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Review

# Functional studies on circulating and disseminated tumor cells in carcinoma patients

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ABSTRACT

Despite numerous clinical studies indicating the clinical relevance of circulating tumor cells (CTCs) in blood and disseminated tumor cells (DTCs) in the bone marrow of cancer patients, the functional properties of these cells are largely unknown. The focus of this review is to emphasize how functional studies on viable CTCs and DTCs can enlarge the spectrum of applications of "liquid biopsies". The low number of CTCs in the peripheral blood and DTCs in the bone marrow and the fact that carcinoma cells are difficult to culture are major challenges. Significant advances in the *in vitro* and *in vivo* expansion of CTCs and DTCs from cancer patients have been achieved, which enable us now to study the functional properties of these cells. Here, we discuss published data about functional studies on CTCs and DTCs using *in vitro* cultivation and *in vivo* xenograft models. Functional analyses on CTCs and DTCs offer the possibility to identify the metastasis-initiating cells. Moreover, CTC-derived cell lines and xenografts might point to new therapeutic targets and can be used for drug development.


## 1. Introduction

Numerous clinical studies have demonstrated strong correlations between circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) counts and clinical outcome in large multi-centre cohort studies on patients with different epithelial tumors such as breast and prostate cancer (Bidard et al., 2014; Goldkorn et al., 2014; Scher et al., 2015). In contrast, the functional properties of CTCs and DTCs are under investigated because these cells occur at very low concentrations in the peripheral blood and bone marrow of cancer patients (Alix-Panabieres and Pantel, 2014). A prerequisite for functional analyses was, therefore, the recent advances in our ability to culture epithelial tumor cells *in vitro* and establish patient-derived xenografts.

Short-term culture of CTCs after leukocytes depletion has been already realized for a decade by the EPISPOT technology (Deneve et al., 2013; Ramirez et al., 2014). Cells are

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cultured for a short time on a membrane coated with antibodies that capture the secreted/released/shed tumor associated proteins that are subsequently detected by secondary antibodies labeled with fluorochromes. Moreover, temporary cultivation of DTCs over several weeks was established 20 years ago (Pantel et al., 1995) and the proliferative activity of DTCs in culture predicted an unfavorable outcome (Solakoglu et al., 2002). More recently, several groups have developed appropriate conditions for long-term culture of CTCs and applied them to cancer patients at very advanced stages with higher amounts of CTCs. In addition, patient-derived xenografts have become a valuable tool in cancer research and have entered the stage of clinical use for testing drug sensitivity in individual cancer patients (Hodgkinson et al., 2014). Extending this technology to CTCs has led to the development of unique *in vivo* models for several tumor entities.

Here, we will emphasize the possibilities and current limitations of functional studies on CTCs and DTCs using *in vitro* and *in vivo* models. We will focus on breast, prostate, colon and lung cancer as the major tumor entities in industrialized countries. However, pilot studies indicate that CTC cultivation can be also achieved in other tumor entities such as pancreatic cancer (Bobek et al., 2014a; Kolostova et al., 2015b), esophageal cancer (Bobek et al., 2014b) and gastric cancer (Kolostova et al., 2015a). Besides insights into the complex biology of metastasis functional CTC and DTC studies can point to new targets and novel strategies for more efficient anti-metastatic therapies.

## 2. Functional studies in solid cancers

### 2.1. Breast cancer

#### 2.1.1. Short-term culture of CTCs (EPISPOT assay)

Using the EPISPOT assay, the release of cytokeratin-19 (CK19) and mucin-1 (MUC1) by breast cancer cells was measured, and the results demonstrated that many breast cancer patients harbored viable DTCs in their bone marrow, even if the tumors were classified as localized (stage $M_0$: 54%) (Alix-Panabieres et al., 2009). Most interestingly, patients with DTC-releasing CK19 in their bone marrow had an unfavorable outcome. In the subsequent study, peripheral blood samples from 194 $M_1$ breast cancer patients were analyzed by the EPISPOT assay. CTCs were identified as CK19-releasing cells (CK19-RC) and were correlated to an unfavorable clinical outcome (Ramirez et al., 2014).

