Mapping the systemic recruitment of ferritin expressing fibroblasts to the angiogenic rim of ovarian tumors

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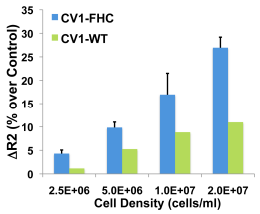
Introduction Recruitment of fibroblasts by solid tumors plays a critical role in initiation, progression and metastatic dissemination. As such, cancer associated fibroblasts (CAFs) are attractive as potential avenues for novel anti-cancer therapies. A number of *in vivo* imaging methods were recently introduced to monitor fibroblast recruitment, including *in vivo* fluorescence, as well as MRI cell labeling and tracking with gadolinium or iron oxide particles (1). Recently, we and others introduced the over-expression of ferritin, a protein responsible for iron homeostasis, for *in vivo* MRI tagging of cells (2-4). However, due to concerns of low sensitivity for detection of ferritin, no study has examined ferritin over-expression for MR imaging of cell recruitment to remote organs. In the present study, we comprehensively examined the potential of ferritin over-expression for MRI tracking of fibroblasts recruitment to human ovarian carcinoma both *in vitro,* and *in vivo*. **Methods** CV-1 fibroblasts, an established cell line, were transfected to stably over-express HA-tagged human ferritin heavy chain (CV1-FHC) or the red fluorescent protein tomato (CV1-tomato). All MR imaging was performed on a horizontal bore 9.4T scanner (Bruker, Ettlingen Germany) using a cylindrical birdcage coil. *In Vitro* CV1-FHC as well as naive CV-1 fibroblasts (WT) were incubated in cell culture medium supplemented with 1mM ferric citrate for 48 hours. Cells were washed and either immediately harvested (D1), or grown for an additional 7 days in normal medium (D7). At each time point, CV1-FHC, WT, and control cells were suspended in 1% agarose at several cell densities (2.5, 5, 10, 20x10^6 cells/ml). R2 was quantified using a multi-slice multi-spin echo MRI pulse sequence (MSME, TE=11ms*60 echoes, TR=3s, FOV=6x4cm, Matrix = 256x256). ΔR2 was calculated for CV1-FHC and WT cells as the percent change over corresponding control cells. Cellular iron content was quantified after MRI using inductively coupled plasma mass spectrometry. *In Vivo Co-injection* Tumors were generated using a human ovarian epithelial carcinoma cell line transfected to express the fluorescent protein eGFP (MLS). 4x10^6 MLS cancer cells were mixed with either 1x10^6 CV1-tomato fibroblasts (Control, n=5), or 1x10^6 CV1-FHC (D1) and 1x10^6 CV1-tomato fibroblasts (Ferritin, n=5), and injected subcutaneously into the hind limb of CD-1 nude female mice. MSME acquisition (TE=7.7ms*30echos, TR=3s, FOV= 2.8x2.8cm, Matrix=256x256, AV=3) was performed on days 3, 5, and 10 post injection in order to quantify tumor R2. The tumor boundary was determined using T2-weighted RARE images acquired following bolus intraperitoneal (IP) injection of gadolinium-DTPA. Whole body *in vivo* fluorescence imaging was performed on days 1, 3, and 10 using an IVIS Spectrum (Caliper Life Sciences, Hopkinton, MA) imaging system. *In Vivo Recruitment* 4x10^6 MLS cells were subcutaneously injected, and four days later either 1x10^6 CV1-FHC (D1) and 1x10^6 CV1-DiR (Ferritin, n=5), or just 1x10^6 CV1-DiR (Control, n=5) fibroblasts were delivered via IP injection. Recruitment of fibroblasts to the tumor was quantitatively monitored using R2 mapping at days 0, 2, 4, 7, and 9 post fibroblast injection, and was confirmed by *in vivo* fluorescence imaging at days 7 and 9.

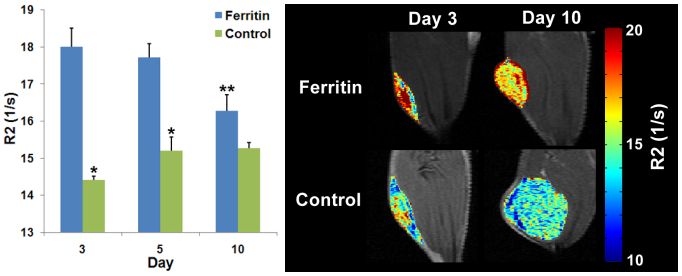
Results *In Vitro quantification of ferritin-expressing fibroblasts.* FHC over-expression was confirmed using Western blot analysis. CV1-FHC fibroblasts demonstrated significantly enhanced cellular iron content immediately (D1: 1.8±0.1 pmoles vs. 0.9±0.3 pmoles WT, p<0.05) and one week (D7: 1.6±0.2 pmoles vs. 0.5±0.1 pmoles WT, p<0.05) after removal of the iron-supplemented medium, which resulted in enhanced ΔR2 at all cell densities compared to WT at both time points. Sample data acquired at D7 at an array of cell densities is illustrated in Fig 1.

