Commentary: Sortilin Inhibition Limits Secretion-induced Progranulin-dependent Breast Cancer Progression and Cancer Stem Cell Expansion

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The genetically conserved glycoprotein progranulin is a pleiotropic factor that is implicated in wound healing, inflammation, neurodegeneration, lysosomal function and tumorigenesis among other biological events1,2. Progranulin is ubiquitously expressed and the fact that progranulin is genetically conserved and has a wide-ranging expression pattern indicates important biological functions. Progranulin acts as a holoprotein that can be cleaved into smaller granulin peptide domains by the activity of proteases, such as elastase and cathepsin L3. Cleavage of progranulin occurs in the extracellular matrix or within the lysosome. Interestingly, cleaved granulin peptides are described to display different biological functions compared to the precursor progranulin in wound healing, inflammation and neurodegeneration4,5. In breast cancer, high progranulin expression is associated with reduced disease-free and overall survival6,7 and is identified as a prognostic marker for ERα positive breast cancer8. Rhost and Hughes et al. recently reported that progranulin is differentially secreted by ERα positive and ERα negative breast cancer cell lines9. ERα negative cells showed high constitutive secretion of progranulin whereas the progranulin secretion fluctuated in ERα positive cells during microenvironmental stress, such as low oxygen. These results imply that subtypes of breast cancer regulate their progranulin secretion differently. Additional data obtained from Rhost et al. using ERα positive ductal carcinoma in situ (DCIS) samples with a hypoxic central area as an in vivo relevant model for hypoxia, revealed a clear gradient of progranulin expression towards the necrotic core (Figure 1). The increase of progranulin was further paralleled by an increase of HIF1α and a decrease of ERα towards the inner edge of the DCIS lesions validating the association between progranulin and hypoxia as well as to ERα. These data are in line with Lu et al. showing that overexpression of progranulin in ERα positive MCF7 cells induced estrogen independent proliferation via activation of cyclin D1 as well as tamoxifen resistance10. Collective data also support that hypoxia is not a solitary and local phenomenon but can actually influence distant biological processes including tumor progression and cancer treatment resistance. Growing evidence that the oxygen content of tumor tissue is an important determinant of metastasis11,12,13 further highlight the importance of the findings reported by Rhost and Hughes et al.

An interesting finding reported by Rhost and Hughes et al., is that recombinant progranulin and the granulin domain A peptide induced breast cancer stem cell propagation, both in vitro and in
vivo, using ERα positive and ERα negative breast cancer cells. These results demonstrate that progranulin and granulin A indeed induce a more malignant form of breast cancer with a significantly increased capacity to form metastasis. The fact that granulin A mediates breast cancer stem cell spreading suggests that enzymatic cleavage of progranulin might play a role in rendering a more aggressive tumorigenesis. Although, previous results published by Sayers et al. describe contrasting results with an association of high secretion of Secretory Leukocyte Protease Inhibitor (SLPI) in highly metastatic cells14. SLPI nevertheless protects progranulin from enzymatic cleavage which indicates that inhibiting cleavage of progranulin induce more metastatic cells. The general findings demonstrate that both progranulin and granulin peptides most likely are involved in tumor progression and future studies need to clarify the exact role for the granulins in the development of metastatic disease.

To improve our understanding of how progranulin and granulin induced breast cancer stem cell propagation, Rhost et al. further investigated the activation of several different kinases during progranulin and granulin treatment using a human phospho-kinase array. Data indicated that both progranulin and granulin A induced phosphorylation of several kinases, such as GSK3α/β EGFR, MSK1/2, AMPKα1, AKT1/2/3, and mTOR, where progranulin was a more potent activator compared to granulin A (Figure 2). These results indicate that both progranulin and granulin A substantially influence several major intracellular signaling pathways. Interestingly, the PI3K/AKT/mTOR pathway is implicated in endocrine resistant ERα positive breast cancer15 where it was shown that AKT activated the estrogen receptor pathway independently of estrogen availability. Further, mTOR inhibitors in combination with endocrine therapy was shown to overcome endocrine therapy resistance. The fact that progranulin induces AKT/mTOR phosphorylation suggests a possible additional link between endocrine therapy resistance and progranulin via the AKT/mTOR pathway.

