Tumor cells can't stand the heat

*Boosting the effectiveness of hyperthermia in cervical carcinoma*

Oei, A.L.

---

Citation for published version (APA):

Chapter 10

Can hyperthermia target cancer stem cells?

Arlene L. Oei, Lianne E.M. Vriend, Przemek M. Krawczyk, Michael R. Horsman, Nicolaas A.P. Franken* and Johannes Crezee*

* These senior authors contributed equally

International Journal of Hyperthermia, 2017
Abstract

Eradication of all malignant cells is the ultimate but challenging goal of anti-cancer treatment; most traditional clinically-available approaches fail because there are cells in a tumor that either escape therapy or become therapy-resistant. A subpopulation of cancer cells, the cancer stem cells (CSCs), is considered to be of particular significance for tumor initiation, progression and metastasis. CSCs are considered in particular to be therapy-resistant and may drive disease recurrence, which positions CSCs in the focus of anti-cancer research, but successful CSC-targeting therapies are limited. Here, we argue that hyperthermia - a therapeutic approach based on local heating of a tumor - is potentially beneficial for targeting CSCs in solid tumors. First, hyperthermia has been described to target cells in hypoxic and nutrient-deprived tumor areas where CSCs reside and ionizing radiation and chemotherapy are least effective. Second, hyperthermia can modify factors that are essential for tumor survival and growth, such as the microenvironment, immune responses, vascularization and oxygen supply. Third, hyperthermia targets multiple DNA repair pathways, which are generally upregulated in CSCs and protect them from DNA-damaging agents. Addition of hyperthermia to the therapeutic armamentarium of oncologists may thus be a promising strategy to eliminate therapy-escaping and -resistant CSCs.

Novelty and impact

Successful cancer therapy requires effective targeting of cancer stem cells. Cancer stem cells are resistant to conventional radiation and chemotherapy. Hyperthermia is an alternative therapeutic strategy to target this resistant cancer cell population.
1. Introduction

Despite advances in cancer treatment, overall survival of cancer patients remains limited and relapse occurs frequently, indicating that there are cancer cells in a tumor that escape from therapy or become therapy-resistant (Bao et al., 2006). It is now generally assumed that at least a fraction of these cells are tumor-initiating cells, or so-called cancer stem cells (CSCs) (Carnero & Lleonart, 2016). CSCs are characterized by reduced or even arrested cell cycle progression and by increased DNA repair capacity, which render CSCs more resistant to therapy than the bulk of the tumor (Cojoc et al., 2015; Orford & Scadden, 2008). CSCs are found in oxygen-deprived (hypoxic) tumor areas, where they are surrounded by a specific microenvironment, the CSC niche, that supports and controls their growth (Conley et al., 2012; Gilbertson & Rich, 2007). CSCs and their niches have recently been visualized histochemically in primary brain tumors in hypoxic areas around arterioles, which are transport vessel and not oxygen- and carbon dioxide-exchanging vessels (Hira et al., 2015; Verbovsek et al., 2015). As CSCs are associated with aggressiveness of tumors and are negatively prognostic for overall survival (Karnoub et al., 2007; Suva et al, 2009), there is an urgent need for therapies targeting this CSC population, but so far only few successful approaches have been developed, such as in leukemia where clinical trials are presently running that are focused on forcing CSCs out of the hematopoietic stem cell niches (Uy et al., 2012).

A successful anti-CSC therapy should be characterized by the ability to (i) target cells in areas populated by CSCs which are often difficult to reach by conventional therapies; (ii) destroy or modify the tumor microenvironment that sustains CSCs; (iii) kill cycling and non-cycling cells; and (iv) overcome the efficient DNA repair pathways that protect CSCs’ genomes. Here we argue that hyperthermia, defined as local heating of the tumor to 40-42.5°C, is one anti-cancer treatment that may fit this challenging bill.

