 4.7 (95% CI 1.8 – 6.7) months for those with pathology confirmation. (**D),** Median overall survival was 6.8 (95% CI 3.0 – 14.1) months for those with pathology confirmation.



Suppl. Fig. 2 Progression-free and overall survival among patients with pathology confirmation of diagnosis and assessment of disease by CT. (A), Median progression-free survival was 4.9 (95% CI .6 – 10.8) months for those fulfilling diagnostic criteria similar to those used in the phase III sorafenib studies [*Llovet et al,* 2008b; *Cheng et al,* 2009]. **(B),** Median survival was 10.8 (95% CI 2.1 – 34.0) months for these patients.

Supplementary Table 1	
HCC-specific modulation frequencies	
Number	Frequency
1	410.2 Hz
2	423.3 Hz
3	427.1 Hz
4	470.2 Hz
5	560.3 Hz
6	642.9 Hz
7	655.4 Hz
8	657.4 Hz
9	668.2 Hz
10	678.0 Hz
11	728.2 Hz
12	806.0 Hz
13	811.9 Hz
14	842.3 Hz
15	843.2 Hz
16	891.9 Hz
17	1250.5 Hz
18	1755.4 Hz
19	1873.5 Hz
20	1924.7 Hz
21	1975.2 Hz
22	2018.0 Hz
23	2053.4 Hz
24	2083.4 Hz
25	2190.7 Hz
26	2221.3 Hz
27	2324.4 Hz
28	2353.5 Hz
29	2362.3 Hz
30	2419.3 Hz
31	2425.2 Hz
32	2430.2 Hz
33	2431.1 Hz
34	2471.3 Hz
35	2478.3 Hz
36	2480.2 Hz

37	2522.3 Hz
38	2744.0 Hz
39	2744.2 Hz
40	2832.0 Hz
41	2843.3 Hz
42	2860.0 Hz
43	2873.5 Hz
44	2886.2 Hz
45	3042.0 Hz
46	3079.0 Hz
47	3086.4 Hz
48	3127.2 Hz
49	3160.9 Hz
50	3161.3 Hz
51	3206.3 Hz
52	3255.2 Hz
53	3267.4 Hz
54	3269.3 Hz
55	3281.4 Hz
56	3457.3 Hz
57	3505.2 Hz
58	3516.3 Hz
59	3530.2 Hz
60	3531.3 Hz
61	3546.3 Hz
62	3572.1 Hz
63	3576.2 Hz
64	3669.5 Hz
65	3923.2 Hz
66	3927.3 Hz
67	4013.9 Hz
68	4071.1 Hz
69	4080.0 Hz
70	4124.0 Hz
71	4162.0 Hz
72	4222.8 Hz
73	4238.4 Hz
74	4256.3 Hz
75	4289.3 Hz

76	4312.9 Hz
77	4376.0 Hz
78	4426.4 Hz
79	4435.2 Hz
80	4471.2 Hz
81	4484.0 Hz
82	4486.4 Hz
83	4556.3 Hz
84	4629.9 Hz
85	4732.2 Hz
86	4876.2 Hz
87	5086.3 Hz
88	5124.1 Hz
89	5133.1 Hz
90	5247.1 Hz
91	5270.8 Hz
92	5340.5 Hz
93	5520.2 Hz
94	5570.2 Hz
95	5882.3 Hz
96	5926.5 Hz
97	6037.3 Hz
98	6180.3 Hz
99	6329.2 Hz
100	6350.3 Hz
101	6361.3 Hz
102	6364.9 Hz
103	6383.3 Hz
104	6461.2 Hz
105	6733.3Hz
106	6758.2 Hz
107	6779.5 Hz
108	6856.2 Hz
109	6877.2 Hz
110	6915.9 Hz
111	6980.5 Hz
112	7019.2 Hz
113	7043.2 Hz
114	7130.3 Hz

115	7144.1 Hz
116	7210.2 Hz
117	7291.2 Hz
118	7482.2 Hz
119	7510.9 Hz
120	7529.2 Hz
121	7549.2 Hz
122	7650.0 Hz
123	7680.5 Hz
124	7692.5 Hz
125	7829.2 Hz
126	7862.2 Hz
127	7932.5 Hz
128	7935.4 Hz
129	7947.4 Hz
130	7979.3 Hz
131	8028.3 Hz
132	8055.9 Hz
133	8072.1 Hz
134	8141.2 Hz
135	8336.4 Hz
136	8394.8 Hz
137	8432.2 Hz
138	8452.1 Hz
139	8460.9 Hz
140	8475.2 Hz
141	8492.2 Hz
142	8542.3 Hz
143	8818.1 Hz
144	8852.3 Hz
145	8853.4 Hz
146	8858.2 Hz
147	8939.2 Hz
148	9332.4 Hz
149	9381.2 Hz
150	9719.3 Hz
151	9740.2 Hz
152	9768.3 Hz
153	9797.3 Hz

154	9819.5 Hz
155	10317.5 Hz
156	10438.5 Hz
157	10443.3 Hz
158	10456.4 Hz
159	10579.4 Hz
160	10863.2 Hz
161	10866.4 Hz
162	11067.4 Hz
163	11149.9 Hz
164	11163.9 Hz
165	11802.8 Hz
166	11953.4 Hz
167	12223.3 Hz
168	12260.9 Hz
169	12265.3 Hz
170	12267.2 Hz
171	12623.2 Hz
172	12633.4 Hz
173	12685.2 Hz
174	12721.4 Hz
175	12785.3 Hz
176	13433.3 Hz
177	14085.2 Hz
178	14333.2 Hz
179	14537.3 Hz
180	14542.4 Hz
181	14655.0 Hz
182	14828.2 Hz
183	15149.2 Hz
184	15237.5 Hz
185	15717.2 Hz
186	16110.9 Hz
187	16144.3 Hz
188	18265.2 Hz
189	18283.3 Hz
190	18863.3 Hz
191	18931.0 Hz
192	19970.3 Hz

193	20330.3 Hz
194	20365.3 Hz
195	

CANCER CELL PROLIFERATION IS IN INHIBITED BY SPECIFIC MODULATION FREQUENCIES

by

JACQUELYN W. ZIMMERMAN, MICHAEL J. PENNISON, IVAN BREZOVICH, NENGJUN YI, CELESTE T YANG, RYNE RAMAKER, DEVIN ABSHER, RICHARD M. MYERS, NIELS KUSTER, FREDERICO P. COSTA, ALEXANDRE BARBAULT, BORIS PASCHE

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Format adapted for dissertation

Background: There is clinical evidence that very low and safe levels of amplitude-modulated electromagnetic fields administered via an intrabuccal spoon-shaped probe may elicit therapeutic responses in patients with cancer. However, there is no known mechanism explaining the anti-proliferative effect of very low intensity electromagnetic fields.

Methods: To understand the mechanism of this novel approach, hepatocellular carcinoma cells were exposed to 27.12 MHz radiofrequency electromagnetic fields using *in vitro* exposure systems designed to replicate *in vivo* conditions. Cancer cells were exposed to tumor-specific modulation frequencies, previously identified by biofeedback methods in patients with a diagnosis of cancer. Control modulation frequencies consisted of randomly-chosen modulation frequencies within the same 100 Hz to 21 kHz range as cancer-specific frequencies.

Results: The growth of hepatocellular carcinoma and breast cancer cells was significantly decreased by hepatocellular carcinoma-specific and breast cancer-specific modulation frequencies, respectively. However, the same frequencies did not affect proliferation of nonmalignant hepatocytes or breast epithelial cells. Inhibition of hepatocellular carcinoma cell proliferation was associated with downregulation of *XCL2* and *PLP2*. Furthermore, hepatocellular carcinoma-specific modulation frequencies disrupted the mitotic spindle.

Conclusion: These findings uncover a novel mechanism controlling the growth of cancer cells at specific modulation frequencies without affecting normal tissues, which may have broad implications in oncology.

Keywords: Hepatocelullar carcinoma, electromagnetic fields, mitotic spindle, *PLP2*, *XCL2*,

Introduction

Treatment of hepatocellular carcinoma (HCC) is a major challenge given the limited number of therapeutic options available (Thomas *et al,* 2005). We have developed a novel approach to treat advanced hepatocellular carcinoma, consisting of intrabuccal administration of very low levels of radiofrequency electromagnetic fields, amplitude-modulated at specific frequencies (RF EMF), and identified using biofeedback methods in patients with cancer (Barbault *et al.,* 2009). The encouraging findings from a feasibility study (Barbault *et al.,* 2009) led to the design of a phase I/II trial in patients with advanced hepatocellular carcinoma, and objective responses assessed by CT-scan and changes in alpha-fetoprotein levels were observed in several patients with biopsy-proven hepatocellular carcinoma (Costa *et al.,* 2011). These findings prompted us to initiate reverse translational experiments to investigate the mechanism of action of amplitude-modulated electromagnetic fields. Two different *in vitro* exposure systems operating at 27.12 MHz were used to expose cells in culture, replicating patient treatment conditions.

Proliferation of both HepG2 and Huh7 hepatocellular carcinoma cells was significantly decreased upon exposure to radiofrequency electromagnetic fields, which were modulated at hepatocellular carcinoma-specific modulation frequencies. To determine how such frequencies modulate cancer cell growth, we assessed differential gene expression with RNA-seq and found that the expression of several genes was significantly downregulated by hepatocellular carcinoma-specific modulation frequencies. Previous reports have shown that low intensity, intermediate frequency, electric fields are capable of inhibiting cancer growth by interfering with the proper formation of the mitotic spindle (Kirson *et al.,* 2004;Kirson *et al.,* 2007). Similarly, we found that electromagnetic fields

that are amplitude-modulated at hepatocellular carcinoma-specific frequencies disrupt the mitotic spindle of hepatocellular carcinoma cells. Thus, we provide novel evidence that very low level of amplitude-modulated electromagnetic fields block the growth of hepatocellular carcinoma cells in a tumor- and tissue-specific fashion.

MATERIALS AND METHODS

In vitro exposure devices

The design and construction of the two *in vivo* exposure devices (Figure 1) used to conduct these experiments is described in the Supplementary Information.

Cell Lines

HepG2 and Huh7 cells, both Biosafety Level 1, were used as representative hepatocellular carcinoma cell lines. HepG2 cells were obtained from ATCC (Manassas, VA), and Huh7 cells were a gift from Dr. Nareej Saxena (Emory University). Normal hepatocytes, THLE-2 cells, were also obtained from ATCC (Manassas, VA). The breast adenocarcinoma cell line MCF-7 was used as a representative non-hepatocellular carcinoma malignant cell line (ATCC; Manassas, VA). The breast epithelial cell line MCF-10A (ATCC; Manassas, VA) was used to represent normal breast cells. Lymphoblastoid cell lines from healthy individuals enrolled in IRB-approved protocols were provided by Dr. Jeff Edberg (UAB).

[^{3}H]thymidine incorporation assay

Growth inhibition was assessed in HCC cells exposed to HCC-specific modulation frequencies as previously described (Rosman *et al.*, 2008).

Luminescent Cell Viability Assay

Cell proliferation was quantitated using the Promega CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), a method to determine the number of viable cells in culture based on ATP quantitation.

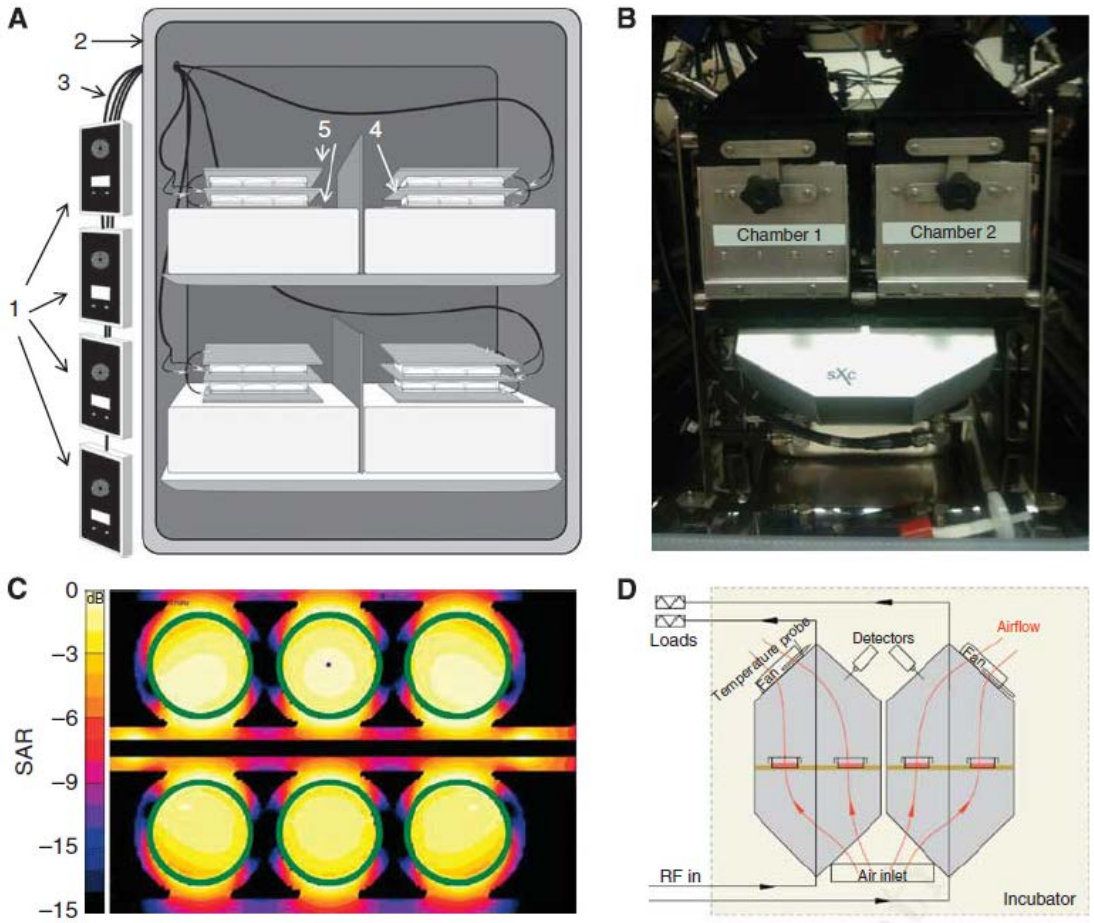

Figure 1. ***In vitro*** **exposure experimental setups. (A)** Parallel plate capacitor. Emitting devices (1) are placed outside the incubator (2). Each device is connected to a coaxial cable (3), which is connected to a set of brass plates inside the incubator. The center brass plate (4) is connected to the inner conductor of the emitting device coaxial cable. The outer two brass plates (5) are connected to the outer conductor of the emitting device coaxial cable. Plates containing cells are placed in between the brass plates. **(B)** Transverse electromagnetic (TEM) cell. The system contains two identical TEM cells placed in an incubator. **(C)** Distribution of the Specific Absorption Rate (SAR) of cell monolayer (1 dB per contour), (D) Schematic showing the air flow through the TEM cell.