Detailed analyses at single-cell level further revealed that progranulin and its receptor sortilin were both associated with signature genes for differentiation and epithelial-mesenchymal transition (EMT) properties demonstrating that differentiated cancer cells respond to progranulin in an autocrine signalling pathway, whereby the sortilin (SORT1) positive subpopulation gradually shifted...
from highly differentiated cell types to more cancer stem cells-like cells. Sortilin itself is reported to be associated with breast cancer aggressiveness. Importantly, the spreading of breast cancer stem cells induced by granulin A could be blocked by the orally bioavailable small molecule AF38469, binding to sortilin. Further, elevated levels of progranulin or granulin A impacted breast cancer stem cells similarly through the receptor sortilin, despite subtype of breast cancer. Progranulin and granulin A induced breast cancer stem cell spreading resulted in a more aggressive cancer with a significant increase in metastasis formation.

To further validate the findings clinically, we performed additional single-cell studies using primary breast cancer cells obtained from surgery to determine the relation between progranulin, sortilin, and subpopulations defined by differentiation and cancer stem cell markers as earlier established. In total, 184 individual cells from an ERα positive breast cancer and corresponding axillary lymph node metastasis were analysed using principal component analysis. SORT1 and GRN clustered with the differentiation associated genes in the primary tumor (Figure 3A) and upon metastatic spread, the expression of SORT1, GRN and a number of proliferative markers nevertheless clustered with cancer stem cell associated genes (Figure 3B). These results suggest that the SORT1 expressing population in the primary tumor was associated with highly differentiated cells, whereas SORT1 expressing cells during disease progression associated with genes related to cancer stem cell features.

Collective data concerning progranulin and sortilin presence in cancer cells and the now reported link to cancer stem cells and cancer progression could be of interest for future cancer therapy design with the potential to develop a unique cancer treatment based on selective targeting cancer stem cell propagation through sortilin. Sortilin is apart from being a receptor to progranulin also described to bind with high-affinity to the proinflammatory cytokines IL6. In breast cancer, IL6 is associated with increased tumor stage, lymph node infiltration, recurrence, and treatment resistance further signifying the role of sortilin in breast cancer progression.

Material and Methods

Single-cell qPCR analysis

Single cell analyses were performed as earlier described. The analyses of primary tumor samples were analysed according to the protocol described in Akrap et al. Human Phospho-Kinase Antibody Array

The phospho-kinase antibody array was performed according to the protocol provided by the manufacturer (R&D systems). Cell lysates from MCF7 cells treated with progranulin (1 µg/ml) or granulin A (1 µg/ml) for 50 minutes were analysed for phosphorylation of several different kinases using the human phospho-kinase antibody array.

Immunohistochemistry

Tissue sections were fixed with 4 % phosphate buffered formaldehyde, embedded in paraffin and cut into 4.5 µm thick sections. Immunohistochemistry was performed using DAKO Autostainer LINK 48 using Envision FLEX+ detection system. Briefly, deparaffinized sections were

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**Figure 3**: Differentiated sortilin positive cells change their transcriptional signature during disease progression in patient derived tumor samples. Single cell analyses of GRN and SORT1 (black) and sets of differentiation (blue), proliferation (green) and cancer stem cell-related (red) transcripts and the parallel gene cluster principal component analysis of (a) a primary ERα positive breast cancer and (b) the corresponding axillary lymph node metastases.
subjected to antigen retrieval by high pressure cocking and DIVA antigen retrieval pH 6.2, followed by blocking with 3% hydrogen peroxide and incubation with primary antibody against HIF1α (ab82832, abcam), progranulin (MA1-187, Thermo scientific), CA-9 (ab15086, Abcam), ER α (M7047, DAKO) at RT for 1 hour. For signal amplification EnVision™ FLEX+ rabbit linker (SM805, DAKO) and EnVision™ FLEX+ mouse linker (SM804, DAKO) was used, respectively. Further EnVision FLEX/HRP visualization reagent EnVision™ FLEX/HRP secondary antibody-coated polymer peroxidase complexes (#SM802, DAKO), followed by DAB substrate/chromogen (DAKO) was used. Slides were counterstained with hematoxylin (DAKO). Stained sections were scanned by Leica SCN400 scanner at 20X and evaluated by the automated image analysis Definiens Developer XD tissue studio program.

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