2. Cancer stem cells

The idea that a unique subset of cancer cells drives tumor (re)growth crystallized only a decade ago (Clarke et al., 2006; Li et al., 2007; Wicha et al., 2006), although the first hints of a unique population was found a decade earlier in acute myelogenous leukemia (Lapidot et al., 1994). It was shown that CD34+/CD38 leukemic cells have the capacity of tumor-initiation and expansion, even though they only represent a small fraction of transformed cells that are hiding in hypoxic hematopoietic stem cell niches. All other leukemic cells that were not CD34+/CD38 lacked the capacity to re-establish the disease (Bonnet & Dick, 1997; Lapidot et al, 1994). The study of Lapidot and colleagues (1994) described typical CSC features that are now routinely used to identify this population.
CSCs share some hallmarks of normal tissue stem cells, like the potential of self-renewal, unlimited cell proliferation and the ability to re-populate and re-establish entire tissue or organ (or, in this case, tumor) structures, even from a single cell (Clarke et al., 2006; Rycaj & Tang, 2014; Vermeulen et al., 2008). They may propagate by cell proliferation or originate from non-CSCs by de-differentiation (Gupta et al., 2011; Kreso et al., 2013; Swanson et al., 2016) and their plasticity is stimulated by the microenvironment (Swanson et al., 2016). Microenvironmental factors contributing to plasticity include low pH (Hjelmeland et al., 2011), hypoxia (Conley et al., 2012) and inflammation (Iliopoulos et al., 2011). Besides microenvironmental factors, de-differentiation of non-CSCs into CSCs is also induced by anti-cancer treatments, such as radiation therapy (Atkinson et al., 2010; Ghisolfi et al., 2012; Gomez-Casal et al., 2013; Lagadec et al., 2012; Salmina et al., 2010) and chemotherapy (Auffinger et al., 2014). Some CSCs are characterized by reduced or arrested cell cycle progression and by increased DNA repair capacity, which makes them more resistant to therapies than the bulk of the tumor (Cojoc et al., 2015; Orford & Scadden, 2008). Glioblastoma CSCs are found in hypoxic tumor areas, supported by the vascular stem cell niche, containing endothelial cells (Conley et al., 2012; Gilbertson & Rich, 2007). Several markers have been shown to identify CSCs in various tumor types (Medema, 2013). Some of these markers seem to characterize CSCs in multiple cancer types, while some tumors have their own stem cell signature which correlates with aggressiveness of the disease and can predict the survival of patients (Suva et al., 2009). CSCs are considered to drive tumor recurrence and therapy resistance and some of the CSC markers, like aldehyde dehydrogenase (ALDH) activity, are indicative of radioresistance (Kurth et al., 2015) and chemoresistance (Hilton, 1984), hinting that this population may be difficult to eradicate by conventional anti-cancer therapies.

3. Hyperthermia

Hyperthermia, defined here as local heating of the tumor to relatively moderate temperatures of 40-43°C for approximately one hour, is an excellent radiosensitizer and chemosensitizer effective in multiple tumor types (Cihoric et al., 2015; Colombo et al., 2003; Harima et al., 2016; Issels et al., 2010; Kim et al., 1988; LaRue & Vujaskovic, 1995; Overgaard et al., 1995; van der Zee et al., 2000; Wessalowski et al., 2013). Clinically, hyperthermia is always combined with either radiotherapy or chemotherapy (Gonzalez Gonzalez et al., 1995; Issels et al., 2016; van der Zee et al., 2000; Wust et al., 2002). When applied prior to conventional therapy, hyperthermia can increase blood flow, improve oxygenation and enhance the therapy effects by stimulating production of oxygen radicals (Horsman, 2016; Sen et al., 2011; Vujaskovic et al., 2000; Winslow et al., 2015). An increased blood flow creates higher oxygen levels, changes the pH in the tumor, influencing hypoxic,
malnourished and acidosis (Song et al., 1980; Vaupel & Kelleher, 2010; Wike-Hooley et al., 1984). When hyperthermia is applied simultaneously with or shortly after radiotherapy, hyperthermia can interfere with the repair of therapy-induced DNA damage, thereby indirectly contributing to tumor cell killing (Kampinga & Dikomey, 2001; Roti Roti, 2004). Hyperthermia can also activate heat shock proteins, triggering protein unfolding, thereby causing loss of functionality and cell death (Dubois et al., 1991). In all cases, hyperthermia can additionally enhance therapy responses by directly targeting the resistant hypoxic population (Horsman & Overgaard, 2007). Whatever the mechanisms, the treatment results in significantly improved local tumor control, reduction of metastasis and improved overall survival. For instance, in sarcoma patients the addition of hyperthermia to radiotherapy resulted in a 50% lower probability to develop distant metastases (Brizel et al., 1996) and in cervical cancer patients it caused fewer recurrences, lower metastasis formation and higher overall survival than radiotherapy alone (van der Zee & Gonzalez, 2002). A phase III clinical trial demonstrated a 15% increase in local progression-free survival for soft-tissue sarcoma patients when hyperthermia was added to chemotherapy (Issels et al., 2010). It is tempting to hypothesize that at least some of these effects are mediated by hyperthermia-mediated suppression of the CSC population via multiple avenues (Dewhirst et al., 2016), which will be discussed next.

