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14. ABSTRACT

We have performed multiple phage screens in search for carcinoma-associated fibroblast (CAF)-targeting peptides utilizing the tumor-penetrating phage display technology combined with next generation phage DNA sequencing. We have identified at least 4 potentially novel CAF/stroma-targeting peptides. One of the peptides, CIS, homed to breast tumor stroma in two mouse models, and the homing was significantly enhanced by iRGD co-injection.

15. SUBJECT TERMS

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1. INTRODUCTION

Tumors grow in an environment that greatly contributes to tumor development and progression. Much attention has been paid recently to tumor vasculature, which has become an important target for tumor therapy. In addition to the vessels, the stromal elements in tumors include immune cells (macrophages polymorphonuclear neutrophils, lymphocytes, dendritic cells), mesenchymal cells (fibroblasts, mesenchymal stem cells), and extracellular matrix. Host-derived stromal cells evolve in parallel with carcinoma cells and can comprise more than half of the cellular content of a tumor. Each of these cell types plays an important role in determining the tumor formation and progression (or conversely, regression). An example is the production of various growth factors and cytokines by tumor macrophages, which can promote tumor cell growth and angiogenesis (Pollard, 2004; Chen et al., 2005). Crosstalk between tumor cells and tumor fibroblasts also promotes tumor growth, and these activated fibroblasts produce extracellular matrix that sometimes becomes so dense that it blocks the access of anti-tumor drugs to tumor cells (Kalluri and Zeisberg, 2006; Sund and Kalluri, 2009). Tumor vessels are well known to contain a multitude of markers not expressed or expressed at much lower levels, in normal vessels (Ruoslahti et al., 2010). Other stromal cells in tumor environment are also thought to be in an activated state and selectively express markers not present in their normal counterparts. However, in contrast to tumor endothelial cells there is a scarcity of markers for tumor macrophages, fibroblasts, and the matrix. Such markers will make it possible to selectively deliver compounds to the stromal elements and tumor stem cells for therapeutic, imaging and research purposes. The method is based on co-administration of a phage library with iRGD, a tumor-penetrating peptide that facilitates penetration of co-administered compounds, including phage, into extravascular tumor tissue (Sugahara et al, 2009; 2010).

We have used a new tumor-penetrating phage screening technology to identify tumor-specific peptides that bind to the non-vascular stromal elements and stem cells in breast cancers.

Body:
The *Specific Aims* approved for the study are:

Aim 1. Perform phage library screenings to identify homing peptides for individual cell types in tumors by using new tumor-penetrating screening technology

Task 1a. Perform tumor-penetrating screens for tumor-homing peptides on orthotopic and MMTV-PyMT *de novo* breast tumors. (Months 1-12)

Responsible PI
Kazuki Sugahara: Screens on carcinoma-associated fibroblasts
Erkki Ruoslahti: Screens on cancer stem cells and tumor-associated macrophages

Task 1b. Perform tumor-penetrating screens for tumor-homing peptides in human breast cancer explants. (Months 1-12)

Responsible PI
Kazuki Sugahara: Screens on carcinoma-associated fibroblasts
Erkki Ruoslahti: Screens on cancer stem cells and tumor-associated macrophages
Sarah Blair: Collection of human breast tumor explants

Task 1c. Optimize and validate the experimental approach and custom-made bioinformatics software for high throughput phage sequencing. (Months 1-12)

Responsible PI
Kazuki Sugahara

Aim 2. To validate the homing specificities of individual phage and synthetic peptides from Task 1 in ex vivo and in vivo tests

Task 2a. Analyze the homing specificity of homing peptides recognizing tumor fibroblasts. (Months 13-24)
Responsible PI
Kazuki Sugahara

Task 2b. Analyze the homing specificity of homing peptides recognizing tumor-associated macrophages (Months 13-24).
Responsible PI
Erkki Ruoslahti

Task 2c. Analyze the homing specificity of homing peptides recognizing cancer stem cells (Months 13-24).
Responsible PI
Erkki Ruoslahti

Summary

We have developed three tumor models in mice for the project and have acquired fresh human breast cancer samples from our clinical collaborator. We have shown using these tumors that the tumor-penetrating peptide iRGD does induce entry of phage into the extravascular tumor tissue as well as penetration of the phage into fresh tumor implants. These results validate our proposed screening approach. We have also established cell lines representing the various cell types we are targeting in tumors and developed methods for the isolation of these cells as primary cells from tumors. We are using the cells to enrich the libraries for phage clones that are capable of binding to the target cell prior to *in vivo* screening and are now conducting *in vivo* screens. Finally, we have set up high throughput sequencing for phage inserts, which will greatly increase the information obtained from the screens and speed up the process.