RNA-seq

We performed RNA-seq as previously described (Reddy *et al.*, 2009). We used HepG2 cells exposed to either HCC-specific modulation frequencies or to randomly-chosen frequencies. We double-selected polyA-containing mRNA from 3 µg of total RNA by using oligo-dT magnetic beads. We fragmented the mRNA with RNA fragmentation buffer and removed free-ions with a G-50 Sepharose spin column. Fragmented mRNA was used as a template to synthesize single-stranded cDNA with SuperScript II reverse transcriptase with random hexamer primers in the presence of RNAseOUT (Invitrogen). We synthesized double-stranded DNA (dsDNA). To prepare dsDNA for sequencing, we ligated Illumina sequencing adapters to blunted and dA extended dsDNA and size selected fragments of 200-300 bp from a 2% Invitrogen gel and purified with a Qiagen Gel Extraction kit. Lastly, we amplified the dsDNA library with 15 rounds of PCR with Illumina sequencing primers. Sequencing was performed on an Illumina GenomeAnalyzer IIx and the paired 36bp reads were mapped to the hg18 reference genome by using ELAND (Illumina), allowing up to two mismatches per read, and 10 or fewer map locations. By using the ERANGE software package, we placed uniquely mapped reads against 29,673 transcripts from NCBI build 36.1 of the human genome. After placing unique reads, ERANGE assigned multiply mapping reads and reads mapping to splice junctions according to the number of unique reads in potential transcripts. Once all reads were mapped, ERANGE reported gene expression in units of reads per kilobase of exon and per million tags sequenced (RPKM).

Quantitative PCR (qPCR)

At the conclusion of the AM-EMF exposure portion of the experiment, RNA extraction (Qiagen, Valencia, CA) and reverse transcription (TaqMan, Applied Biosystems by Lite Technologies Corporation, Carlsbad, California) was performed to generate cDNA. Experiments comparing gene expression in HCC cells receiving HCC-specific AM-EMF to gene expression in HCC cells not receiving any exposure were conducted using Applied Biosystems pre-designed TaqMan Gene Expression Assays (*PLP2,* Cat# Hs01099969_g1; *XCL2,* Cat# Hs00237019_m1; Applied Biosystems by Lite Technologies Corporation, Carlsbad, California). Real-Time quantitation was completed out in quadruplicate according to the manufacturer's instructions using an ABI 7900HT Real-Time PCR System, with analysis performed using ABI SDS2.2 software. Quantitative values of gene expression were determined by comparing PCR amplification curves to a known standard curve generated in tandem with the experimental samples. Each sample was individually normalized to the average corresponding to endogenous expression of *GAPDH* (*GAPDH,* Cat# Hs99999905_m1). Averages of the normalized values from each condition were then used to compare the relative gene expression between the experimental groups. The standard error of the mean (SEM) was determined for each experimental condition.

Confocal Laser Scanning Microscopy

Cells undergoing mitosis were imaged using the Zeiss LSM 710 Confocal Laser Scanning Microscope (Carl Zeiss, Inc., Thornwood, NY). For imaging experiments, 22 mm square microscope cover glass (Corning Life Sciences, Lowell, MA, Cat. #2865-22) were flame-sterilized with 200-proof ethanol and placed in 6-well or 35 mm Falcon tissue cul-

ture plates. Approximately 300uL of cell suspension/growth media was added directly to the top of the cover slips, and cells were plated at varying concentrations ($4x10^5 – 5x10^5$ cells/mL) on separate cover slips for each assay to control for variability in antibody affinity between different experiments. Once the cells were given 8-18 hours to attach to the cover slips, 3 mL of complete growth media was added to each well containing a cover slip. Following RF EMF exposure, indirect immunofluorescent microscopy compared cells receiving HCC-specific modulation frequencies with cells not receiving any exposure (Microtubule Marker (AE-8) sc-73551, Fluorescent Secondary Alexa Fluor® 488 goat anti-mouse IgG (H+L): A-11001; Santa Cruz Biotechnologies).

Karyotype Analysis

To determine whether these changes were associated with karyotypic changes, HepG2 cells exposed to HCC-specific modulation frequencies or unexposed were harvested, slides prepared, and metaphase chromosomes G-banded using standard methods. The chromosomes were analyzed and the karyotype described according to the International System for Cytogenetic Nomenclature (Brothman *et al*, 2009).

Statistical Analyses

One sample 2-sided t test was performed to test the significance of cell proliferation exposed to radiofrequency electromagnetic fields amplitude-modulated at tumor-specific or randomly-chosen frequencies. ANCOVA analysis: For the long-term (7 week) growth inhibition analysis and the growth inhibition analysis for varying SAR values (0.05 W/kg, 0.1 W/kg, 0.4 W.kg, and 1.0 W/kg), data was fit to a linear model and time point and

dosage level, respectively, were considered as covariates in determining significance.

RESULTS

Assessment of cell proliferation in the presence of RF EMF

Cell proliferation assays were conducted after 7 days, i.e. 21 hours of exposure to amplitude-modulated RF EMF. Treatment with HCC-specific modulation frequencies (Suppl. Table 1) significantly reduced the proliferation of HepG2 and Huh7 cells using both the parallel plate capacitor and the transverse electromagnetic (TEM) setups (Figure 1). The observed growth inhibitory effect on HepG2 cells was of the same magnitude when using a tritium incorporation assay and a bioluminescence assay based on ATP consumption (Figure 2A). Having shown similar results with two different assays, the remainder of the cell proliferation experiments were conducted with the more commonly used tritium incorporation assay. Cell proliferation of HepG2 and Huh7 cells exposed to HCC-specific modulation frequencies was significantly lower than the proliferation of cells exposed either to randomly-chosen frequencies (Suppl. Table 2) or not exposed to RF EMF (Figure 2A, columns 1-3). When HepG2 cells were exposed for only one hour daily, we did not observe any significant inhibition of cell proliferation (Figure 2B). Daily exposure for 6 instead of 3 hours resulted in the same level of cell proliferation inhibition (Figure 2B). To determine when HCC-specific modulation frequencies begin to exert anti-proliferative effects on HepG2 cells, we assessed cell proliferation following three days (9 hours) of exposure and did not find any significant difference between cells exposed to HCC-specific modulation frequencies and unexposed cells (Figure 2B).

To determine further whether the growth inhibitory effect of HCC-specific modulation frequencies persists over time and results in a decrease in the total number of tumor cells, we counted the number of HepG2 cells following treatment with HCC-specific modulation frequencies and that of untreated HepG2 cells weekly for up to seven weeks. Cells that were either exposed to HCC-specific modulation frequencies or not exposed were split weekly at the same ratio over a period of seven weeks. As shown in Figure 2C, when compared to unexposed HepG2 cells, the number of HepG2 cells following exposure to HCC-specific modulation frequencies decreased steadily over 7 weeks, resulting in a cumulative loss of 1.71×10^6 cells/mL at week 7.

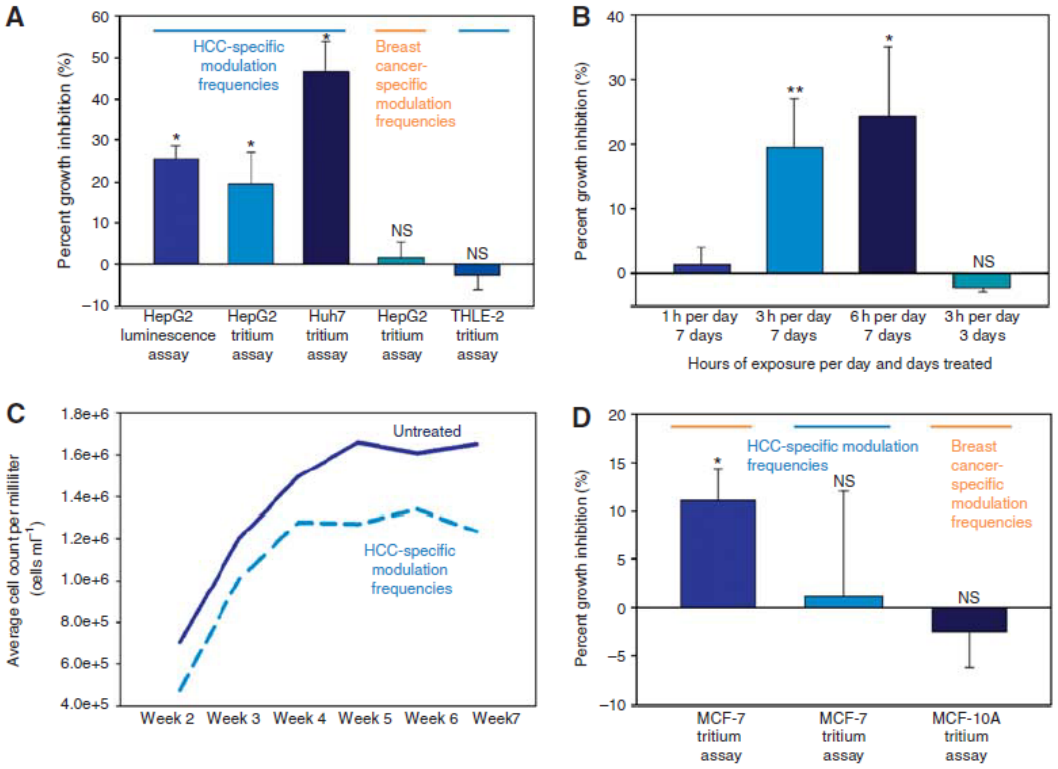

Figure 2. Cell proliferation assays of cell lines exposed to HCC-specific or breast cancer-specific modulation frequencies. (A) Cells were not split after initial seeding; medium was exchanged every 48 hours. Experiments were performed with both equipment setups. Left to right columns: 1: HepG2 cells exposed to HCC-specific modulation frequencies with growth inhibition (GI) evaluated with a luminescence assay, 25.46±3.22% GI (p=0.0009). 2: HepG2 cells exposed to HCC-specific modulation frequencies with GI evaluated using tritium incorporation, 19.44±7.60% GI (p=0.00993). : Huh7 cells exposed to HCC-specific modulation frequencies, 47.73±7.14% GI (p=0.018). 4: HepG2 cells are not significantly inhibited when exposed to breast cancer-specific modulation frequencies, 1.49±3.99% GI (p=0.8815). 5: THLE-2 cells are not affected by HCC-specific modulation frequencies, -2.54±3.54% GI (p=0.6550). Values represent average percent growth inhibition (n=6) ± percent standard error (%STERR). **(B)** Cell proliferation assays exposing cells for varying hours per day. Left to right: 1 hour/day 1.36±2.77% (p=0.8508); 3 hours/day 19.44±7.60% (p=0.0099); 6 hours/day 24.46±10.75% (p=0.0301); 3 hours/day for three days -2.12±0.66% (p =0.4067). Values represent average percent growth inhibition (n=6) ± %STERR. **(C)** Cumulative decrease in cell counts over time when HepG2 cells are exposed to HCC-specific modulation frequencies. Samples were subcultured by volume every 7 days (1:20 split by volume). Average total cells/mL per week: week 2: $7.07x10^5$, $4.75x10^5$; Week 3: $1.20x10^6$, $1.01x10^5$; Week 4: $1.50x10^5$, $1.28x10^5$; week 5: $1.66x10^5$, $1.22x10^5$; week 6: $1.61x10^6$, $1.34x10^6$; week 7: $1.65x10^6$, $1.24x10^6$ for untreated and treated samples, respectively. For the duration of the 7 week experiment with time considered as a covariate: p= 0.005751. **(D)** Left to right columns: 1: MCF-7 cells exposed to breast tumor-specific modulation frequencies, 11.08±3.30% GI (p=0.0230). 2: MCF-7 cells are not significantly inhibited when exposed to hepatocellular carcinoma-specific modulation frequencies, 1.49±3.99% (p=0.8815) GI, respectively. 3: MCF-10A cells are not affected by breast tumor-specific

modulation frequencies, -2.46±3.75% GI (p=0.8579). Values represent average percent growth inhibition (n=6) ± percent standard error (%STERR).

The average Specific Absorption Rate (SAR) for cells exposed in the parallel capacitor plate system is 0.03 W/kg (Suppl. Information). All initial experiments conducted with the TEM system were conducted at a SAR of 0.4 W/kg. To determine the range of SARs within which significant growth inhibition was observed, additional cell proliferation experiments were performed at 0.05, 0.1 and 1.0 W/kg. A significant antiproliferative effect was observed at all SARs ranging from 0.05 to 1.0 W/kg ($p = 0.0354$). All subsequent assays with the TEM system were conducted at an SAR of 0.4 W/kg.

Inhibition of cell proliferation is tumor and tissue-specific

Our previous clinical observations revealed that patients with hepatocellular carcinoma had biofeedback responses to specific modulation frequencies that were different from those identified in patients with other types of cancer such as breast cancer (Barbault *et al*, 2009). To experimentally assess the relevance of these findings on the proliferation of tumor cells, we determined the specificity of frequencies identified in patients with these two tumor types given the documented objective clinical responses that included one complete and one partial response in two patients with metastatic breast cancer (Barbault *et al*, 2009) and three partial and one near-complete responses in four patients with hepatocellular carcinoma (Costa *et al.*, 2011). A total of 194 breast cancer-specific modulation frequencies ranging in the same modulation frequency band from 100 Hz to 21 kHz have been identified in patients with a diagnosis of breast cancer (Suppl. Table 3). Nine (4.6%) of the HCC-specific modulation frequencies are identical to breast cancer-specific modulation frequencies.

The two patients with metastatic breast cancer who had experienced an objective

response to breast cancer-specific modulation frequencies had tumors that over-expressed estrogen receptor (ER+) and progesterone receptor (PR+) but did not over-express ERBB2 (ERBB2-) (Barbault *et al,* 2009). We therefore chose the MCF-7 cell line as it represents the same tumor phenotype, i.e. ER+, PR+, ERBB2-. While the growth of MCF-10A breast cells was unaffected by exposure to breast cancer-specific modulation frequencies, exposure of MCF-7 breast cancer cells to breast cancer-specific modulation frequencies significantly inhibited cell proliferation (Figure 2D). However, exposure of HepG2 cells to the same breast cancer-specific modulation frequencies did not affect cell proliferation (Figure 2A). Similarly, the proliferation of MCF-7 cells was not affected by exposure to HCC-specific modulation frequencies (Figure 2D). Consequently, the observed antiproliferative effect on hepatocellular carcinoma and breast cancer cells was observed only upon exposure to tumor-specific modulation frequencies previously identified in patients with a diagnosis of hepatocellular carcinoma and breast cancer, respectively, despite the fact that more than 57% of the modulation frequencies only differed by less than 1% (Suppl. Table 1 and 3).

Having demonstrated that the antiproliferative effect of amplitude-modulated frequencies was tumor specific, we sought to determine whether the HCC-specific modulation frequencies have an effect on the proliferation of THLE-2 normal hepatocytes. As shown in Figure 2A, exposure of THLE-2 cells to HCC-specific modulation frequencies did not have any measurable effect on cell proliferation. These findings provide strong support for the novel notion that a combination of narrowly-defined, specific modulation frequencies identified in a group of patients with the same type of cancer is capable of inhibiting cell proliferation in a tumor- and tissue-specific fashion.