4. Targeting the pro-CSC factors by hyperthermia

4.1. Quiescence and resistance to therapies targeting cycling cells
Like normal stem cells, CSCs exploit protective mechanisms that contribute to their survival and therapy resistance. Among these mechanisms is the maintenance of a quiescent state. Quiescent stem cells are characterized by their low RNA content and their lack of proliferation markers (Cheung & Rando, 2013). Quiescence may be a protective response triggered, at least in part, by hypoxia (see also paragraph 4.2) and poor nutrient supply (Vaupel, 2004) or a high cell density (Stoker, 1972). Since non-cycling cells are generally less susceptible to radiation and chemotherapeutic DNA-damaging agents (Baguley, 2010; Barendsen et al., 2001; Clevers, 2011), quiescence may be an important contributor to CSC therapy resistance (Orford & Scadden, 2008). Paradoxically, eradication of the cycling tumor cells arrests disease progression but it can also stimulate proliferation of quiescent cells that evaded therapy, which can then repopulate the tumor (Kim et al., 2011; Phillips et al., 2006; Vlashi et al., 2009).

Subsequently, cells enter S-phase, which is associated with increased (radio) resistance (Baumann et al., 2008). A combination of irradiation with hyperthermia activates cells from quiescence to S-phase (Zolzer et al., 1993). Proliferating cells have been found to be sensitive to hyperthermia (Bhuyan, 1979) and these
cells are also sensitive to chemotherapeutic agents and radiation. Although not all effects of hyperthermia are completely clear, previous studies have demonstrated that hyperthermia prolongs the cell cycle at G1/S transition (Higashikubo et al., 1989; Nishita et al., 1998; Sisken et al., 1965) and block cells in G2 (Oei et al., 2015a). Moreover, hyperthermia has been found to decrease the hypoxic fraction of quiescent cells (Masunaga et al., 2009; Masunaga et al., 1997). Therefore, hyperthermia in combination with conventional therapy may push CSCs out of quiescent state, after which they become more vulnerable to anti-cancer therapies.

4.2. Hypoxia
Oxygen supply is a key factor that affects tumor homeostasis and microenvironment. Rapidly dividing solid malignancies struggle to maintain sufficient oxygenation due to poor vascularization, increasing the likelihood of hypoxic regions (Brown & Giaccia, 1998; Li et al., 2009; Mao et al., 2013; Vaupel & Harrison, 2004; Vaupel et al., 1989). Tumor types in which hypoxia is common include malignant brain tumors (Rampling et al., 1994), melanomas (Lartigau et al., 1997), cervical carcinomas (Fyles et al., 2002), and soft tissue sarcomas (Nordsmark et al., 2001). Hypoxia is also correlated with poor prognosis and metastasis (Ratcliffe, 2013; Semenza, 2006; Semenza, 2013). For instance, patients with hypoxic soft tissue sarcomas have a significantly higher incidence of lung metastasis and shorter disease-free survival than those with less hypoxic tumors (Brizel et al., 1996; Nordsmark et al., 2001). Similarly, patients with hypoxic lymph node-negative cervical cancer treated with radiotherapy have a significantly higher risk of distant metastasis than patients with better oxygenated tumors (Fyles et al., 2002). Since oxygen stimulates induction of DNA damage by ionizing radiation and some chemotherapeutic agents (Horan et al., 1999), hypoxia can decrease the efficacy of treatment and thus protect tumor cells from therapy (Littlewood, 2001; Rockwell et al., 2009; Teicher et al., 1981).

