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Abstract* – *A subtype of metastatic breast cancer has recently been identified, in which netrin-1 is over-expressed. Netrin-1 binds to dependence receptors inducing survival, while its absence actively triggers apoptosis. An anti-netrin-1 therapy is under development, which disrupts ligand-receptor interaction and induces apoptosis. To identify potential responders for such a targeted therapy, netrin-1 expression has to be analyzed. Molecular imaging with ultrasound (US) is able to detect the expression of a specific protein when using targeted contrast agents. Thus, we aim to develop an US molecular imaging approach using microbubbles (MB) functionalized with an anti-netrin-1 antibody. Binding was tested on purified netrin-1 protein in static and dynamic conditions. Anti-netrin-1 MB were validated in in-vitro assays and will be further tested in pre-clinical studies using metastatic breast cancer animal models.

Index terms - Contrast agents, Molecular Imaging, Ultrasound.

I. INTRODUCTION

Breast cancer is one of the most frequent causes of cancer death in women and forms a large group of heterogeneous diseases [1]. Traditional treatments for breast cancer (surgery, radio-, drug therapy) cause strong side effects by attacking normal cells and are inefficient against relapse of aggressive metastatic cancers. Thus, new targeted therapies are required specifically attacking cancer cells with a metastatic profile while omitting normal cells.
Netrin-1 is a diffusible protein with metastatic signaling activity. As a ligand of so-called dependence receptors, netrin-1 triggers survival upon binding to these receptors [2]. In absence of ligand, dependence receptors actively trigger apoptosis conferring them a tumor suppressor function. During tumor progression, tumor cells can lose their dependence receptors, inactivate the downstream signaling, or induce an autocrine expression of ligand to maintain survival signaling. The latter has been shown for metastatic breast cancer [2] and motivated the development of an anti-netrin-1 therapy (data unpublished).
Molecular imaging has the potential to reveal the expression of a specific marker in a non-invasive and quick manner; thereby paving the way to personalized medicine by screening patients effectively expressing the targeted molecular abnormality. Different molecular imaging modalities are currently under development whereof ultrasound (US) imaging has a huge potential for clinical use with a wide availability, spatial and temporal resolution, and sensitivity for contrast agents [3].
Classical US imaging provides anatomical information, while contrast-enhanced US (CEUS) provide functional information on vascularization and blood volume. For the latter, microbubble (MB) contrast agents are used that are limited to the vascularization due to a size of 1-4 µm. They are composed of a gas core for echogenic response, which is stabilized by a lipid monolayer shell. Targeting ligands can be grafted onto the shell e.g. via non-covalent interaction using biotin-avidin. After injection and a short binding period, ultrasonography of targeted MB reveals the expression level of the target.
In our project, we aim at the development of a netrin-1-targeted US molecular imaging approach by loading an anti-netrin-1 antibody (Ab) on lipid-coated MB.

II. MATERIALS AND METHODS

II.1. **Functionalization of microbubbles**

A humanized anti-netrin-1 monoclonal IgG1 Ab (Netris-Pharma) was biotinylated using hydrazide biotin (Ref #21339, Thermo Scientific). The degree of biotinylation was determined with HABA/Avidin Reagent (Ref #H2153, Sigma). US contrast agent Vevo MicroMarker® Target-Ready (Ref #VS-11915, VisualSonics) were loaded with biotinylated anti-netrin-1 Ab according to the manufacturer. Titration of free anti-netrin-1 Ab was done via enzyme-linked immunosorbent assay: 96-well plates were coated with Fc-netrin-1 fusion protein (AG-40B-0075, AdipoGen) and incubated with the infranatant of centrifuged anti-netrin-1 MB. Captured anti-netrin-1 Ab was revealed with Avidin-HRP (Ref #A-115, Boston Biochem) and TMB substrate (Ref #0440, Sigma), and quantified using a FluoroMax-4 spectrometer (Horiba).

II.2. **Static and dynamic binding assays**

For static binding experiments, MB were incubated in 24-well plates, either coated with netrin-1 protein or blocked with BSA (15% w/v), by gentle rocking. Plates were

washed on a vortex. Binding was analyzed with an Axiovert 200M microscope (Zeiss) in scan slide acquisition mode. For blocking experiments, non-biotinylated anti-netrin-1 Ab was employed.
Dynamic binding experiments were performed using a Parallel Plate Flow Chamber (Ref #31-001, GlycoTech) adapted to 35 mm Corning dishes, which were previously coated with netrin-1 or blocked with BSA. Shear stress was set with a syringe pump (PHD ULTRA, Harvard Apparatus). Binding was analyzed with a Laborlux S microscope (Leitz) in time lapse acquisition mode.

III. RESULTS

On average, 4.7 hydrazide biotin molecules were added to the carbohydrates of each Ab, to allow their coupling to the avidin molecules of the MB shell.
To functionalize MB, different Ab concentrations were tested and free Ab was quantified. A maximum concentration of 10 µg/mL was coupled to a vial of MB without leaving detectable free Ab in solution.
Binding specificity of netrin-1-targeted MB was tested in a static binding assay. Binding on netrin-1, at concentrations varying from 0 to 4 µg/mL, showed the highest coverage with MB for the highest concentration of netrin-1 (Figure 1). Unspecific binding was neither observed on BSA control protein nor on netrin-1 that was previously blocked with a blocking Ab. Thus, binding occurred specifically to the expected epitope.

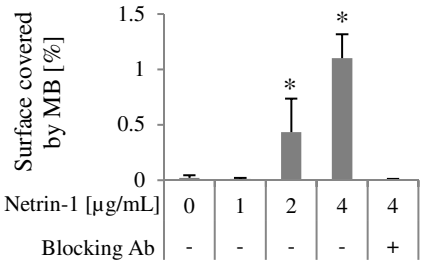

Figure 1: Static binding assay with netrin-1-targeted MB. *: Binding was significantly different ($P<0.05$) from controls.

Dynamic binding of anti-netrin-1 MB was tested at physiological shear stresses of 1 and 2 dynes/cm². Binding increased with ongoing inflow of MB in netrin-1-coated areas. No binding occurred in BSA-blocked areas. The number of attached MB was higher at the lower shear stress.

IV. DISCUSSION – CONCLUSION

Targeted molecular US imaging has been developed for markers like VEGFR2 and $\alpha_v\beta_3$-integrin, which are associated with angiogenesis [4]. Our approach targets a marker associated with metastasis. Thus, visualization of netrin-1-expressing cancers might give complementary information about aggressiveness.

Our molecular imaging approach is developed in parallel to a netrin-1-targeted therapy. Patient selection for such targeted therapies is extremely important, due to the high level of heterogeneity among cancers and within one cancer. Our molecular imaging technique could therefore help to identify potential responders and exclude patients not expressing netrin-1.
Future cancer treatments will require combinations of targeted therapies to fight all cancer cells. Thus, the adaptability of the presented MB functionalization approach is beneficial as further appropriate ligands can be selected and coupled. But a first targeted US contrast agent has to be clinically-approved which might then promote further developments.
For breast cancer, contrast-enhanced US is already part of a routine clinical diagnosis [5], and US molecular imaging could be an appealing modality to extend and improve the clinical algorithm for the diagnosis and triage of patients.
In our study, anti-netrin-1 MB were functional in static and dynamic in-vitro binding assays. Binding capacity and specificity have to be further validated on cells and in the presence of serum to simulate more physiologic conditions. Then, a pre-clinical study will serve to evaluate the feasibility of the molecular imaging approach in a metastatic breast cancer animal model.

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