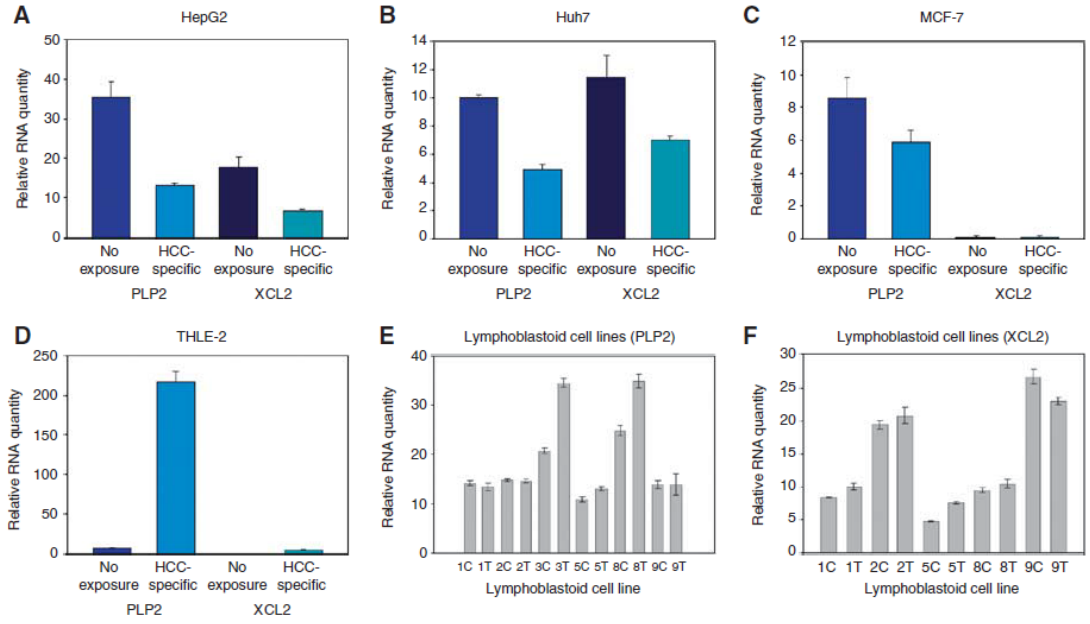

Figure 3. Expression of *XCL2* and *PLP2* receiving HCC-specific RF EMF compared to cells not receiving exposure. (A) HepG2: *PLP2* (35.46±3.85; 13.17±0.70) and *XCL2* (17.87±2.49; 6.52±0.48) (p= 9.0371e-3 and p=0.0179, respectively). (B) Huh7: *PLP2* (10.02±0.19; 4.95±0.35) and *XCL2* (11.52±1.49; 7.02±0.29) (p=9.4981e-5 and p=0.0536, respectively). (C) MCF-7: *PLP2* (8.52±1.30; 5.84±0.77) and *XCL2* (levels not detectable). (D) THLE-2: *PLP2* (7.11±0.14; 216.89±13.18) and *XCL2* (0.03±0.01; 4.55±1.04) in THLE-2 cells exposed to HCC-specific modulation frequencies (p=5.5108e-4 and p=0.0221, respectively). (E) Expression levels of *PLP2* in lymphoblastoid cell lines (C=unexposed; T=HCC-specific exposure) (for all cell lines compiled p=0.418), LCL 3 expression was significant p=0.0021 as was LCL8 p=0.0159 (F) Expression levels of *XCL2* in lymphoblastoid cell lines (for all cell lines compiled (p=0.899), LCL 1 expression difference was significant p=0.0002. Values represent average relative RNA expression (n=4) ± s.e.m. Levels were normalized to levels of GAPDH.

Tumor-specific modulation frequencies and gene regulation

To study the mechanism by which tumor-specific modulation frequencies inhibit cell proliferation, we assessed the gene expression profile of HepG2 cells exposed to HCC-specific modulation frequencies using RNA-seq as it provides a more comprehensive assessment of differential gene expression across a broader range of expression levels than does microarray-based analysis (Wang *et al,* 2009). Overall, we did not observe statistically significant differences in transcript levels when comparing two HepG2 cultures exposed for one week, 3 hr a day to HCC-specific modulation frequencies to two HepG2 cultures exposed to randomly-chosen modulation frequencies (Suppl. Fig. 1). However, we did observe a small number of genes with an absolute fold-change >1.5 and a minimum mean RPKM of 1.5 following exposure to HCC-specific modulation frequencies. Two genes with an absolute fold-change >1.8 appeared to be downregulated in HepG2 cells exposed to HCC-specific modulation frequencies, *PLP2* and *XCL2,* and were considered to be candidates worthy of further experiments. We validated with qPCR the downregulation of *PLP2* and *XCL2* in both HepG2 as well as Huh7 cells exposed to HCC-specific modulation frequencies (Figure 3A and 3B). There was no significant downregulation of *PLP2* and *XCL2* in MCF-7 breast cancer cells (Figure 3C). Similarly, there was no downregulation of *PLP2* and *XCL2* in nonmalignant cells, i.e. in THLE-2 normal hepatocytes (Figure 3D), or in lymphoblastoid cell lines from healthy individuals (Figure 3E and 3F). These findings support the novel notion that the demodulation effects of RF EMF amplitude-modulated at specific frequencies inhibit cell proliferation and affect the expression of several genes in a tumor- and tissue-specific fashion.

Tumor-specific modulation frequencies and disruption of the mitotic spindle

There is evidence that the proliferation of several rodent and human cancer cell lines is arrested by exposure to sinusoidal electric fields of 100 – 200 V/m at a frequency of 100-300 kHz (Kirson *et al,* 2004). This approach has also shown efficacy in animal human tumor models as well as promising results in the treatment of patients with cancer (Kirson *et al.*, 2004;Kirson *et al.*, 2007;Salzberg *et al.*, 2008;Kirson *et al.*, 2009). The antitumor effect of this therapeutic approach appears to be caused by disruption of the mitotic spindle mediated by interference of spindle tubulin orientation and induction of dielectrophoresis (Kirson *et al.*, 2004;Kirson *et al.*, 2007). In contrast to the sinusoidal signals (Kirson *et al,* 2004), the carrier frequency of the signal applied in our experiments is more than hundred times higher, the peak E-field amplitude of the carrier at 0.4 W/kg corresponds to approximately 35 V/m inside the cell medium when the signal is sinusoidally amplitude-modulated at specific frequencies with 85% modulation depth (Kirson *et al,* 2004).

Despite these significant differences, confocal laser scanning microscopy revealed pronounced disruption of the mitotic spindle in more than 60% of HepG2 cells exposed for one week, 3 hr per day to HCC-specific modulation frequencies while there was no disruption of the mitotic spindle in unexposed HepG2 cells (Figure 4A and 4B). Specifically, the observed cytoskeletal disruption in cells exposed to HCC-specific modulation frequencies was apparent in cells in mitosis, in which we saw centrosomal distortion and poor chromosomal separation at anaphase (Figure 4D). We found no evidence of karyotypic differences between HepG2 cells exposed to HCC-specific modulation frequencies and unexposed HepG2 cells.

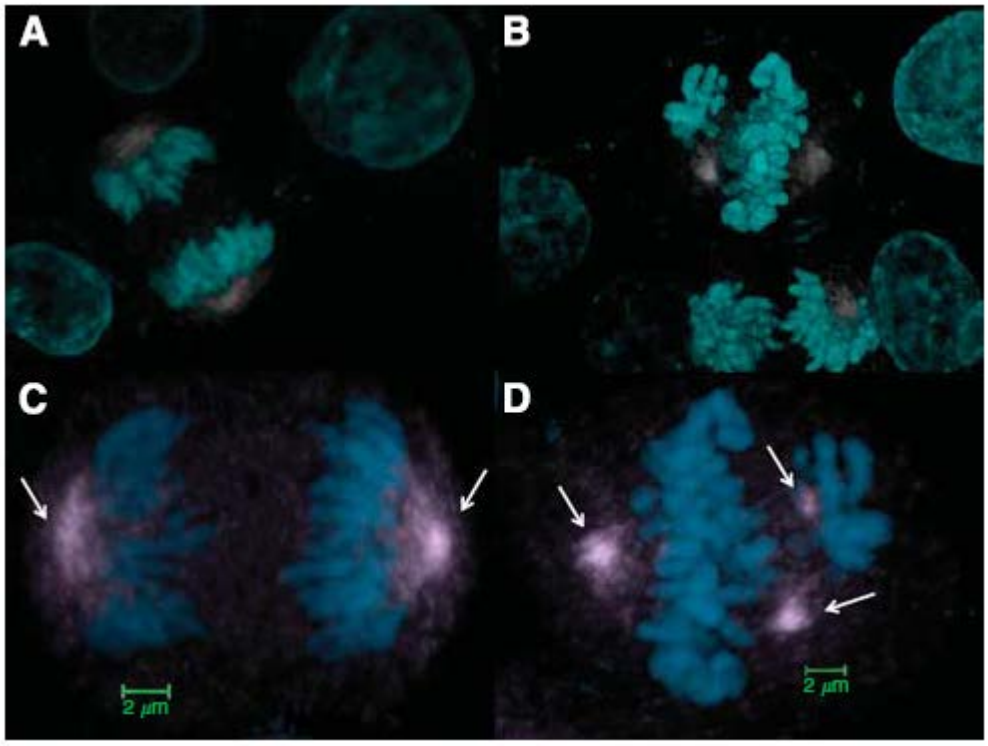

Figure 4. Mitotic spindle disruption in cells receiving HCC-specific RF EMF compared to cells not receiving exposure. (A) HepG2 efficiently assemble a bipolar mitotic spindle, allowing cells to pass through the mitotic assembly checkpoint and successfully progress from metaphase to anaphase, (B) More than 60% of dividing HepG2 cells exposed to HCC-specific modulation frequencies exhibit microtubule-associated anomalies, (C) high magnification of unexposed HepG2 cell in mitosis (D) high magnification of HepG2 cell exposed to HCC-specific modulation frequencies shows errors such as tripolar spindle formation. (Cyan=DAPI; Gray=Microtubules; Arrows=mitotic spindle)

DISCUSSION

By exposing hepatocellular carcinoma cells to 27.12 MHz RF EMF sinusoidally amplitude-modulated at specific frequencies, which were previously identified in patients with a diagnosis of HCC (Barbault *et al*, 2009) and result in therapeutic responses in patients with HCC (Costa *et al.*, 2011), we demonstrate a robust and sustained antiproliferative effect. This effect was seen within SARs ranging from 0.03 to 1.0 W/kg, i.e. within the range of exposure in humans receiving treatment administered intrabuccally (Barbault *et al.*, 2009;Costa *et al.*, 2011). HCC-specific modulation frequencies began to hinder cell proliferation after 7 days of exposure and the antiproliferative effect increased over a 7 week period. The antiproliferative effect HCC-specific modulation frequencies was only observed in HCC cells, not in breast cancer cells or normal hepatocytes.

The specificity of modulation frequencies is exemplified by the fact that two sets of similar modulation frequencies (breast cancer-specific and randomly-chosen) within the same range, i.e. from 100 Hz to 21 kHz, did not affect the proliferation of hepatocellular carcinoma cells. Similarly, the proliferation of breast cancer cells was only affected by breast cancer-specific modulation frequencies, neither by hepatocellular carcinoma-specific nor by randomly-chosen modulation frequencies. The fact that more than 50% of the modulation frequencies from these three programs differed by less than 1% provides strong experimental evidence that the biological effects are only mediated by a combination of narrowly defined, tumor-specific modulation frequencies.

The modulation-frequency specific laboratory findings are consistent with the clinical observation of a complete response in a patient with breast cancer metastatic to the adrenal gland and the bone while, during the same time, a primary malignancy of the

uterus continued to grow (Barbault *et al,* 2009). This suggests that a combination of precise tumor-specific modulation frequencies is needed to block cancer growth *in vitro* and in patients with a diagnosis of cancer. The clinical results reported by Barbault et al. (Barbault *et al.*, 2009) and Costa et al. (Costa *et al.*, 2011) as well as laboratory evidence included in this report provide support for the novel and transformational concept that the growth of human tumors arising from the same primary tissue may be effectively blocked by identical modulation frequencies. Three White and one Black patients with advanced had partial responses while receiving treatment with HCC-specific modulation frequencies (Costa *et al.*, 2011). Furthermore, proliferation of the Huh7 hepatocellular carcinoma cell line, which is derived from a Japanese patient's tumor (Nakabayashi *et al,* 1982), exhibited the most pronounced response to HCC-specific modulation frequencies (Figure 2A). This indicates that the frequency signature and biological effects of HCC-specific modulation frequencies are likely independent of ethnic status.

There is no known biophysical mechanism accounting for the effect observed in these experiments; however, other modulation frequency dependent effects have been observed in biological systems at similarly low exposure levels. Documented effects have occurred in cellular processes controlling cell growth, proliferation, and differentiation (Blackman, 2009). Further, modulation of the signal appears to be a critical factor in the response of biological systems to electromagnetic fields (Blackman, 2009). The amount of electromagnetic energy delivered is far too low to break chemical bonds or cause thermal effects, necessitating alternative mechanistic explanations for observed biologic outcomes. Several theories have been put forth to explain biologic responses to electromagnetic fields. Some reports have shown that low levels of electromagnetic fields can

alter gene expression and subsequent protein synthesis by interaction of the electromagnetic field with specific DNA sequences within the promoter region of genes (Blank *et al*., 2008; Blank *et al.,* 2009). Such changes have been demonstrated in the family of "heat shock" proteins that function in the cell stress response (Blank *et al.,* 2009).

To thoroughly interrogate gene expression changes in cells exhibiting decreased cell proliferation, we used high-throughput sequencing technologies in order to sequence the cells' cDNA, a technique that has become invaluable in the study of cancer (Maher *et al.*, 2009). Tumor cell growth inhibition was associated with downregulation of *PLP2* and *XCL2* as well as with disruption of the mitotic spindle. *PLP2* encodes an integral membrane protein that localizes to the endoplasmic reticulum in epithelial cells. The encoded protein can multimerize and may function as an ion channel (Breitwieser *et al,* 1997). *PLP2* enhances chemotaxis of human osteogenic sarcoma cells (Lee *et al,* 2004), and *PLP2* downregulation is associated with decreased metastasis in a mouse model of cancer (Sonoda *et al,* 2010). *XCL2* encodes for a protein that enhances chemotactic activity for lymphocytes and downregulation of *XCL2* has been shown to be associated with good prognosis in patients with breast cancer (Teschendorff *et al.,* 2007;Teschendorff and Caldas, 2008). The pronounced disruption of the mitotic spindle seen in the majority of HepG2 cells exposed to HCC-specific modulation frequencies undergoing mitosis is not associated with karyotypic changes but may be a major determinant of the antitumor effects of HCC-specific modulation frequencies accounting for the therapeutic responses seen in patients receiving the same modulation frequencies (Costa *et al.,* 2011).

Exposure of hepatocellular carcinoma cells to the same RF EMF modulated at slightly different modulation frequencies did not result in changes in gene expression,

which demonstrates that inhibition of cell proliferation is associated with changes in gene expression levels.

In conclusion, we show that very low level of 27.12 MHz radiofrequency electromagnetic fields, which are comparable to the levels administered to patients, inhibit tumor cell growth when modulated at specific frequencies. These exciting findings presented in this report suggest that the anti-proliferative effect of modulation frequencies is both tumor- and tissue-specific and is mediated by changes in gene expression as well as disruption of the mitotic spindle. These findings uncover a new alley to control tumor growth and may have broad implications for the treatment of cancer.