Hypoxic areas are indispensable in any type of stem cell niche - either embryonic, adult or cancer. Hypoxia preserves the undifferentiated state of stem cells, reduces oxidative DNA damage (Gilbertson & Rich, 2007) and stimulates upregulation of markers associated with a stem-like phenotype (Heddleston et al., 2010). Importantly, the self-renewal and differentiation capacity of CSCs has been found to increase in regions deprived of oxygen (Gilbertson & Rich, 2007). In addition, hypoxia-inducible factor 1α (HIF1α), a protein abundantly expressed after radiation exposure (Moeller et al., 2004) and under hypoxic conditions, contributes to CSC proliferation and survival (Schwab et al., 2012). HIF1α can directly upregulate the activity of Notch signaling, a pathway promoting survival and self-renewal of CSCs (Quail et al., 2012), and CSCs with a reduced HIF1 activity were unable to form tumors in vitro (Jogi
et al., 2002). As a consequence, hypoxia has been suggested to promote the phenotype of CSCs, to enhance their tumorigenicity and to be a biomarker of radioresistance (Peitzsch et al., 2014; Vinogradov & Wei, 2012).

Hyperthermia appears to counteract some of the effects of hypoxia, primarily by stimulating the blood flow and increasing the leakiness of tumor blood vessels (Song, 1978). Hyperthermia has been shown to improve tumor oxygen supply in several clinical studies (Jones et al., 2004; Oleson, 1995; Song et al., 1996). This was not only evident in chronically hypoxic areas, where hyperthermia decreased the amount of quiescent cells considerably, but was also detectable in the total tumor cell population (Masunaga et al., 1997). In addition, re-oxygenation induces microenvironmental changes, such as decreased acidosis and induces cell cycle arrest, resulting in autophagy and eventually apoptosis (Wu et al., 2012).

4.3. Immune responses
Tumorigenesis can only occur when cancer cells evade the immune system. The immune response is critical for eliminating cancer cells based on the expression of tumor-associated antigens (Vesely et al., 2011). Additionally, inflammatory responses of the immune system can increase the burden of DNA damage and, as a consequence, induce tumorigenesis (Sakurai et al., 2008). On the other hand, inflammation can cause an increase in body temperature, which may activate specific heat shock proteins (HSPs), such as HSP70 (Bechtold et al., 2000). HSPs are potent immune modulators and can lead to stimulation of both the innate and adaptive immune responses against transformed cells. However, once a malignancy develops, immune cells, such as tumor-associated macrophages (TAMs), can also promote malignant cell growth by stimulating angiogenesis, invasion and metastasis (Condeelis & Pollard, 2006), and high levels of TAMs are correlated with poor prognosis (Murdoch et al., 2008). CD8+ and IFNα-producing helper T cells are also present frequently in the tumor microenvironment and they exert tumor and metastasis: promoting effects (Aspord et al., 2007; DeNardo et al., 2009; Hanada et al., 2006; Roberts et al., 2007). A recent study demonstrated that tumor-bearing mice had higher CSC-specific IgG levels in serum after vaccination with CSC lysate-activated dendritic cells (DCs). When administered complement, these mice eradicated CSCs more efficiently than mice vaccinated with whole tumor-activated DCs (Ning et al., 2012). Furthermore, CSCs were targeted more efficiently in vitro by cytotoxic T cells from these CSC-vaccinated mice. CSC-derived antigens can thus directly stimulate anti-CSC immune responses. On the other hand CSCs have been reported to suppress immune system functionality (Di Tomaso et al., 2010; Kawakami et al., 2012; Wei et al., 2010).
Anti-tumor immune responses can be stimulated by radiotherapy or chemotherapy (Calderwood et al., 2006) and enhanced by mild hyperthermia. Hyperthermia triggers the immune system by stimulating the production of HSPs (Wang et al., 2013) and their release by necrotic tumor cells (Basu et al., 2000; Sauter et al., 2000). HSPs, in turn, can trigger maturation of DCs, resulting in systemic anti-tumor response (Bleifuss et al., 2008; Chen et al., 2009; Schueller et al., 2003). Furthermore, hyperthermia can enhance antigen display, facilitating recognition of malignant cells by the immune system (Shi et al., 2006; Takahashi et al., 1995). In animal experiments, hyperthermia also increased levels of natural killer cells, resulting in significant tumor growth inhibition (Burd et al., 1998). In conclusion, these results suggest that hyperthermia can be exploited to potentiate anti-tumor and anti-CSC immune responses.