The establishment of primary cultures from CTCs of breast cancer patients were reported for the first time by Zhang *et al.*; in this study CTCs from patients in advanced stage with brain metastases were cultured over weeks but no permanent cell lines were established (Zhang et al., 2013). In $EpCAM^{(-)}$ CTCs, a potential signature of brain metastasis comprising "brain metastasis selected markers (BMSMs)" $HER2^{(+)}/EGFR^{(+)}/HPSE^{(+)}/Notch1^{(+)}$ was identified. Cultured CTCs that expressed the BMSM signature were highly invasive and able to generate brain and lung metastases after xenografting into nude mice.

#### 2.1.2. Establishment of CTC lines

Recently, Yu *et al.* reported on oligoclonal CTC cultures that were sustained *in vitro* for more than 6 months. The cultured CTCs were isolated from 6 patients with metastatic luminal subtype breast cancer (Yu et al., 2014). Three of the five CTC lines tested were tumorigenic in mice. CTC lines revealed pre-existing mutations in the PIK3CA gene and newly acquired mutations in the estrogen receptor gene (ESR1), PIK3CA gene, and fibroblast growth factor receptor gene (FGFR2). Through drug sensitivity testing of the established CTC lines multiple mutations could be revealed as potential new therapeutic targets.

#### 2.1.3. Xenograft CTC assays ("avatars")

In the first xenograft model, CTCs from patients with metastatic luminal breast cancer were injected into the tibial bone of immunodeficient mice and gave rise to bone, lung and liver metastases (Baccelli et al., 2013). These metastases uniformly expressed EpCAM, CD44, CD47 and MET, which might be important for engraftment and metastatic outgrowth of CTCs. In a subsequent validation cohort, the number of $EpCAM^{(+)}CD44^{(+)}CD47^{(+)}MET^{(+)}$ CTCs, but not all $EpCAM^{(+)}$ CTCs, correlated with increased number of metastatic sites and poor prognosis. Although this report suggests that a special subset of CTCs might have potential metastasis-initiating activity, it should be noted that xenografts could be only obtained from advanced stage patients with high count of CTCs. Thus, future studies need to include early stage patients correlate their potential metastasis-initiator phenotype to the development of metastases. Obviously, this investigation will require long-term follow-up as well as more sensitive CTC assays.

Rossi *et al.* confirmed that CTCs have the potential to grow in immunodeficient mice (NOD/SCID) in a small pilot study on two breast cancer patients (Rossi et al., 2014). In contrast to Baccelli *et al.* who injected CTCs into the tibia, Rossi *et al.* injected CTCs subcutaneously. Thus, both routes of injection seem to work for the establishment of CTC xenografts in breast cancer and CTCs appear to sustain their migratory capacity in immunodeficient mice models.

### 2.2. Prostate cancer

#### 2.2.1. Short-term culture of CTCs (EPISPOT assay)

In the EPISPOT assay, prostate-specific antigen (PSA) secretion was used as marker to detect PSA-secreting cells in prostate cancer patients. In total, 83% and 42% of $M_1$ & $M_0$ cancer patients, respectively, and higher CTC counts were observed in metastatic patients as compared to earlier disease stages (Alix-Panabieres et al., 2005). Importantly, a fraction of CTCs secreted fibroblast growth factor-2 (FGF-2), a known stem cell growth factor (Alix-Panabieres et al., 2005, 2007). Further studies will show whether this subset of CTCs has an increased ability to initiate overt metastases.

Recently, Kolostova *et al.* used size-based filtration of CTCs and were able to culture CTCs from patients with localized prostate cancer for 7–28 days (Kolostova et al., 2014). Cytokeratin-positive cells with a proliferative capacity were observed in 18 of 28 CTC-positive patients.



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