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Supplementary Table 1
HCC-specific modulation frequencies

Number	Frequency
1	410.231 Hz
2	423.321 Hz
3	427.062 Hz
4	470.181 Hz
5	560.32 Hz
6	642.932 Hz
7	655.435 Hz
8	657.394 Hz
9	668.209 Hz
10	677.972 Hz
11	728.232 Hz
12	806.021 Hz
13	811.924 Hz
14	842.311 Hz
15	843.22 Hz
16	891.901 Hz
17	1250.504 Hz
18	1755.402 Hz
19	1873.477 Hz
20	1924.702 Hz
21	1975.196 Hz
22	2017.962 Hz
23	2053.396 Hz
24	2083.419 Hz
25	2190.731 Hz
26	2221.323 Hz
27	2324.393 Hz
28	2353.478 Hz
29	2362.309 Hz
30	2419.309 Hz
31	2425.222 Hz
32	2430.219 Hz
33	2431.094 Hz
34	2471.328 Hz
35	2478.331 Hz
36	2480.191 Hz

37	2522.328 Hz
38	2743.995 Hz
39	2744.211 Hz
40	2831.951 Hz
41	2843.283 Hz
42	2859.891 Hz
43	2873.542 Hz
44	2886.232 Hz
45	3042.012 Hz
46	3078.983 Hz
47	3086.443 Hz
48	3127.232 Hz
49	3160.942 Hz
50	3161.331 Hz
51	3206.315 Hz
52	3255.219 Hz
53	3267.433 Hz
54	3269.321 Hz
55	3281.432 Hz
56	3457.291 Hz
57	3505.229 Hz
58	3516.296 Hz
59	3530.188 Hz
60	3531.296 Hz
61	3546.323 Hz
62	3572.106 Hz
63	3576.189 Hz
64	3669.513 Hz
65	3923.221 Hz
66	3927.331 Hz
67	4013.932 Hz
68	4071.121 Hz
69	4079.951 Hz
70	4123.953 Hz
71	4161.889 Hz
72	4222.821 Hz
73	4238.402 Hz
74	4256.321 Hz
75	4289.296 Hz

76	4312.947 Hz
77	4375.962 Hz
78	4426.387 Hz
79	4435.219 Hz
80	4471.188 Hz
81	4483.889 Hz
82	4486.384 Hz
83	4556.322 Hz
84	4629.941 Hz
85	4732.211 Hz
86	4876.218 Hz
87	5086.281 Hz
88	5124.084 Hz
89	5133.121 Hz
90	5247.142 Hz
91	5270.834 Hz
92	5340.497 Hz
93	5520.218 Hz
94	5570.234 Hz
95	5882.292 Hz
96	5926.512 Hz
97	6037.311 Hz
98	6180.334 Hz
99	6329.195 Hz
100	6350.333 Hz
101	6361.321 Hz
102	6364.928 Hz
103	6383.321 Hz
104	6461.175 Hz
105	6733.331 Hz
106	6758.232 Hz
107	6779.482 Hz
108	6856.222 Hz
109	6877.183 Hz
110	6915.886 Hz
111	6980.525 Hz
112	7019.235 Hz
113	7043.209 Hz
114	7130.323 Hz

115	7144.142 Hz
116	7210.223 Hz
117	7291.21 Hz
118	7482.245 Hz
119	7510.92 Hz
120	7529.233 Hz
121	7549.212 Hz
122	7650.028 Hz
123	7680.518 Hz
124	7692.522 Hz
125	7829.231 Hz
126	7862.209 Hz
127	7932.482 Hz
128	7935.423 Hz
129	7947.392 Hz
130	7979.308 Hz
131	8028.339 Hz
132	8055.942 Hz
133	8072.134 Hz
134	8141.174 Hz
135	8336.383 Hz
136	8394.793 Hz
137	8432.181 Hz
138	8452.119 Hz
139	8460.944 Hz
140	8475.221 Hz
141	8492.193 Hz
142	8542.311 Hz
143	8818.104 Hz
144	8852.329 Hz
145	8853.444 Hz
146	8858.179 Hz
147	8939.212 Hz
148	9332.397 Hz
149	9381.221 Hz
150	9719.314 Hz
151	9740.219 Hz
152	9768.331 Hz
153	9797.294 Hz

154	9819.511 Hz
155	10317.499 Hz
156	10438.495 Hz
157	10443.311 Hz
158	10456.383 Hz
159	10579.425 Hz
160	10863.209 Hz
161	10866.382 Hz
162	11067.418 Hz
163	11149.935 Hz
164	11163.895 Hz
165	11802.821 Hz
166	11953.424 Hz
167	12223.329 Hz
168	12260.933 Hz
169	12265.295 Hz
170	12267.233 Hz
171	12623.191 Hz
172	12633.372 Hz
173	12685.231 Hz
174	12721.423 Hz
175	12785.342 Hz
176	13433.323 Hz
177	14085.222 Hz
178	14333.209 Hz
179	14537.331 Hz
180	14542.432 Hz
181	14655.03 Hz
182	14828.234 Hz
183	15149.213 Hz
184	15237.489 Hz
185	15717.221 Hz
186	16110.932 Hz
187	16144.343 Hz
188	18265.238 Hz
189	18283.323 Hz
190	18863.292 Hz
191	18930.995 Hz
192	19970.311 Hz

193	20330.294 Hz
194	20365.284 Hz
195	

Supplementary Table 2
Randomly-chosen modulation frequencies

Number	Frequency
1	504.288 Hz
2	601.388 Hz
3	758.079 Hz
4	801.055 Hz
5	1579.861 Hz
6	1686.129 Hz
7	1732.231 Hz
8	1777.676 Hz
9	1816.165 Hz
10	1999.19 Hz
11	2060.824 Hz
12	2117.172 Hz
13	2118.13 Hz
14	2126.078 Hz
15	2172.715 Hz
16	2177.378 Hz
17	2182.699 Hz
18	2230.497 Hz
19	2270.095 Hz
20	2291.76 Hz
21	2409.804 Hz
22	2443.455 Hz
23	2573.901 Hz
24	2586.187 Hz
25	2588.825 Hz
26	2661.239 Hz
27	2667.744 Hz
28	2673.24 Hz
29	2674.203 Hz
30	2718.46 Hz
31	2726.164 Hz
32	2771.084 Hz
33	2774.56 Hz
34	2777.798 Hz

Each modulation frequency is at least 0.4 Hz higher or lower than any HCC-specific (Suppl. Table 1) or breast cancer-specific (Suppl. Table 3) modulation frequencies.

35	2814.508 Hz
36	2940.689 Hz
37	2942.388 Hz
38	3018.394 Hz
39	3018.632 Hz
40	3145.88 Hz
41	3154.706 Hz
42	3174.855 Hz
43	3177.169 Hz
44	3178.166 Hz
45	3191.69 Hz
46	3214.895 Hz
47	3218.57 Hz
48	3302.561 Hz
49	3346.213 Hz
50	3386.881 Hz
51	3395.087 Hz
52	3439.955 Hz
53	3477.036 Hz
54	3477.464 Hz
55	3526.946 Hz
56	3534.597 Hz
57	3612.538 Hz
58	3671.955 Hz
59	3800.538 Hz
60	3803.02 Hz
61	3814.561 Hz
62	3881.652 Hz
63	3883.206 Hz
64	3884.425 Hz
65	3900.955 Hz
66	4024.069 Hz
67	4035.5 Hz
68	4036.464 Hz
69	4037.745 Hz
70	4166.946 Hz
71	4577.652 Hz
72	4611.675 Hz
73	4619.808 Hz

74	4645.103 Hz
75	4662.242 Hz
76	4681.953 Hz
77	4718.225 Hz
78	4722.427 Hz
79	4743.75 Hz
80	4744.241 Hz
81	4806.447 Hz
82	4918.306 Hz
83	4932.277 Hz
84	4935.022 Hz
85	5013.21 Hz
86	5092.935 Hz
87	5205.432 Hz
88	5294.062 Hz
89	5333.86 Hz
90	5562.3 Hz
91	5594.909 Hz
92	5636.492 Hz
93	5646.433 Hz
94	5696.275 Hz
95	5725.188 Hz
96	5728.435 Hz
97	5771.856 Hz
98	5797.917 Hz
99	5874.546 Hz
100	6059.997 Hz
101	6072.239 Hz
102	6101.533 Hz
103	6127.257 Hz
104	6170.599 Hz
105	6189.464 Hz
106	6317.311 Hz
107	6338.888 Hz
108	6417.29 Hz
109	6429.727 Hz
110	6470.521 Hz
111	6489.2 Hz
112	6519.163 Hz

113	6562.089 Hz
114	6641.042 Hz
115	6794.29 Hz
116	6798.367 Hz
117	6885.025 Hz
118	6923.269 Hz
119	6962.114 Hz
120	6997.453 Hz
121	7001.42 Hz
122	7075.988 Hz
123	7107.292 Hz
124	7181.377 Hz
125	7250.347 Hz
126	7264.92 Hz
127	7327.056 Hz
128	7406.664 Hz
129	7434.055 Hz
130	7457.43 Hz
131	7502.744 Hz
132	7588.962 Hz
133	7606.907 Hz
134	7614.849 Hz
135	7642.973 Hz
136	7721.158 Hz
137	7747.529 Hz
138	7972.361 Hz
139	8045.29 Hz
140	8262.012 Hz
141	8282.156 Hz
142	8304.133 Hz
143	8399.157 Hz
144	8415.03 Hz
145	8443.098 Hz
146	8612.154 Hz
147	8648.429 Hz
148	8728.965 Hz
149	8742.131 Hz
150	8791.497 Hz
151	8817.564 Hz

152	8831.172 Hz
153	8837.559 Hz
154	8879.347 Hz
155	8955.291 Hz
156	9170.021 Hz
157	9234.272 Hz
158	9285.749 Hz
159	9297.33 Hz
160	9307.038 Hz
161	9322.743 Hz
162	9341.412 Hz
163	9410.744 Hz
164	9521.482 Hz
165	9699.914 Hz
166	9776.888 Hz
167	9779.743 Hz
168	9833.133 Hz
169	9960.676 Hz
170	10034.941 Hz
171	10047.505 Hz
172	10265.636 Hz
173	10622.538 Hz
174	10714.24 Hz
175	10745.164 Hz
176	10758.081 Hz
177	10777.023 Hz
178	10801.462 Hz
179	11000.996 Hz
180	11038.765 Hz
181	11040.509 Hz
182	11349.248 Hz
183	11360.871 Hz
184	11416.707 Hz
185	11449.28 Hz
186	11487.642 Hz
187	11502.021 Hz
188	11506.807 Hz
189	11637.367 Hz
190	11953.02 Hz

191	12089.99 Hz
192	12174.159 Hz
193	12280.284 Hz
194	12676.699 Hz
195	12899.888 Hz
196	12983.103 Hz
197	13083.597 Hz
198	13088.188 Hz
199	13148.766 Hz
200	13189.52 Hz
201	13345.41 Hz
202	13445.661 Hz
203	13491.824 Hz
204	13493.956 Hz
205	13634.291 Hz
206	13713.74 Hz
207	13885.51 Hz
208	13953.754 Hz
209	14063.876 Hz
210	14145.498 Hz
211	14499.838 Hz
212	14529.908 Hz
213	14776.655 Hz
214	15493.744 Hz
215	15766.529 Hz
216	15991.064 Hz
217	15996.675 Hz
218	16120.533 Hz
219	16311.057 Hz
220	16438.714 Hz
221	16454.99 Hz
222	16664.134 Hz
223	16761.237 Hz
224	16976.962 Hz
225	17037.895 Hz
226	17288.9437 Hz
227	17722.025 Hz
228	17758.777 Hz
229	18297.264 Hz

230	18328.755 Hz
231	20091.761 Hz
232	20111.655 Hz
233	20749.621 Hz
234	20824.094 Hz
235	21967.342 Hz
236	22363.323 Hz
237	22401.812 Hz
238	

Supplementary Table 3
Breast tumor-specific modulation frequencies

Number	Frequency
1	181.821 Hz
2	414.817 Hz
3	430.439 Hz
4	628.431 Hz
5	655.435 Hz
6	677.972 Hz
7	721.313 Hz
8	752.933 Hz
9	813.205 Hz
10	818.342 Hz
11	825.145 Hz
12	839.521 Hz
13	841.211 Hz
14	843.312 Hz
15	891.901 Hz
16	929.095 Hz
17	929.131 Hz
18	958.929 Hz
19	1021.311 Hz
20	1156.79 Hz
21	1372.207 Hz
22	1372.934 Hz
23	1588.721 Hz
24	1624.802 Hz
25	1670.699 Hz
26	1821.729 Hz
27	1836.219 Hz
28	2193.937 Hz
29	2221.323 Hz
30	2278.312 Hz
31	2332.949 Hz
32	2357.832 Hz
33	2417.323 Hz
34	2423.292 Hz
35	2450.332 Hz

36	2551.313 Hz
37	2556.221 Hz
38	2598.853 Hz
39	2621.322 Hz
40	2740.191 Hz
41	2823.428 Hz
42	2831.386 Hz
43	2851.347 Hz
44	2919.273 Hz
45	3074.333 Hz
46	3115.188 Hz
47	3249.529 Hz
48	3405.182 Hz
49	3432.274 Hz
50	3434.693 Hz
51	3594.231 Hz
52	3647.619 Hz
53	3657.931 Hz
54	3742.957 Hz
55	3753.382 Hz
56	3830.732 Hz
57	3855.823 Hz
58	3916.321 Hz
59	3935.218 Hz
60	3975.383 Hz
61	3993.437 Hz
62	4153.192 Hz
63	4241.321 Hz
64	4243.393 Hz
65	4253.432 Hz
66	4318.222 Hz
67	4375.962 Hz
68	4393.419 Hz
69	4394.134 Hz
70	4417.243 Hz
71	4481.463 Hz
72	4495.138 Hz
73	4549.808 Hz
74	4558.306 Hz

75	4779.451 Hz
76	4838.674 Hz
77	4871.513 Hz
78	4895.296 Hz
79	4962.213 Hz
80	4969.224 Hz
81	4979.321 Hz
82	5027.231 Hz
83	5059.792 Hz
84	5118.094 Hz
85	5176.287 Hz
86	5365.222 Hz
87	5376.392 Hz
88	5426.323 Hz
89	5431.542 Hz
90	5536.242 Hz
91	5739.422 Hz
92	5745.218 Hz
93	5821.975 Hz
94	6037.432 Hz
95	6044.333 Hz
96	6086.256 Hz
97	6208.932 Hz
98	6212.808 Hz
99	6231.031 Hz
100	6280.321 Hz
101	6329.391 Hz
102	6476.896 Hz
103	6477.098 Hz
104	6497.319 Hz
105	6504.983 Hz
106	6651.276 Hz
107	6657.913 Hz
108	6757.901 Hz
109	6758.321 Hz
110	6855.286 Hz
111	6858.121 Hz
112	6898.489 Hz
113	6915.886 Hz

