A molecular signalling network in breast cancer metastasis:
Finding new targets, designing new drugs.

A Sister's Hope project summary

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Clinical treatment of breast cancer has shown great improvements over the last decades, however several subtypes of breast tumours remain for which currently few successful treatment options are available, triple negative breast cancers being foremost among them. Due to their aggressive nature these tumours are prone to form metastases, often the leading cause of mortality.

In our previous work we have given new definition to an already well-known protein in the field of cancer: Fos-related antigen 1 (Fra-1). Though numerous publications have described a role for Fra-1 in various types of cancers [1, 2], we were the first to discover the prognostic potential of this transcription factor in the context of metastatic breast cancer. Many metastatic breast cancer subtypes overexpress Fra-1. We have developed a Fra-1 target gene expression classifier that can accurately predict breast cancer recurrence, and which was stratified in a large collection of available patient datasets. Besides having a prognostic value, we were also able to demonstrate *in vitro* that genetically inhibiting Fra-1 strongly reduces the migratory and invasive capacities of the triple negative breast cancer cell line MDA-MB-231. *In vivo,* Fra-1 depletion strongly decreased the formation and outgrowth of metastases in the lung, which almost doubled the survival time of these animals [3].

Seeing that Fra-1 has such a powerful role in breast cancer metastasis, the necessary step to undertake was finding a drug that could repress Fra-1 action. Unfortunately, there is no inhibitor of Fra-1 and pursuing development of such an inhibitor would be highly time-consuming, because transcription factors typically are poor drug targets. Having established that Fra-1 can activate a multitude of genes, we performed a functional screen to identify which Fra-1 targets contribute to breast cancer metastasis. The results of this screen yielded a list of target genes. We have reason to believe that some of them reside in a signalling network, which we are currently pursuing. Generation of this list and the pursuit of the discovered targets therein form the basis of our project, funded by A Sister's Hope.

The target genes on our hit list that we gave most attention are those that have readily available inhibitors on the market, or those that are molecules for which it is possible to develop inhibitors (like receptors or enzymes). This makes for a fairly straightforward approach to test, both *in vitro* and *in vivo*, whether these individual Fra-1 target genes have a comparable role in breast cancer progression/metastasis to Fra-1 itself.

The first target gene we tested was the Adenosine A_2B receptor (ADORA2B). We have shown that through genetic inhibition of ADORA2B we observed an effect on the migration and invasion properties of triple negative MDA-MB-231 breast cancer cells *in vitro*, and also could reduce tumour burden in the lungs of an *in vivo* experimental lung metastasis model – similar to the effects of genetic inhibition of Fra-1. Chemical inhibition of ADORA2B is clinically already applied to treat certain forms of asthma. It also was published that inhibition of ADORA2B could improve the effects of the standardised chemotherapeutic Paclitaxel to treat melanoma *in vivo* [4]. We have applied this same strategy in our *in vivo* model of lung metastasis and treated with the combination of Docetaxel – a common chemotherapy in breast cancer – with the ADORA2B inhibitor Theophylline. The results of this experiment showed that Theophylline was capable of improving the effects of Docetaxel by seven-fold. Besides that we have shown that chemical inhibition with theophylline acts through a similar mechanism compared to genetic inhibition with short-hairpin RNA technology. These results were confirmed by using other described and more specific inhibitors of ADORA2B and thus we can say that ADORA2B forms an important chain-link in the Fra-1 target network that particularly interferes with the breast cancer cells' ability to migrate and invade, as demonstrated by the reduction in the formation of filopodia – cellular protrusions responsible for direction and movement (Figure 1) [3].

In the meantime, knowing that the effects of chemical inhibition of ADORA2B was not enough to nullify tumour burden by itself, or even in combination with Docetaxel, we have started investigating the other candidate genes on our hit list in order to find other, complimentary effectors of breast cancer progression that through inhibition could potentially synergise with chemotherapy and/or ADORA2B inhibition.

One particular gene on our list has drawn our attention because the effects of genetically inhibiting this gene with shRNAs are remarkably strong on the outgrowth of lung metastases *in vivo*, but most striking is that knocking down this gene also has a very strong reducing effect on the growth of primary breast tumours *in vivo*. Genetic knockdown caused the tumour burden in the lungs and the primary tumour growth to be suppressed by at least ten-fold, in some cases even showing a complete absence of tumour formation (Gallenne et al., man. in prep.). These data suggest that this target gene represents a very good candidate for drug development. The protein, being an enzyme, allows for the design of a chemical compound capable of inhibiting it.

