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, 20x10⁵ cells/ml). R2 was quantified using a multi-slice multi-spin echo MRI pulse sequence (MSME, TE=11ms*60 echoes, TR=3s, Matrix = 256x256). AR2 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). 4x10⁶ MLS cancer cells were mixed with either 1x10⁶ CV1-tomato fibroblasts (Control, n=5), or 1x10⁶ CV1-FHC (D1) and 1x10⁶ CV1-tomato fibroblasts (Ferritin, n=5), and injected subcutaneously into the hind limb of CD-1 nude female mice. MSME acquisition (TE=7.7ms*30echoes, 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 4x10⁶ MLS cells were subcutaneously injected, and four days later either 1x10⁶ CV1-FHC (D1) and 1x10⁶ CV1-DiR (Ferritin, n=5), or just 1x10⁶ CV1-DiR (Control, n=5) fibroblasts were delivered via IP injection. Recruitment of fibroblasts to the 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 WT, p<0.05) after removal of the iron-supplemented medium, which resulted in enhanced AR2 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.

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).

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 SPION 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). AR2 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.

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.