114	7092.219 Hz
115	7120.218 Hz
116	7127.311 Hz
117	7156.489 Hz
118	7208.821 Hz
119	7224.197 Hz
120	7282.169 Hz
121	7285.693 Hz
122	7376.329 Hz
123	7488.742 Hz
124	7577.421 Hz
125	7621.085 Hz
126	7627.207 Hz
127	7650.939 Hz
128	7668.231 Hz
129	7691.212 Hz
130	7842.184 Hz
131	7849.231 Hz
132	7915.423 Hz
133	7932.482 Hz
134	7949.196 Hz
135	7967.311 Hz
136	8021.229 Hz
137	8070.181 Hz
138	8114.032 Hz
139	8149.922 Hz
140	8194.19 Hz
141	8245.801 Hz
142	8328.322 Hz
143	8330.534 Hz
144	8355.987 Hz
145	8408.121 Hz
146	8431.184 Hz
147	8452.119 Hz
148	8548.324 Hz
149	8749.383 Hz
150	8784.424 Hz
151	8894.222 Hz
152	8923.1 Hz

153	8923.361 Hz
154	8935.752 Hz
155	8936.1 Hz
156	9060.323 Hz
157	9072.409 Hz
158	9131.419 Hz
159	9199.232 Hz
160	9245.927 Hz
161	9270.322 Hz
162	9279.193 Hz
163	9393.946 Hz
164	10227.242 Hz
165	10340.509 Hz
166	10363.313 Hz
167	10456.383 Hz
168	10468.231 Hz
169	10470.456 Hz
170	10472.291 Hz
171	10689.339 Hz
172	11525.121 Hz
173	11541.915 Hz
174	11812.419 Hz
175	11840.323 Hz
176	12267.281 Hz
177	12294.283 Hz
178	12629.222 Hz
179	12648.221 Hz
180	13315.335 Hz
181	13735.241 Hz
182	13853.232 Hz
183	13915.231 Hz
184	13990.123 Hz
185	14519.232 Hz
186	14543.128 Hz
187	15651.323 Hz
188	17352.085 Hz
189	17970.122 Hz
190	18524.419 Hz
191	18619.331 Hz

192	18662.112 Hz
193	19385.893 Hz
194	19406.211 Hz
195	

CURRENT AND FUTURE DIRECTIONS

Introduction

Hepatocellular carcinoma is a global problem for which additional therapeutic options are sorely needed [34, 35]. We have previously demonstrated a phenotype in HCC cell lines exposed to amplitude-modulated RF EMF that includes proliferative inhibition, modulation of gene expression, and mitotic spindle disruption [36]. We have also demonstrated the clinical efficacy of amplitude-modulated RF EMF in a Phase I/II trial in 41 hepatocellular carcinoma patients [37]. The purpose of recent and ongoing studies has been to gain a mechanistic understanding of RF EMF, which will allow for optimal implementation and maximal clinical effect. The established differences between malignant and nonmalignant cells are becoming an ever lengthier list, suggesting that there are a myriad of possible differences which may be exploited and modulated by RF EMF therapy [38].

We have used both RNA-Seq and miRNA array analysis to identify possible pathways and cellular functions that may be altered by RF EMF exposure. Based on these findings, we followed global analysis with a functional examination of the IP3/DAG signaling pathway as well as an evaluation of cell death mechanisms and Ca^{2+} flux.

Simultaneously, we have evaluated the safety and efficacy of RF EMF *in vivo* using a murine subcutaneous xenograft model of HCC as well as in a spontaneous murine

model of breast cancer. Future *in vivo* studies aim to continue to evaluate and characterize the systemic effects of RF EMF, including an examination of the immune response.

Recent experiments have also begun to evaluate the effects of RF EMF in malignancies other than HCC in an effort to establish proof-of-principle. In the future, we hope to expand *in vitro* experiments to tissue obtained from patients enrolled in clinical trials and receiving RF EMF.

Molecular Experiments

In Vitro Experimental Methods

In vitro RF EMF exposure equipment and exposure protocol were previously described [36]. Briefly, cells were seeded in 35 mm dishes (BD Falcon) and were given approximately 4 hours for adherence. Cells received 3 hours of daily exposure to amplitude-modulated RF EMF. Control samples did not receive any exposure. Cells received exposure for 7 days (21 total hours) prior to collection for endpoint assays.

Cell Death

Consistent growth inhibition in HCC cell lines exposed to HCC-specific RF EMF warranted investigation of cell death pathways [36]. Further, decreased expression of *PLP2* prevents inhibition of Fas-initiated release of cytochrome *c*, promoting apoptosis [39]. We also showed mitotic spindle disruption following exposure to HCC-specific RF EMF, a phenotype similar to that described by Kirson *et al.,* after cell exposure to alternating currents [36, 40, 41]. To determine the functional relevance of these findings,

we designed experiments to evaluate possible effects of RF EMF on cell death and cell cycle progression.

Cell death pathways were evaluated by Western blot, flow cytometry and fluorescence microscopy. We evaluated apoptosis by Western blot, examining levels of FasL, Bcl2, and cleaved caspase. Additionally, we used flow cytometry and fluorescence microscopy to provide a more sensitive and quantitative evaluation of modest cell death. For microscopy and flow cytometry experiments we used the ApoAlert Annexin V flow cytometry kit (Clontech).

Preliminary experiments suggest increased levels of apoptosis in HepG2 cells treated with RF EMF as compared to controls. This was visualized microscopically by evaluating levels and localization of fluorescence, which correspond to the position of phosphatidylserine in the plasma membrane (Figure 1). Distribution of phosphatidylserine in the outer layer of the plasma membrane is evidence of early apoptosis [42-45]. Preliminary flow cytometry experiments have also suggested increased apoptosis, as there was a greater population of Annexin V positive cells in the RF EMF treated samples of HepG2 cells, as compared to controls (average 33.6% and 49.6%, for control and RF EMF treated, respectively). These data also demonstrated a slight difference in the cell cycle distribution of cells treated with RF EMF with more cells in the G2/M phase (average 6.3% and 7.5% for control and RF EMF treated, respectively) alluding to possible effects on cell cycle progression and corresponding with previously published mitotic spindle disruption [36]. Increased cleaved caspase in RF EMF treated HepG2 cells as compared to controls were not detected by Western blot,

suggesting a caspase-independent pathway or early apoptosis prior to caspase activation [46].

We also evaluated apoptosis in HepG2 cells simultaneously exposed to camptothecin (100 μM for 3 hours, Sigma) and RF EMF. Camptothecin is a topoisomerase I inhibitor that induces lethal DNA strand breaks and is frequently used as an inducer of apoptosis [47]. Using fluorescence microscopy, it appeared that apoptosis is enhance by the dual sources of cellular stress (Figure 1A-B). This finding corresponds to other reports of synergistic activity between chemotherapeutic agents and EMF exposure both clinically and *in vitro* [48-51]. However, these data will need further quantitation for a definitive determination of apoptosis levels.

We have evaluated autophagy using ATG family proteins as well as Beclin and LC3A and LC3B (Autophagy Sampler Kit, Cell Signaling). Western blot led to the identification of subtly increased levels of ATG3, ATG7, and Beclin-1 in HepG2 cells treated with RF EMF (Figure 2). Elevation of other autophagic proteins was not identified.

The interplay between apoptosis and autophagy is poorly understood, as each cellular function can have either inhibitory or promoting effects on the other [52-54]. For example, a recent study reported that Beclin-1 is critical for the engulfment of apoptotic cells [55]. Moreover, autophagy may incite either pro-death or pro-survival responses to cellular stress [54]. ATG7 is a key protein in the autophagic process, as it modulates p53 activity in regulating cell cycle and death in settings of cellular stress and is necessary for two ubiquitination-like reactions that are necessary for the maturation of the autophagosome [56, 57]. Beclin-1 is a regulator of autophagic processes and low levels

in tumors are associated with metastasis and poor prognosis, while elevated levels have demonstrated an anti-proliferative effect in tumors [58-61]. The identification of the earliest stages of apoptosis may be a consequence of a direct effect of RF EMF on the apoptotic process, as cells are harvested for evaluation immediately following RF EMF exposure. Our current data indicate that both processes may be occurring at low levels as a consequence of RF EMF exposure and that this effect may be enhanced by adding a second, independent source of cellular stress, such as camptothecin.

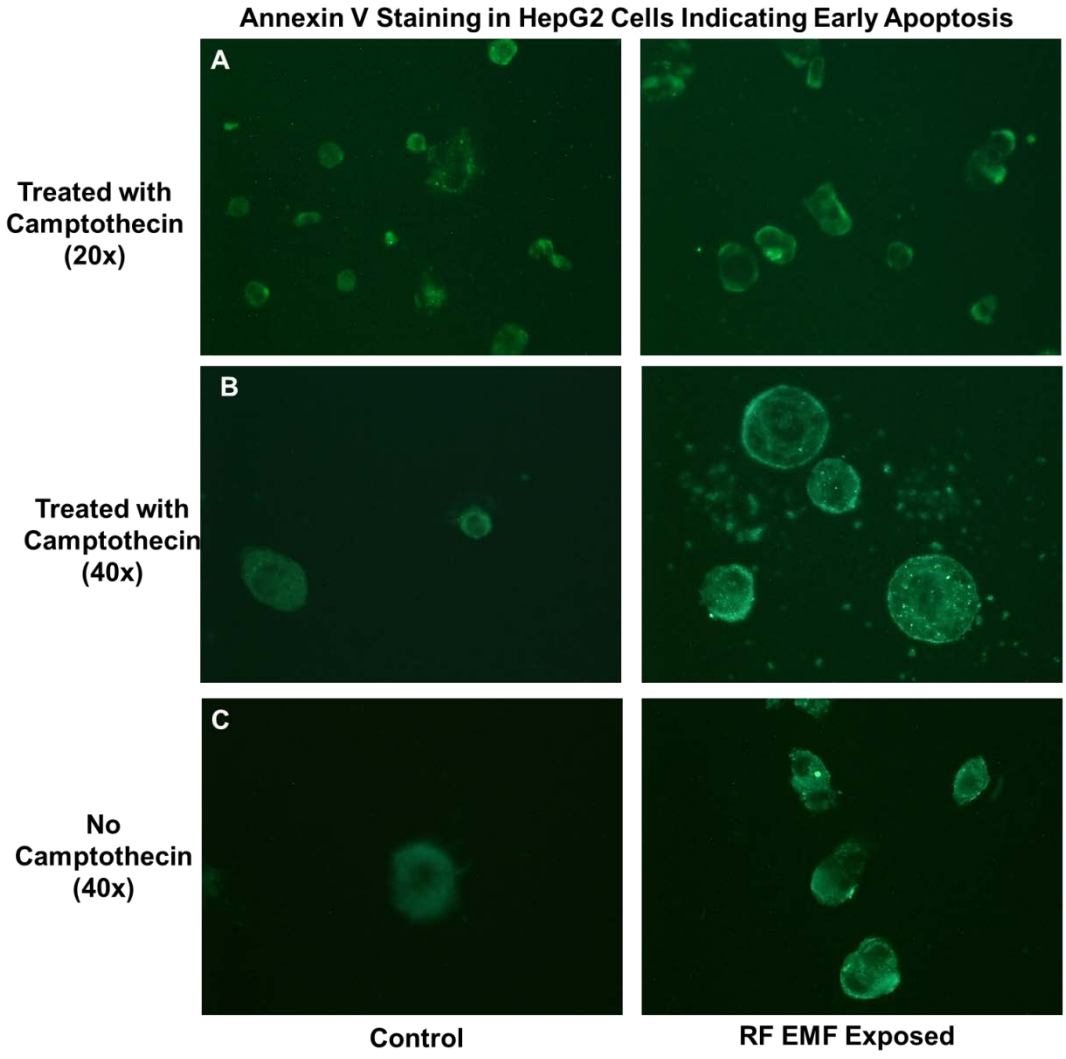

Figure 1. Evidence of early apoptosis in HepG2 cells exposed to RF EMF. Membrane blebbing and greater fluorescence intensity in the cell membrane indicate apoptotic processes. **(A)** Annexin V staining to indicate early apoptosis in induced by camptothecin (100 µM for 3 hours). Cells were either simultaneously treated with RF EMF or were in the control group not receiving exposure. Magnification 100x. **(B)** Annexin V staining to indicate early apoptosis induced by camptothecin (100 µM for 3 hours). Cells were either simultaneously treated with RF EMF or were in the control group not receiving exposure. Magnification 200x. **(C)** Annexin V staining to indicate early apoptosis in HepG2 cells treated with RF EMF as compared to control. Magnification 200x. Images acquired using an $EVOS_{fl}$ LED digital inverted microscope (Advanced Microscopy Group).

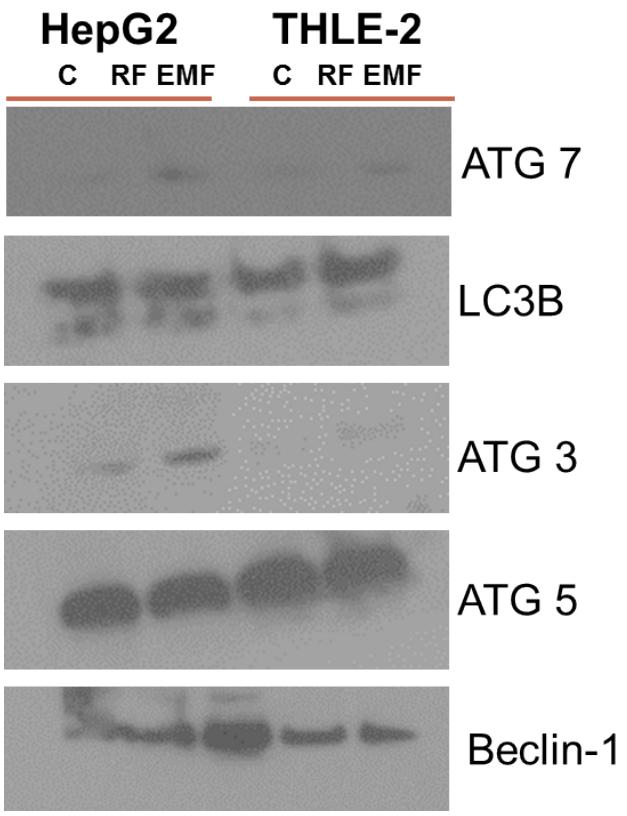

Figure 2. Evaluation of autophagy in HepG2 cells exposed to RF EMF.

Effects on IP3/DAG Signaling

Gene Expression

Recently, an updated analysis of RNA-Seq data led us to evaluate the impact of EMF on the phosphatidylinositol 3-kinase (PI3K) pathway, specifically through IP3/DAG signaling. PI3K signaling, both through AKT and IP3/DAG, is frequently perturbed in HCC [62-64]. Furthermore, the downstream effects of the pathway include: cell cycle progression, proliferation, inhibition of apoptosis, and cell migration, each of which may be affected either directly or indirectly in the phenotype we previously established [36, 65]. In a collaborative effort with Dr. Dongquan Chen, a bioinformatician at the UAB Comprehensive Cancer Center specializing in pathway analysis, we reanalyzed RNA-Seq data using an updated local instance of Galaxy lined to the UAB high performance GRID (www.uab.edu/galaxy) [66]. Several newly identified differentially expressed genes function in IP3/DAG signaling such as *NFAT5, MBOAT1,* and *ARHGDIB* [67, 68].

MicroRNAs (miRNAs) are being increasingly implicated in HCC carcinogenesis and progression [69-72]. Additionally, miRNA alterations are observed in both human and rodent models following both direct and indirect exposure to ionizing radiation at various doses [73]. While RF EMF are non-ionizing, they are capable of causing a stress response in cells, similar to the cellular response to ionizing radiation [73-75]. Data suggest that RF EMF are potent modifiers of miRNAs that target key components of the IP3/DAG arm of the PI3K pathway.