Our institute lacks the expertise it takes to develop such a drug and so we have attracted a partner to team up with that focuses on the chemical and structural biology aspects. Together with MRC-T UK we now have an on-going program to identify and establish small molecule inhibitors of this enzyme, which was met with a lot of enthusiasm from the scientific board and staff of MRC-T UK. Their team consists of about fifteen multi-disciplinary experts that have shown great progress over the last two years. During our collaboration in 2012 we have managed to obtain large quantities of purified enzyme. As a first step to discover which chemical

compounds might be capable of inhibiting its enzymatic activity, this purified enzyme will be screened accordingly against several tens of thousands of chemical compounds which are available through the many compound-libraries accessible to MRC-T.

The screening of the available libraries has taken the larger part of 2013 to perform, but by now are all finished. Through periodic updates between MRC-T and NKI we learned that their screening efforts have resulted in a list of compounds that could potentially inhibit the enzyme. However, these compounds are often not suitable (yet) for direct use *in vitro* or *in vivo* and so MRC-T is now researching which of the generated hit compounds can be used for further study, and which would require certain chemical modification.

As a next step, we will use available hit compounds *in vitro* to determine whether they have anti-tumorigenic activity in a panel of breast cancer cell lines. Parameters to test are for instance proliferation, survival, migration and invasion, as was also done for Fra-1 and ADORA2B. In parallel with the drug-screens performed at MRC-T, their team of cell biologists as well as our group at NKI have employed a multitude of *in vitro* assay systems that can assess such parameters on a relatively large scale ranging from high- to medium-throughput and should provide us with an efficient way of screening hit compound functionality in a cell-based setting.

Once compounds fit the criteria that we need to address, we can expand our efforts in the pre-clinical setting and validate them *in vivo* in both our experimental lung metastasis model, but surely also the primary breast tumour growth model. In this phase we will examine the anti-tumour effect of the discovered compound as standalone treatment, but also as a combination treatment with chemotherapy like Docetaxel as before, and/or other substances like the ADORA2B inhibitor.

Our team at NKI eagerly awaits the coming of the first compounds from MRC-T. While this is all on-going, we have developed an alternative approach to target this enzyme *in vitro* and *in vivo*. While this approach cannot be translated directly into clinical treatment options, it nonetheless holds clinical relevance. We have developed a vector with an inducible shRNA cassette against the enzyme, this means that under doxycycline treatment the tumour cells infected with our construct will express shRNAs that genetically silence the target gene. The clinical relevance of this system is that we can now start targeting the enzyme once a tumour – or lung metastasis – has already formed, which of course mimics the patient situation much more closely. In other words: with this system we will be able to tell whether the target enzyme only holds relevance for the initiation of a tumour, or whether it is important for tumour maintenance, the latter naturally being of great importance for determining if (clinical) treatment with an inhibitor would be beneficial, and will give us an insight on the application of any potential inhibitor that comes our way.

As a final part of our project we are trying to link all the Fra-1 target genes of our list together in order to understand more of the molecular mechanics in our proposed network of genes that regulate breast cancer progression/metastasis. We have

already experimentally determined that genetic inhibition of one of the components has an effect on most of the other genes in the network and by linking these genes and their effects on each other through bioinformatics we were able to identify some of the strands that tie the network together and also a few central transcription factor nodes that could be the key downstream effectors of transcriptional regulation in this network of genes. With our long-standing collaborators at the MGH Cancer Centre/Harvard Medical School in Cambridge, US (who have been our partners throughout the publication of our Fra-1/ADORA2B paper), we have designed an elaborate experiment to determine whether indeed our network-hypotheses are correct. We have compared the profiles of RNA sequencing and chromatin immuno-precipitation sequencing when individually inhibiting the central nodes of our proposed network as compared to the normal situation with the nodes still intact. The datasets that were generated from the sequencing results have been analysed and we are currently finalising the manuscript that describes our findings and follows up on the Fra-1 publication.

References:

[1] Milde-Langosch, K. (2005) **The Fos family of transcription factors and their role in tumourigenesis.** Eur J Cancer 41(16):2449–2461.

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[3] Desmet, C., Gallenne, T., *et al*. (2013) **Identification of a pharmacologically tractable Fra-1/ADORA2B axis promoting breast cancer metastasis.** Proc Natl Acad Sci U S A. Mar 26;110(13):5139-44.

[4] Lentini A, *et al*. (2010) **Antitumor activity of theophylline in combination with Paclitaxel: A preclinical study on melanoma ex- perimental lung metastasis.** Cancer Biother Radiopharm 25(4):497–503.