Figure 1. *In vitro* detection of enhanced relaxation in fibroblasts which over-express ferritin (D7). Ferritin over-expression resulted in greater ΔR2 compared to WT cells. MRI measurements suggested a close relationship between cell density and ΔR2.



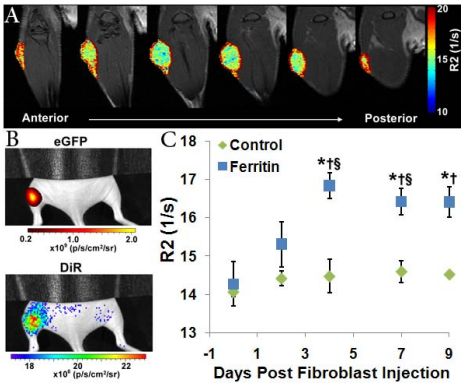
In Vivo rearrangement of co-innoculated ferritin-expressing fibroblasts. Tumors initiated with CV1-FHC fibroblasts (Ferritin) demonstrated significantly higher mean R2 compared to Control tumors at days 3 and 5 after injection (Fig 2). R2 mapping suggested migration of CV1-FHC fibroblasts from the core to the tumor rim by day 10 (Fig 2).

Figure 2. Spatial re-arrangement of fibroblasts co-inoculated with tumor cells. (Left) Mean tumor R2 was significantly enhanced in Ferritin vs. Control mice at 3-5 days after injection (*p<0.05 vs. Ferritin, **p<0.05 vs. Days 3&5). (Right) Representative R2 maps following co-injection of FHC-over expressing (Ferritin) or control fibroblasts demonstrate enhanced relaxation in Ferritin tumors.



In Vivo systemic Recruitment of ferritin-expressing fibroblasts R2 mapping (Fig 3A) illustrated preferential recruitment of CV1-FHC cells to the tumor rim, in agreement with prior SPIO particle labeling studies (1). Fluorescence imaging confirmed spatial co-localization of CV1-DiR signal with MLS-EGFP signal (Fig 3B). The time-course of tumor R2 measurements illustrated recruitment of CV1-FHC cells as early as 2 days after IP injection, with maintained recruitment at subsequent time points (Fig 3C). ΔR2 between tumors and skeletal muscle on the opposing leg served as an internal control and mirrored R2 measurements. Additionally, there existed a statistically significant correlation between R2 and distance from the tumor core in Ferritin, but not control tumors.

Figure 3. In vivo detection of systemic recruitment of ferritin tagged fibroblasts by a remote tumor. (A) Representative R2 maps (day 7 post injection) of a hind limb tumor illustrate preferential recruitment of FHC-over expressing fibroblasts to the tumor rim. (B) In vivo fluorescence imaging confirmed the recruitment of CV-1 fibroblasts (DiR) to the tumor (eGFP), and not to the opposing limb. (C) Tumor R2 measurements elucidate the time-course of fibroblast recruitment (*p<0.05 vs. Control, †p<0.05 vs. Day 0, §p<0.05 vs. Day 2).



Conclusions Over-expression of ferritin, with proper *ex vivo* preparation, can be used to label and track fibroblasts involved in tumor growth and metastasis, and could be used to more quantitatively examine novel therapies which seek to inhibit tumor metastasis by modulating fibroblast recruitment. Our results further suggest that this method could be expanded to a number of disease models in which cell recruitment plays an important role.

(1) Granot et al. Cancer Res. 2007;67(19):9180-9. (2) Campan et al. AJP Heart. 2011;300(6):2238-2250. (3) Naumova et al. Mol Imaging. 2010;9(4):201-10. (4) Cohen et al. Neoplasia. 2005;7(2):109-17. Acknowledgements: R01 CA75334 US National Cancer Institute, European Commission 7th Framework Integrated Project ENCITE, and European Research Council Advanced grant 232640-IMAGO to MN, Whitaker International Scholarship to MV.