Similar to RNA-Seq reanalysis, we used Galaxy for miRNA analysis and identified increased levels of miRNAs that target mRNAs used to synthesize proteins

important in the early steps of the PI3K pathway, specifically IP3/DAG signaling: has-miR-148a targets *NFAT5, PLCB1*; Hs-miR-1246 targets *PLCXD3, PLCB4*; has-let-7g targets *PLCB2, PLCD4* [68]. Additionally, the miRNAs identified target 20 of the 65 frequently mutated genes recently identified by exome sequencing of 10 patients with hepatitis B-associated HCC [81]. These genes were also differentially expressed in the primary tumor as compared to the portal vein tumor thrombosis, suggesting specific effects on HCC metastatic processes [81].

Functional data suggests that RF EMF are not directly impacting signal transduction through AKT, so emphasis has remained on IP3/DAG signaling. Western blot analysis in HepG2 cells evaluated modulation of the AKT arm of the PI3K pathway and demonstrated no change in pAKT or PI3K (p85 regulatory subunit). These findings in addition to global differential gene expression identification further justified the investigation of the IP3/DAG arm of the pathway as well as cytosolic Ca^{2+} levels.

Calcium Efflux

IP3/DAG signaling also regulates calcium (Ca^{2+}) efflux from the endoplasmic reticulum (ER) membrane. Though EMF-induced changes in Ca^{2+} efflux from the endoplasmic reticulum have been extensively documented and debated, such changes have not previously been evaluated for the frequency range we employ [77-79].

Preliminary fluorescence microscopy experiments in HepG2 cells suggest that Ca^{2+} localization is impacted by RF EMF exposure, resulting in a greater level of cytosolic Ca^{2+} within the cell (Figure 3). However, surprisingly this has been observed

without identifying significant corresponding changes in the levels of proteins that function upstream and downstream of Ca^{2+} release from the endoplasmic reticulum (ER).

Previous studies demonstrated that weak EMF are capable of interacting with electrons, presumably because of their high charge to mass ratio [80]. This was shown in Na,K-ATPase function for which there are transiently available electrons in the region of the enzyme because hydrogen bonds continuously form and break as the conformation of the protein changes [81]. A similar mechanism may hold true for Ca^{2+} channels in the ER membrane, which play an important role in IP3/DAG signaling and downstream cellular metabolism. Moreover, the effects of different EMF frequency ranges on Ca^{2+} efflux has been previously documented both *in situ* and *in vitro* [77-79]. This physiological effect is appealing because it does not require charging of the membrane, an EMF effect that is heavily debated [74]. Additionally, PLP2 has been characterized as an ER membrane ion channel, presumably for Ca^{2+} [82]. Finally, *S100B* and *ANXA1*, two genes that affect available intracellular Ca^{2+}, are differentially expressed in HCC cells following RF EMF exposure [83, 84]. Though more definitive evidence is necessary, current data suggest that Ca^{2+} flux is directly affected by RF EMF without altering protein levels in the IP3/DAG signaling pathway.

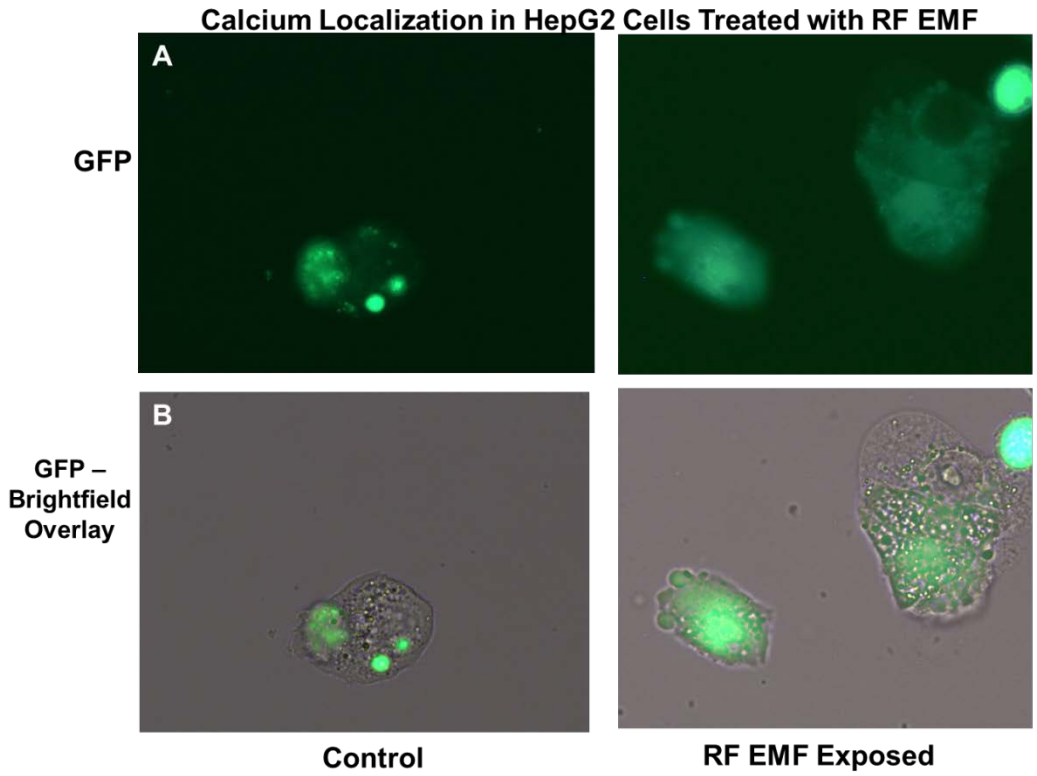

Figure 3. Distribution of calcium in HepG2 cells exposed to RF EMF. Calcium was more diffusely distributed in the cytoplasm of HepG2 cells exposed to RF EMF than in controls not receiving exposure. **(A)** Oregon Green 488 BAPTA-AM was used to identify calcium localization using the GFP channel of the microscope **(B)** Overlayed brightfield and GFP images to view calcium localization in combination with the entire cell structure. Images acquired using an $EVOS_{fl}$ LED digital inverted microscope (Advanced Microscopy Group). Magnification 200x.

Murine models of Cancer

Though RF EMF exposure has already been evaluated in human cancer patients, it was imperative to investigate the systemic effects of RF EMF on tumor growth *in vivo*. We have used a subcutaneous xenograft model of HCC in non-obese diabetic severe combined immunodeficient (NOD SCID) mice (Jackson Labs, Bar Harbor, ME) to evaluate tumor size and rate of tumor growth in mice exposed to HCC-specific RF EMF as compared to mice not receiving RF EMF. We have also implemented the MMTV-PyMT spontaneous model of breast cancer to evaluate the effects of RF EMF in a malignancy of murine origin [85]. All exposure equipment and protocols were approved for use by the Institutional Animal Care and Use Committee (IACUC).

In Vivo Exposure Methods

An optimized system for controlled exposure of mice to 27.12 MHz radiofrequency (RF) electromagnetic fields was developed, manufactured, and characterized by the Foundation for Research in Information Technologies in Society (IT'IS Foundation, Zurich, Switzerland). The system is composed of two identical chambers, each capable of housing 8 cages, with each cage holding 2 mice (Figure 4). The exposure system is composed of a stripline, with 350 mm ground-septum spacing, used in a half wave resonator configuration. This configuration provides high levels of field homogeneity across the exposure volume and a considerable enhancement of the maximum SAR level for a given RF input power. The control unit contains 4 DDS frequency sources, 2 for the carrier frequency, and 2 for the modulation frequencies. All measured and control parameters are stored in a log file. The system also allows for

blinded exposure schemes using active and inactive modulation frequencies. For blinded exposures the computer selects which chamber receives the active signal, and this information is included in the log file.

Numerical dosimetry was completed using anatomical models to evaluate more than 100 murine body parts. Analysis was completed for scenarios with both one and two mice housed in the same cage. The greatest impact on Specific Absorbed Rate (SAR) was the rearing of the mouse, and this was taken into consideration as phantoms were evaluated when oriented horizontally as well as at 45°.

Animals have ad lib access to food and water while housed in the exposure system. Water is provided in sterile hydropacs located outside the exposure system with medical-grade tubing as a conduit to the cage. Lixits are placed in the wall of the cage, providing water through the same general mechanism used by mice throughout the animal facility. Food is placed directly in the cages. The back side of the exposure system is composed of metal in a cross-hatched pattern, so the animals in the exposure system receive standard light/dark cycles.

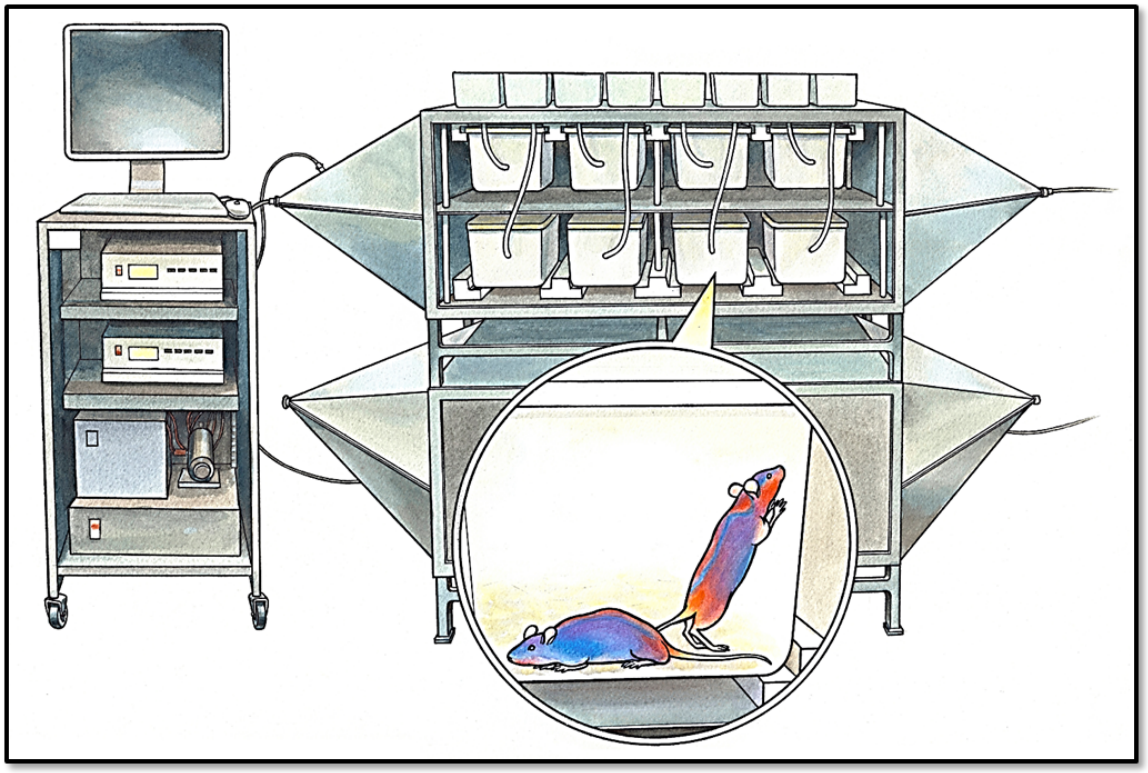

Figure 4. ***In vivo*** **RF EMF exposure system.** Extensive dosimetry was completed in over 100 distinct murine body parts to assure frequency delivery at levels consistent with those received by humans. Animals receive standard light and dark cycles by way of the transparent back. Two mice can be housed in each of the 8 cages per chamber. Mice have ad lib water access through medical grade rubber tubing connected to a lixit placed in the cage side. Hydropacs are placed along the top on the outside the chamber. Dimensions of each chamber: 39 cm x 76.5 cm x 185 cm.

Xenograft Model

We have used both HepG2 and Huh7 cells as subcutaneous cellular xenograft models of HCC, as previously described [86, 87]. Xenograft size was monitored daily using calipers. Mice were euthanized upon reaching excessive tumor burden in compliance with the UAB IACUC Policy of Surveillance and Euthanasia of Rodents Bearing Solid Tumors (Updated 8/31/11). Tumor and non-tumor tissue were collected, formalin fixed, and paraffin embedded for IHC evaluation.

Tumor Growth Rate

Using this model, we have demonstrated near complete tumor regression in a mouse engrafted with HepG2 cells and treated with HCC-specific RF EMF (Figure 5). This took place over approximately 14 weeks and was followed with an additional 4 weeks of observation. Additionally, mice engrafted with Huh7 cells and treated with HCC-specific RF EMF showed significantly less increase in tumor volume over a 5 week treatment period than did control mice ($p=0.00158$) (Figure 6). Tumor volume was calculated as the average of 3 weekly caliper measurements.

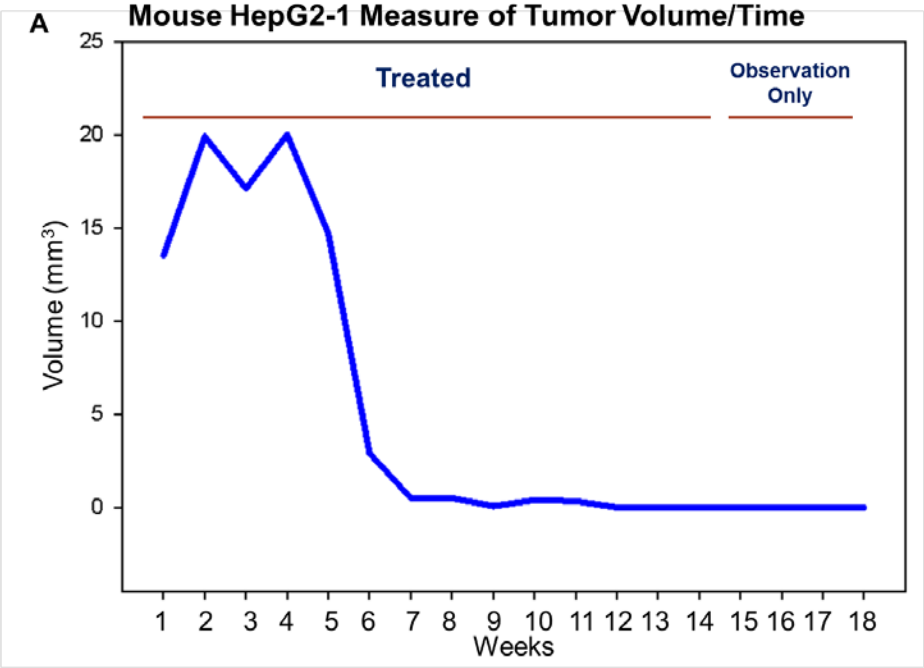

Figure 5. Mouse engrafted with HepG2 cells and treated with RF EMF.
(A) Mouse was injected with $1x10^7$ HepG2 cells subcutaneously in the flank. RF EMF exposure began when tumor was palpable. Volume measurements were calculated weekly, based on 3 average measurements (length x $width^2/2$). Mouse was treated for 14 weeks and then observed for an additional 4 weeks. **(B)** Residual tumor mass (box). **(C)** H&E of residual tumor.