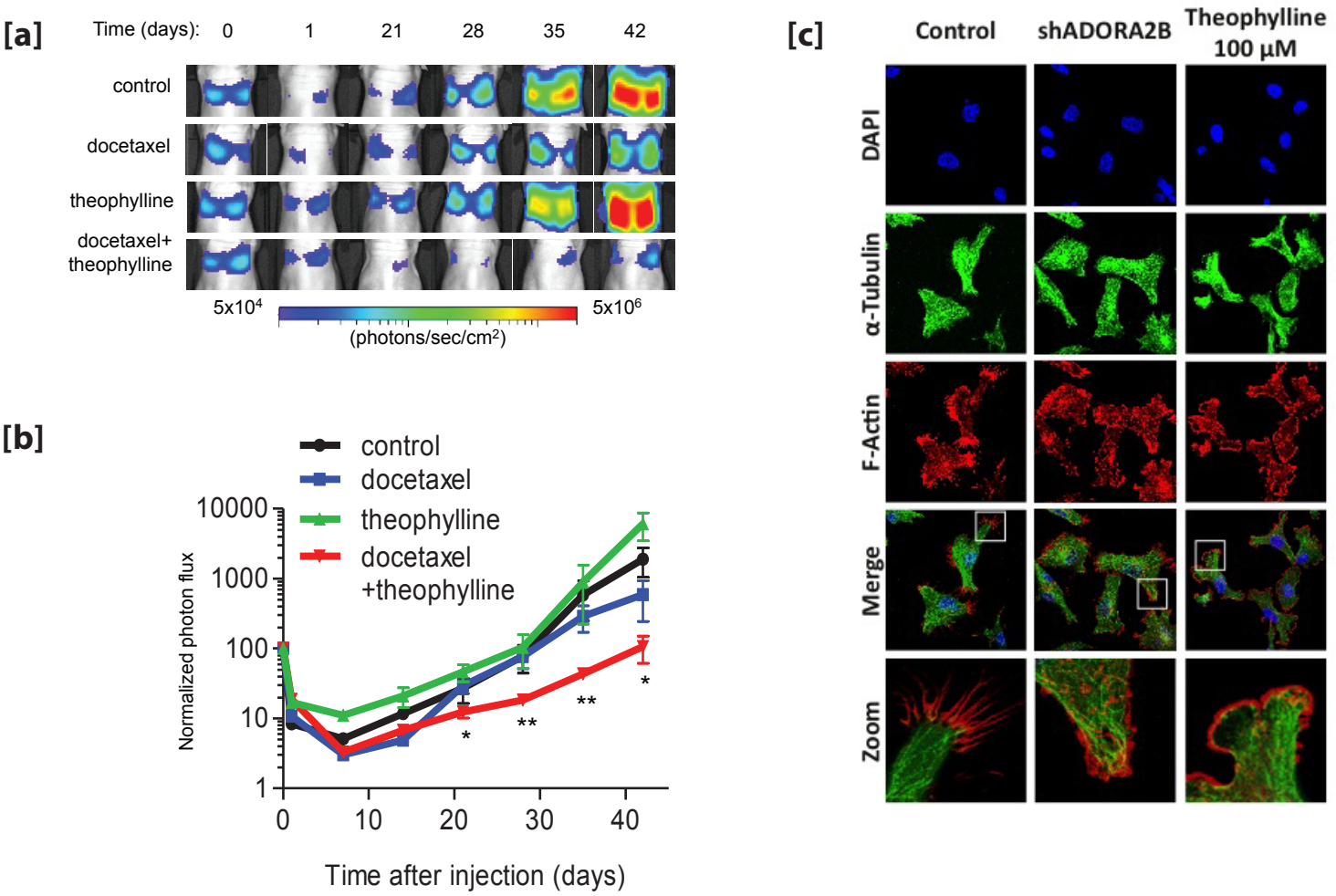

Figure 1 | [a] Visual representation of the effect of the ADORA2B inhibitor theophylline on tumour burden in the lungs. **[b]** Quantification of the signal shown in *[a]*: Theophylline can significantly enhance the effect of Docetaxel on metastasis formation in the lungs. **[c]** Confocal microscopy images of the effect of genetic inhibition (middle panel) and chemical inhibition with Theophylline (right panel) on metastatic breast cancer cells in vitro: ADORA2B inhibition shows a dramatic reduction in the formation of filopodia (shown in red).

Overview of the use of funds from A Sister's Hope

Total funding awarded: € 270,000.00

Period: April 2011 – February 2014

Material budget	
Chemicals	€ 5,325.85
Reagents	€ 19,181.31
Consumables	€ 34,077.16
Mice	€ 9,451.75
Internal facilities	€ 9,570.00
Material budget total	**€ 77,606.06**

Personnel budget	
Salary	€ 150,652.23
Other	€ 683.59
Personnel budget total	**€ 151,335.82**