4.4. Microenvironment
The CSC population co-exists in a symbiotic relationship with the tumor microenvironment, which preserves and regulates their plasticity, but is also affected by their presence (Calabrese et al., 2007; Swanson et al., 2016). Many mechanisms are involved in this bi-directional communication, as summarized by Plaks and colleagues (Plaks et al., 2015) Endothelial cells play an essential role in CSC proliferation (Fessler et al., 2015). They secrete nitric oxide, activating the Notch signaling pathway (Charles et al., 2010) which controls cell fate, self-renewal, angiogenesis, and endothelium interactions in the CSC microenvironment. In glioblastoma, the Notch signaling causes radioresistance in CSCs (Wang et al., 2010) and inhibition of this pathway or angiogenesis can deplete the CSC population (Folkins et al., 2007; Hovinga et al., 2010). Furthermore, fibroblasts that inhabit the tumor microenvironment are essential for cell proliferation and for promotion of angiogenesis (Ingber, 2002).

Both endothelial cells and fibroblasts have been found to be extremely sensitive to hyperthermia (Kalamida et al., 2015). Hyperthermia can additionally inhibit endothelial cell adhesion (Nakabe et al., 2007) and cell proliferation and promote apoptosis (Basile et al., 2008). The resulting reorganization of CSC microenvironment can deprive CSCs of the factors that are essential for the maintenance of their stem-like potential and that promote their resistance to therapy.

4.5. Viral infections
Approximately 15% of human cancers are caused by infections with oncoviruses (zur Hausen, 1991). Infections with hepatitis B virus (Beasley & Hwang, 1984), Epstein-barr virus (Epstein et al., 1964), papilloma virus (zur Hausen et al,
1975) or herpes virus-8 (Chang et al., 1994) can initiate cancer. Fortunately, in most cases a healthy immune system is capable of eliminating these viruses. However, in a small number of cases, viral infections can slowly progress into cancer, which can take years or even decades (Liao, 2006).

Human papilloma virus (HPV) is a pathogen that has been shown to affect CSCs homeostasis. Recent studies suggest that HPV plays an important role in stimulating CSCs. This may be caused by production of the viral oncoproteins E6 and E7 that interfere with tumor suppressor proteins p53 and retinoblastoma protein (Rb), abolishing cell cycle regulation and apoptosis (Jin & Xu, 2015; Scheffner et al., 1990; Wise-Draper & Wells, 2008). Overexpression of E6 was found to promote stemness and self-renewal in HPV-cervical CSCs (Tyagi et al., 2016) and the ALDH1+ CSC population in HPV+ tumors was considerably higher than in HPV tumors (Zhang et al., 2014). Furthermore, various transcription factors that are overexpressed in non-CSCs and CSCs, such as Oct4, Nanog and Notch - essential for self-renewal and for re-establishing pluripotency - are altered by HPV infections (Swanson et al., 2016). HPV+ and HPV tumors differ in therapy sensitivity, which may be at least partly due to CSC fractions in these tumors (Lewis et al., 2012; Schwartz et al., 1998; Weinberger et al., 2006). Our recent study showed that hyperthermia temporarily inactivates the viral oncoprotein E6 (Oei et al., 2015a). Since E6 normally suppresses p53-mediated cell death in cervical cancer, it is plausible that hyperthermia may also block HPV-induced immortality.