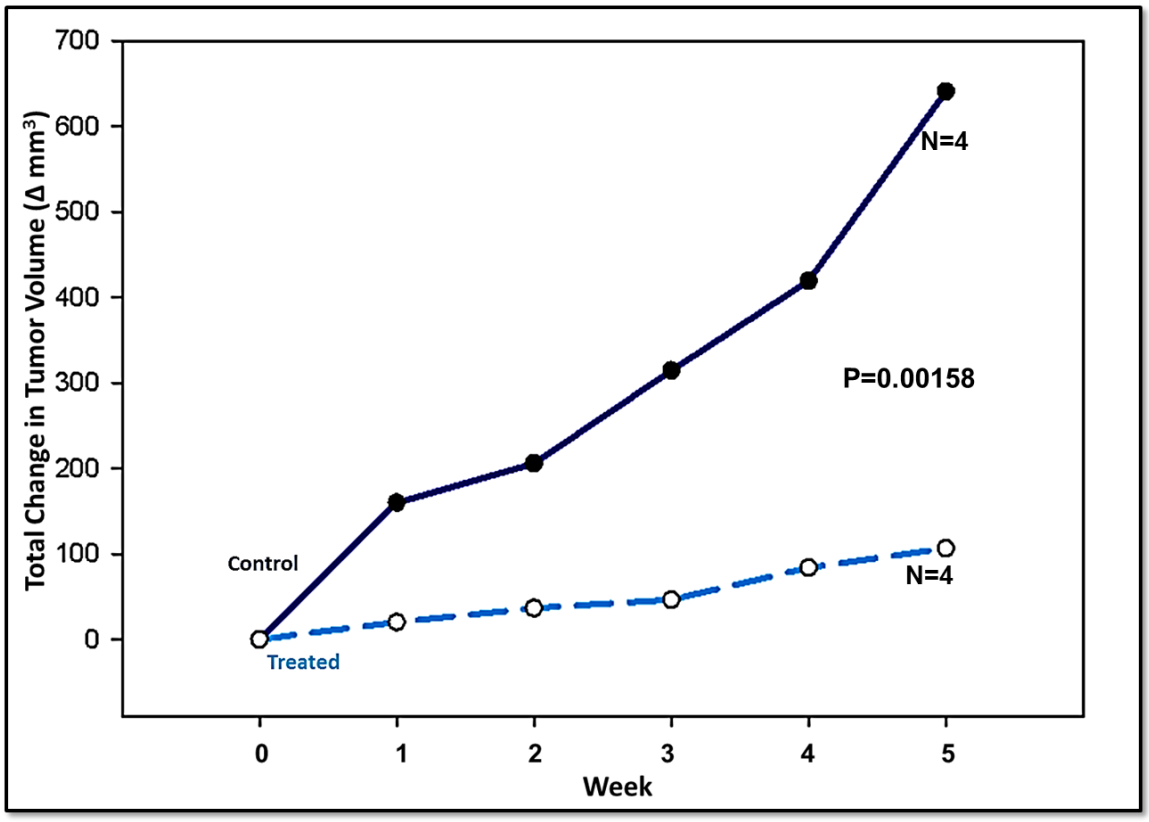

Figure 6. Total change in tumor volume in mice carrying Huh7 subcutaneous cellular xenografts. Mice were injected with $1x10^7$ Huh7 cells subcutaneously in the flank. RF EMF exposure began when tumors were palpable. Volume measurements were calculated weekly, based on 3 average measurements (length x $width^2/2$). Each the treatment and control group had 4 animals. P=0.00158, as determined by a two-sided t-test.

Tumor Immunohistochemistry

These models have provided extensive amounts of tumor tissue and surrounding stroma for analysis, which is not readily available from human patients receiving treatment. Post-mortem histological analysis included hematoxylin and eosin (H&E) staining to assess differences in tumor morphology. H&E staining of tumors extracted from mice carrying Huh7 xenografts revealed a phenotype of increased fibrous connective tissue surrounding and infiltrating the tumor tissue in animals treated with RF EMF (Figure 7A). We hypothesize that this fibrotic change impacted the tumor growth and proliferation of the tumor by a mechanism yet to be identified.

We used immunohistochemistry (IHC) to assess apoptosis and autophagy (slides pending) in tumors from animals receiving RF EMF exposure. Using a TUNEL assay (Apotag Peroxidase in situ Apoptosis Detection Kit, Millipore) to evaluate late apoptosis and caspase 3 to evaluate early apoptosis, we found that there were more apoptotic cells in tissue collected from mice receiving RF EMF (Figure 8). We also evaluated tumor vasculature using CD31 and tumor cell proliferation using ki-67. Staining revealed diminished ki-67 staining in tumor samples extracted from mice treated with RF EMF (Figure 7B). There were not significant differences noted in tumor vasculature between the treatment and control groups (Figure 7C).

Safety Validation

We also validated the safety of RF EMF exposure *in vivo.* In examining normal tissue from animals exposed to RF EMF, we demonstrated that normal tissue architecture remained intact (Figure 9). In order to evaluate systemic effects on proliferation, we used

ki-67 to examine proliferation in small intestine tissue of mice exposed to RF EMF (Figure 10), as this tissue is one of the most rapidly proliferating in the body. There was not a significant difference in the proliferation of the cells in the crypts of the small intestine, suggesting that RF EMF exposure does not systemically affect proliferative rate.

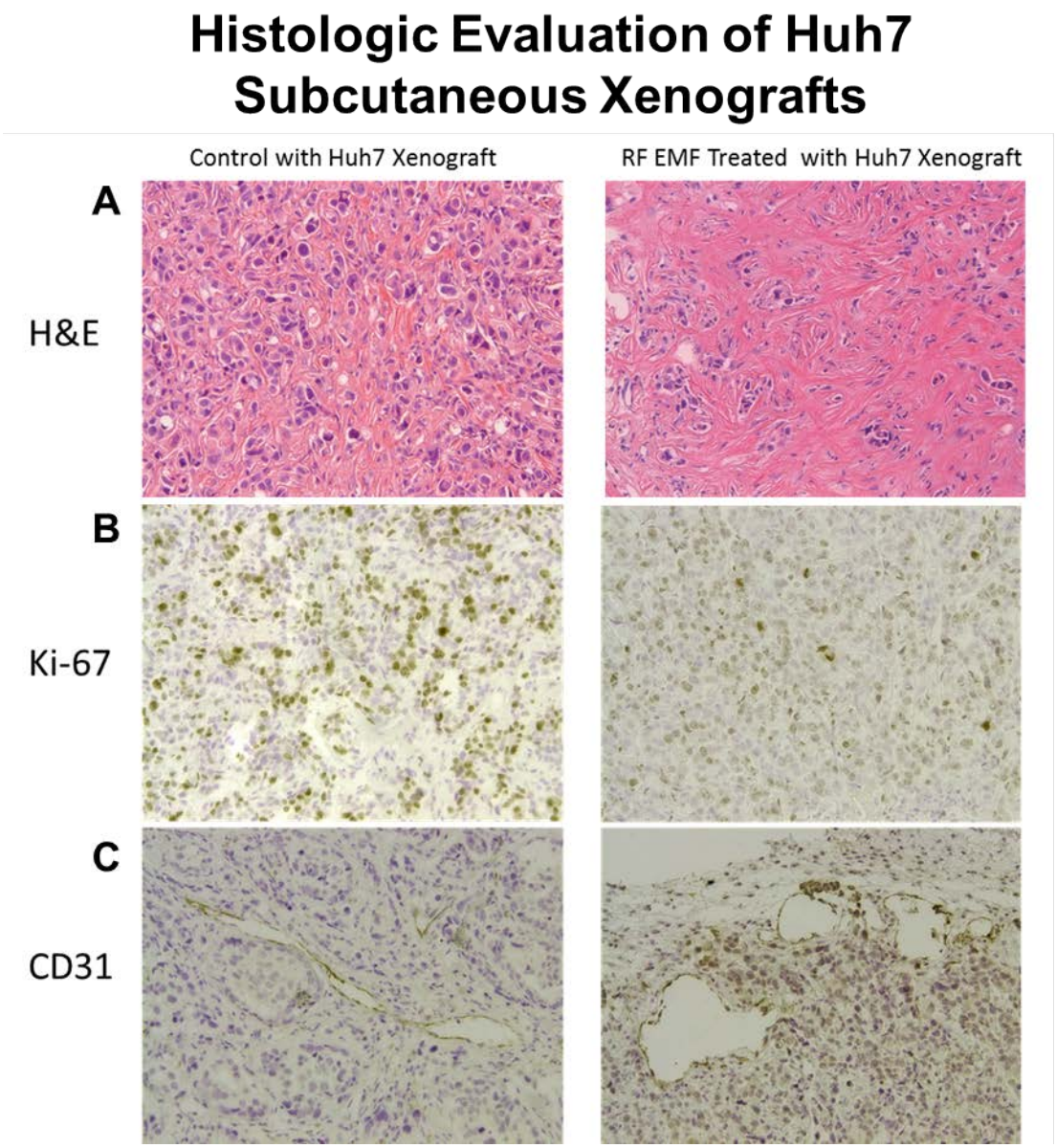

Figure 7. Immunohistochemistry from mice carrying Huh7 cellular xenografts. (A) H&E stain demonstrating increased fibrotic change in mice treated with RF EMF. **(B)** Ki-67 stain reveals decreased proliferating cells in mice treated with RF EMF. **(C)** Significant vasculature changes were not identified between the treated and controlled mice. Magnification 200x.

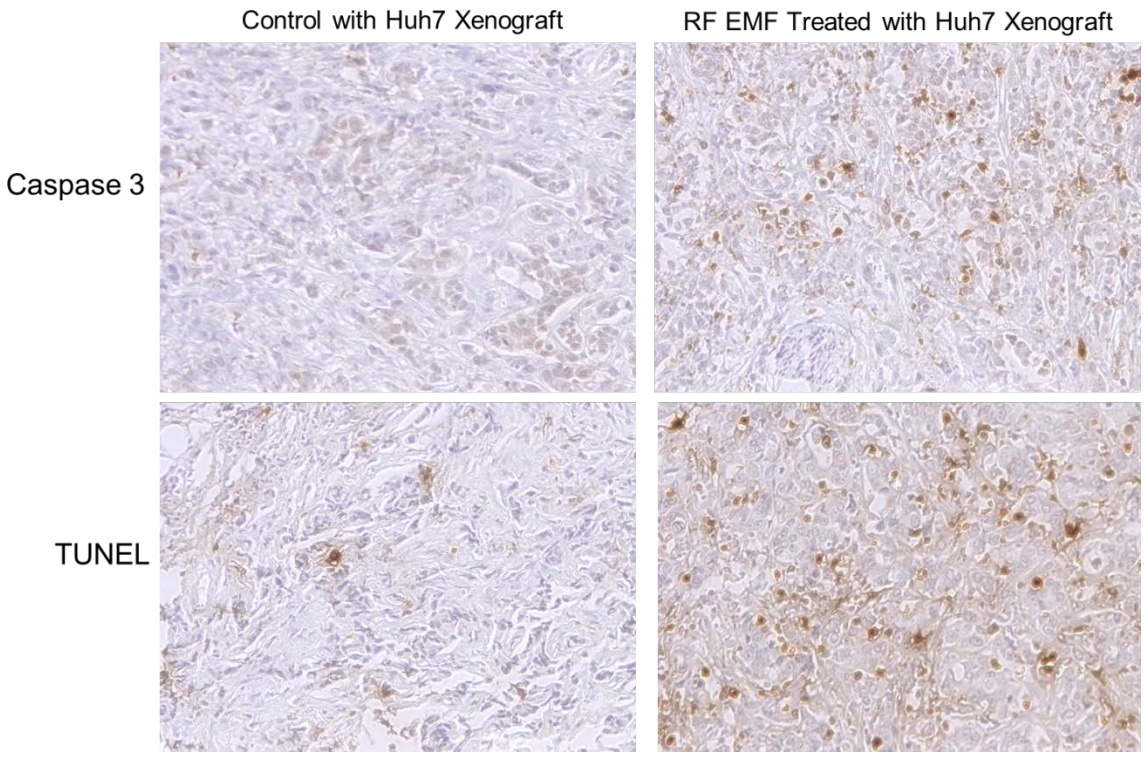

Figure 8. Evaluation of apoptosis in tumors from mice carrying Huh7 cellular xenografts. Indication of early apoptosis is identified using caspase 3, while late apoptosis is identified using a TUNEL assay. Brown staining indicates the apoptotic process (Magnification 200x).

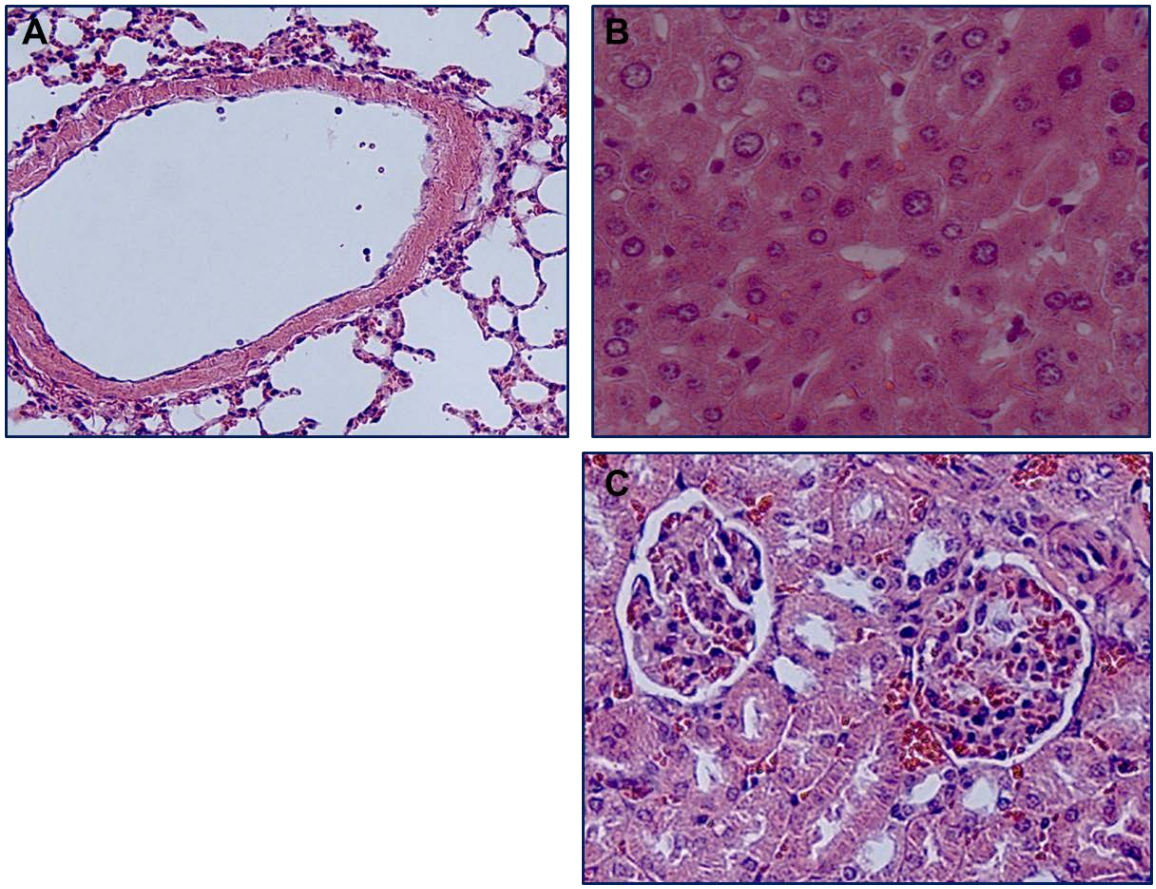

Figure 9. Normal tissue architecture in mice exposed to RF EMF. (A) H&E stain demonstrating normal bronchiolar tissue (Magnification 200x). **(B)** H&E stain of normal hepatocytes (Magnification 400x). **(C)** H&E stain of kidney cortex displaying normal glomerular structure (Magnification 200x).