Hyperthermia stimulates immune function and tumor immunogenicity which may affect the tumor microenvironment, and other viruses may be tackled successfully as well (Repasky et al., 2013). However, further investigations are needed to elucidate the anti-viral effects and eradication of viral related cancer cells.

4.6. DNA repair
CSCs are often characterized by enhanced activity of DNA repair pathways (Bao et al., 2006; Diehn et al., 2009). Increased expression of major DNA repair proteins, including BRCA1, ATR and ATM, has been observed in pancreatic, breast and prostate CSCs (Mathews et al., 2011a; Mathews et al., 2011b; Maugeri-Sacca et al., 2012). Reduced DNA repair capacity in CSCs has also been reported (reviewed by Wang and colleagues (Wang, 2015)). In any case, DNA-damaging therapies targeting CSCs certainly benefit from inhibition of DNA repair as well as from increasing the total DNA damage burden. Hyperthermia can likely perform both. First, elevated temperatures have been shown to inhibit pathways that are responsible for repairing DNA lesions that are relevant in clinical cancer treatment (Corry et al., 1977). More specifically, hyperthermia induces degradation of the BRCA2 protein and thereby inactivates homologous recombination, one of the major pathways responsible for
repairing DNA double-strand breaks (Eppink et al., 2012; Krawczyk et al., 2011). Furthermore, hyperthermia (above 43°C) has been found to interfere with base excision repair (Kampinga, 2006; Kampinga et al., 2004), leading to more severe breaks. Interference with DNA repair pathways is important to increase levels of DNA breaks, causing accumulation of unrepaired DNA lesions, resulting in cell death. Hyperthermia has been found to decrease the CSC population due to inhibition of DNA repair (Atkinson et al., 2010; Dewhirst et al., 2016; Pelicci et al., 2011; van Oorschot et al., 2016). Hyperthermia not only inhibits repair of DNA damage caused by radiation but also suppresses AKT signaling, a radiation-induced survival mechanism preferentially utilized by the CSC population (Man et al., 2015).

Second, it is becoming clear that hyperthermia can induce DNA damage by causing protein denaturation and interfering with DNA replication (reviewed by Oei and colleagues (Oei et al., 2015b)). There has been a long debate whether hyperthermia induces DNA damage or not, as increased (unrepaired) DNA damage is observed after combinational therapies that include hyperthermia in nearly all studies that tested the effectiveness of hyperthermia. Direct methods to detect immediate induction of DNA breaks failed in most of the studies as mild hyperthermia interferes predominantly with DNA repair pathways. Therefore, the effects of hyperthermia on (unrepaired) DNA damages may therefore be caused by indirect effects ((Takahashi et al., 2016) and reviewed by Oei and colleagues (Oei et al., 2015b)).

5. Summary and future perspectives

Conventional radiotherapy and chemotherapy target the bulk of the tumor but not the therapy-resistant CSCs, often resulting in tumor relapse (Figure 1). After tumor relapse, conventional therapies may be combined with hyperthermia, thereby increasing effectiveness of therapy. We argue that first-line conventional treatment combined with hyperthermia may directly sensitize CSCs by inducing leaky vessels that allow chemotherapeutics to reach deeper regions of the tumor; oxygen levels will rise (re-oxygenation) thereby increasing sensitivity to radiotherapy; increased number of DNA breaks and decrease of DNA repair cause accumulation of DNA damages; quiescent cells may repopulate after first doses of therapy and the immune system may be triggered to interfere with the CSCs microenvironment. All these factors may also prevent CSCs from acquiring resistance to therapy.