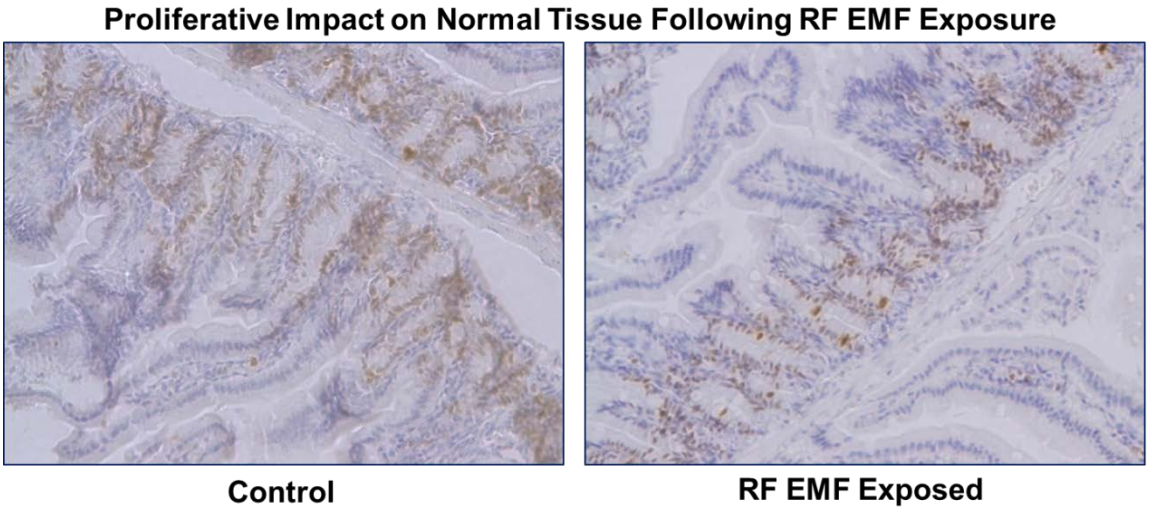

Figure 10. Normal proliferating small intestinal tissue in mice carrying Huh7 xenografts. Ki-67 staining demonstrates comparable proliferation in the crypts of the small intestine of mice exposed to RF EMF as compared to control mice. Magnification 200x.

Spontaneous Model

Since there is not a readily available transgenic model of HCC or an inducible model with a timely induction period, we evaluated the effects of breast cancer-specific amplitude-modulated RF EMF on a spontaneous model of breast cancer. This choice was justified by one of the first clinical reports of the efficacy of RF EMF, which was in a patient with metastatic breast cancer [48]. The MMTV-PyMT model uses the polyomavirus middle T oncogene controlled by a mammary promoter and causes mammary fat pad hyperplasia that ultimately culminates in metastatic breast cancer in less than 4 months [85, 88-92]. The timeline for tumor development is reasonably predictable for this model, so animals began receiving RF EMF at approximately 6 weeks of age. Mice were euthanized upon development of excessive tumor burden.

Effects on Tumor Growth

Caliper measurements are not reliable for tumor burden evaluation of the MMTV-PyMT model, as many multifocal tumors develop preventing caliper measurement of individual masses. Tumor mass was evaluated indirectly by weighing the mice weekly. Mice were also weighed at euthanasia, and the mass of the total tumor burden was also determined following tumor extraction. This allowed for the calculation of percent mass of the mouse that was attributable to tumor burden. Thus far, a significant difference in tumor burden has not been identified between the treatment and control group, though a portion of the cohort is still being evaluated. This finding, if it persists, is important, as it suggests that the amplitude-modulated RF EMF identified in cancer patients may be

specific for human malignancy. This finding will also guide future decisions regarding *in vivo* models.

Histologic Impact

Tumor samples were stained with H&E and tumor morphology was evaluated. There was evidence of squamous metaplasia in the tumor samples, which appears to be more abundant in tumors from the control group (Figure 11). Squamous metaplasia has been noted in the MMTV-PyMT model of breast cancer, specifically in cells expressing matrix metalloproteinase 13 [92]. Squamous metaplasia is a histological sign of extremely advanced disease, so further evaluation may provide evidence that RF EMF does ultimately produce to a lower grade malignancy, despite no appreciable difference in tumor volume [93].

Immune Effects

Additionally, we compared whole blood in RF EMF treated and control mice. Blood was collected by cardiac puncture at euthanasia and complete blood count was determined using a Hemavet (Hemavet 1700, Drew Scientific). Since these are transgenic mice that are not immunocompromised like those in our xenograft models, complete blood count allows for a gauge of the basic immune response. White blood cell counts were elevated, presumably in response to the tumor burden, both in RF EMF treated and control animals. However, no one cellular component of the blood was significantly different between the treated and control groups.

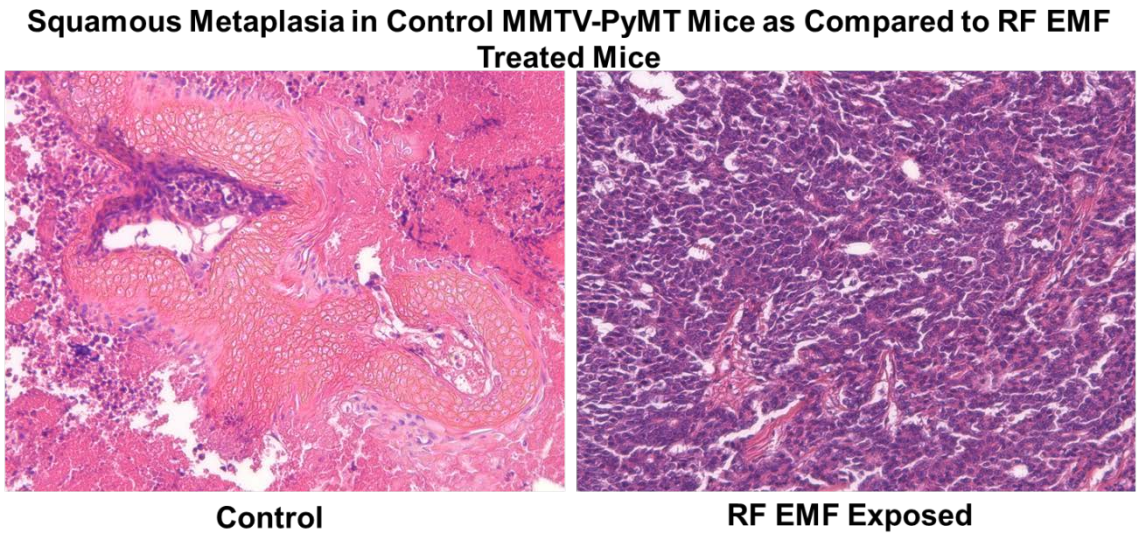

Figure 11. Squamous metaplasia in control MMTV-PyMT mice as compared to mice treated with RF EMF. H&E staining demonstrates squamous metaplasia in control MMTV-PyMT mice. Mice treated with RF EMF still demonstrate high grade malignancy, but metaplastic changes were not noted. Magnification 200x.

Conclusions

The understanding of the molecular basis of cancer and the pathways involved in initiation, promotion, metastasis is rapidly evolving [38]. We have reported clinical and *in vitro* evidence that amplitude-modulated RF EMF impact cancer cells in a tumor specific manner, identifying another possible novel pathway leading to an antitumor effect specific to malignant cells [34, 35, 48].

We have also demonstrated treatment safety and efficacy using a subcutaneous cellular xenograft model of HCC. This was associated with increased fibrotic appearance in tissue collected from mice treated with RF EMF. Further, we have preliminary evidence that the anti-tumor effects of RF EMF are specific for malignancies of human origin, as the MMTV-PyMT spontaneous model of breast cancer has not demonstrated a significant difference between treated and control groups. Though, these experiments are ongoing.

At the molecular level, we have recently identified differential expression of mRNAs that are ultimately translated into proteins that function in IP3/DAG signaling as well as miRNAs that target players in this pathway. We have also demonstrated increased levels of cytosolic Ca^{2+} in HepG2 cells exposed to RF EMF. These findings support RF EMF effects on the IP3/DAG signaling pathway. Increased cytosolic Ca^{2+} levels correspond with a significant body of literature proposing that RF EMF has an impact on Ca^{2+} signaling [77-79, 94-103]. Ultimately, elevated cytosolic Ca^{2+} results in significant cellular stress that forces cells down autophagic and apoptotic pathways [52].

Though we have identified differential mRNA and miRNA expression following

RF EMF exposure, we have thus far not identified significant differences in the levels of proteins that have seminal functions in IP3/DAG and Ca^{2+} signaling, though additional targets have yet to be evaluated. Therefore, we posit that differential gene expression and alterations in miRNA levels may be a compensatory effort carried out by the cells to adapt to increased cytosolic Ca^{2+} levels but that compensation does not carry through to translation and mature protein generation. Evidence of effects on Ca^{2+} localization without evidence that protein levels within the IP3/DAG signaling pathway are directly impacted indicates that the rate of Ca^{2+} flux is impacted by RF EMF. This may be due to RF EMF modulation of the conformation of Ca^{2+} channels in the ER membrane impacting Ca^{2+} flow between the organelle and the cytoplasm. A similar hypothesis was previously put forth by Blank and Goodman who suggested that RF EMF interacts with free electrons transiently available as receptor and channel conformation changes [80]. However, the dynamic process described in this hypothesize is difficult to test.

We have also reported that the antitumor effects of RF EMF act in a tumor-specific manner without impacting normal tissue. Cancer cells have a wide range of metabolic perturbations ranging from the dependence on glycolysis to the dysfunction of many signaling pathways [104-108]. Calcium signaling is also aberrant in cancer cells [109, 110]. Therefore, cancer cells must already adapt to cellular stress prior to exposure to RF EMF. We hypothesize that nonmalignant cells are more adept at compensating for Ca^{2+} flux, explaining the lack of effects identified in nonmalignant cells. However, more experiments in nonmalignant cells are necessary to better establish this explanation.

Future Directions

Significant characterization and mechanistic studies are still necessary for a more complete understanding of the biological impacts of amplitude-modulated RF EMF. In an effort to more completely bridge the gap between bench and bedside, it will be ideal to have access to tissue samples from patients receiving RF EMF treatment to correlate physiologic responses with those previously reported *in vitro*[36]. Patient samples would also allow for the evaluation of differences in primary tumor samples, in an effort to better understand why there is frequency specificity among tumors from different primary tissues of origin [48]. This will also allow for determination of the germline and tumor genomic features associated with response to RF EMF therapy.

Additionally, since we have preliminary data suggesting that Ca^{2+} localization is altered by exposure to RF EMF, we plan to more thoroughly examine the localization of Ca^{2+} by using thapsigargin to inhibit the ER Ca^{2+} ATPase [111, 112]. Thapsigargin prevents Ca^{2+} uptake in the ER leading to increased cytosolic Ca^{2+} and depletion of Ca^{2+} stores. This will allow us to validate the fluorescence microscopy localization we have observed. Moreover, thapsigargin will induce ER stress by preventing the final step of autophagy, the fusion of the lysosome with the autophagosome [113]. This will allow for the visualization of the vacuoles characteristic of autophagy while the cell is in a temporary “static” state. Thus, the use of thapsigargin will allow us to visualize effects on autophagy as well as cytosolic Ca^{2+} following exposure to RF EMF. These visualization experiments are critical for better understanding the cellular effects of RF EMF, especially since thus far we have not seen appreciable alterations in protein levels

within Ca^{2+} signaling pathways. If additional physiologic experiments are necessary, electrophysiology experiments can be completed in order to more specifically identify potential changes in cellular depolarization or hyperpolarization following RF EMF exposure.

The possibility of synergistic effects from simultaneous exposure to RF EMF while receiving anticancer pharmacologic agents also needs additional examination. Sorafenib is a multikinase inhibitor used to treat patients with unresectable HCC [114, 115]. A recent study suggests that a more potent sorafenib formulation induces both autophagy and apoptosis in HCC cells [116]. Therefore, we plan to evaluate autophagic and apoptotic activity in HCC cells exposed to RF EMF while treated with sorafenib to determine if there is enhancement of the effects seen following exposure to RF EMF.

Finally, we have described the effects of RF EMF in two *in vivo* murine models. Though we have not observed specific activation of the immune system, a more complete evaluation of immune response should be undertaken given the complex role of the immune system in both tumor suppression and promotion [38].

CONCLUSIONS

Cancer continues to be a significant public health concern in the United States [117, 118]. Though there have been improvements in the mortality rate for some primary malignancies, there is still critical need for additional therapeutic options. We have identified use of amplitude-modulated RF EMF as a novel option for treating cancer [119-121]. This therapeutic approach has demonstrated minimal adverse effects, and clinical responses have been reported in breast, ovarian, and thyroid cancer as well as in hepatocellular carcinoma [119, 120]. We propose that this modality affects cytosolic Ca^{2+} levels through the modulation of Ca^{2+} channel conformation ultimately leading to cancer cell death through autophagic and apoptotic mechanisms.

Though *in vitro, in vivo,* and clinical results are promising, there are several challenges associated with carrying out these studies. First, there is very limited precedent for experimental methods to evaluate the effects of RF EMF at the levels in which we employ. Additionally, studies that evaluate biological effects cannot be carried out in real-time. For example, we are not able to evaluate Ca^{2+} flux while cells are receiving RF EMF, as the exposure equipment structure does not permit real-time imaging during exposure. Finally, though we have extensive dosimetric studies validating the power delivered *in vitro* and *in vivo,* studies of RF EMF exposure don't allow traditional dose-response experiments with known concentrations of pharmacologic agent or lead compound to cause a known or anticipated biologic outcome. The biologic effects evaluated following exposure to RF EMF are far more

subtle, adding to the challenge in trying to identify a molecular mechanism. Yet, ultimately the use of amplitude-modulated RF EMF offers the possibility of exploiting novel cancer-specific properties to generate an antitumor effect with minimal adverse effects.

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APPENDIX

IACUC APPROVAL FORMS

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: January 13, 2012

TO: BORIS C PASCHE, MD
NP -2566 3300
FAX: (205) 975-2669

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee (IACUC)

SUBJECT: Title: TGFBR1 signaling in colorectal cancer
Sponsor: NIH
Animal Project Number: 120108941

As of January 13, 2012, the animal use proposed in the above referenced application is approved. The University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) approves the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	A	0
Mice	B	0
Mice	C	0

Animal use must be renewed by January 12, 2013. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 120108941 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at (205) 934-7692.

Institutional Animal Care and Use Committee
CH19 Suite 403
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205.934.7692
FAX 205.934.1188

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