Hyperthermia is successful in sensitizing tumors with hypoxic areas to radiotherapy and chemotherapy. The question is whether the effectiveness of hyperthermia on hypoxic tumor tissue is mediated through a direct effect of hyperthermia on CSCs or by a change in the tumor microenvironment after
Figure 1: *Schematic overview of targeting CSCs by hyperthermia*. The tumor consists of many different cells types including CSCs. Conventional first-line radiotherapy and chemotherapy is able to destroy all tumor cells and especially CSCs that often reside in hypoxic areas. These cells are considered to drive tumor recurrence and metastasis formation. Combined with conventional first-line treatment, hyperthermia can target CSCs via multiple avenues, including stimulation of blood flow and re-oxygenation of hypoxic areas, causing blood vessel leakage, triggering the immune response and inhibiting DNA repair.
which the CSCs become more sensitive to radiotherapy or chemotherapy treatment, or both. One would expect that in case of a direct effect, hyperthermia affects the CSC population even without re-oxygenation of the CSC niche. However, clinical data seem to suggest that this re-oxygenation is needed for the effectiveness of hyperthermia (Jones et al., 2004). But we cannot be certain that this precludes a direct effect as radiotherapy is less effective in the absence of oxygen radicals and chemotherapy may not be delivered to poorly-perfused hypoxic areas. In other words, even when hyperthermia directly eliminates CSCs, the traditional modalities remain ineffective without reperfusion and re-oxygenation, and these modalities are needed to eliminate the non-CSC tumor cell population.

This suggests that hyperthermia can sensitize CSCs directly to traditional cancer therapy and that this sensitization may be the underlying mechanism responsible for at least part of the clinical success of hyperthermia. However, a thorough understanding of the mechanisms driving this sensitization is lacking. It is difficult to derive conclusions from the presently available data because any effects of hyperthermia on the CSCs are difficult to distinguish from other suggested mechanisms that can account for the established effects of hyperthermia on hypoxic tumor areas. This distinction requires further research, that focuses on determining reliable and specific CSC markers in preclinical in vitro and in vivo tumor models under various microenvironmental conditions.

Isolation of CSCs on the basis of expression of specific CSCs markers, can help to dissect how hyperthermia affects CSCs. However, it is essential to study the microenvironmental factors to eliminate and target the complete tumor. In vivo experiments should be focused on comparison of the effectiveness of radiotherapy and hyperthermia in aerobic and hypoxic tumor regions. The first important step to accomplish this is to visualize these hypoxic areas (Horsman et al., 2012) to facilitate investigations whether hyperthermia indeed targets the cells in these areas. Imaging the dynamics of tumor responses in different parts of the tumor is also important. Furthermore, specific markers of CSCs are necessary to detect in order to observe whether CSCs are preferentially located in the tumor areas targeted by hyperthermia and to monitor whether the frequencies of cells that show stemness characteristics decrease sufficiently in numbers that they lose their ability to repopulate tumors.

Unravelling this mechanism may have major clinical impact as it will permit more effective multi-modality strategies against therapy-resistant tumor types.
Acknowledgements
The authors wish to thank prof. dr. C.J.F. van Noorden for discussion and useful suggestions on the manuscript. This work was supported by the Dutch Cancer Society (grants #UVA 2008-4019, #UVA 2012-5540 and #UVA 2011-4962), NWO Medium grant (#40-00506-98-16015) and the Maurits en Anna de Kock foundation.
References


Diehn M, Cho RW, Lobo NA, Kalisky T, Dorie MJ, Kulp AN, Qian D, Lam JS, Ailles LE, Wong M,


Hanada T, Kobayashi T, Chinen T, Saeki K, Takaki H, Koga K, Minoda Y, Sanada T, Yoshioka


Ingber DE (2002) Cancer as a disease of epithelial-mesenchymal interactions and extracellular matrix regulation. *Differentiation; research in biological diversity* 70(9-10): 547-60


Kampinga HH, Dynlacht JR, Dikomey E (2004) Mechanism of radiosensitization by hyperthermia (> or = 43 degrees C) as derived from studies with DNA repair defective mutant cell lines. *International journal of hyperthermia : the official journal of European Society*


Song CW (1978) Effect of hyperthermia on vascular functions of normal tissues and experimental tumors; brief communication. *Journal of the National Cancer Institute* 60(3): 711-3