RESULTS

Aim 1. ***Perform phage library screenings to identify homing peptides for individual cell types in tumors by using new tumor-penetrating screening technology***

General

In Year 1, we created a breast tumor mouse model by orthotopically injecting MCF10CA1a human breast cancer cells into nude mice. The MCF10CA1a tumors contain high amounts of tumor stroma, which iRGD efficiently penetrates (refer to Fig. 5). We also bred MMTV-PyMT mice to have enough animals for screens and subsequent characterization assays. With help of our collaborator, Dr. Sarah Blair at the University of California, San Diego, we collected 5 primary tumors, 2 metastatic lymph nodes, and 2 normal breast tissue samples from human patients. We also reported in Year 1 development of the tumor-penetrating *in-vivo* phage screening and high-throughput phage insert sequencing methods that were proposed in the original application. These resources were then used to perform the work proposed in the Specific Aims as reported below.

1a1. *Screens on carcinoma-associated fibroblasts*

In Year 1, we reported the findings listed below:

- Development of tools and key technologies required for the proposed project (i.e., mouse tumor models, immortalized human breast carcinoma-associated fibroblasts (CAFs), CAF isolation techniques).
- Co-penetration of a tumor-penetrating peptide, iRGD, and co-applied phage particles into human breast tumor explants.
- Preliminary results of *in vitro*, *ex vivo*, and *in vivo* phage display screens.
- Co-penetration of of phage library to human breast cancer explants in the presence of the iRGD peptide.
- The novel finding that iRGD efficiently targets breast CAFs, and that the iRGD receptor, NRP-1, is a potential CAF marker in breast tumors.

During Year 2, we made significant progress in identifying novel peptides that potentially target CAFs.

Aim 1.

To identify novel breast CAF-targeting peptides, we performed various types of phage display screens as reported last year (e.g., *in vitro* + *in vivo*, *ex vivo* with human tumor samples, *in vivo*). Among them, a combination of *in vitro* and *in vivo* display has yielded peptides that potentially target breast CAFs.

First, four rounds of *in vitro* phage panning on cultured breast CAFs were performed. A cyclic CX_7C peptide library displayed on T7 phage was used (diversity approximately 10^9). The CAFs, i6011, were generated by immortalizing primary breast CAFs isolated from Stage II invasive lobular breast cancer using human telomerase reverse transcriptase (hTERT). The resulting phage pool bound to i6011 approximately 7 fold more than a phage clone expressing an inert peptide, CG_7C (Fig. 1A).

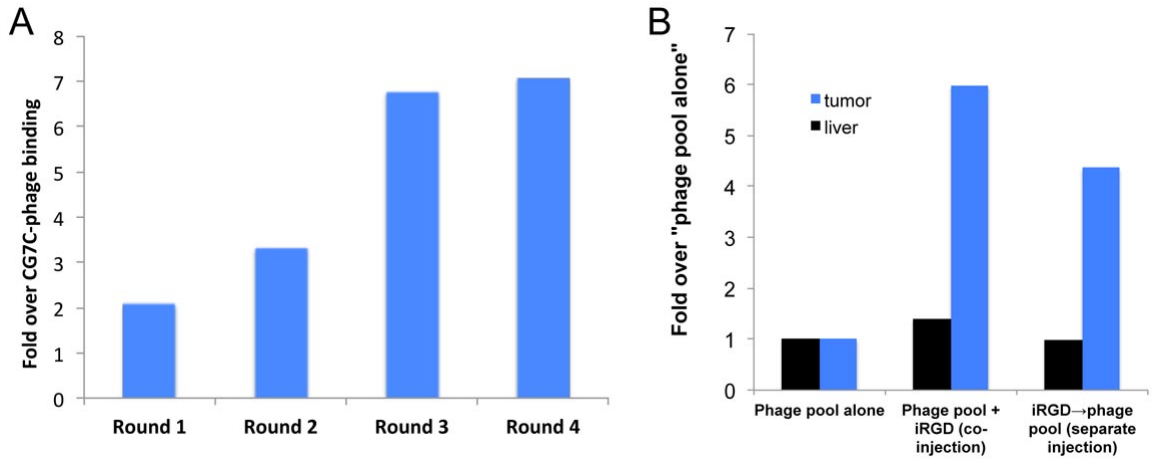

Figure 1. *In vitro* and *in vivo* phage screen for CAF binding peptides. A, A T7 phage pool expressing a cyclic CX_7C peptide library was applied to cultured i6011 immortalized human breast CAFs to enrich phages that bind to the CAFs. **B**, The phage pool from "Round 3" in panel A was injected alone, together with 75 mg of iRGD, or 15 min after iRGD injection into the tail vein of mice bearing orthotopic MCF10CA1a human breast cancer xenografts. Phages that accumulated in the tumor (and liver as a control) were recovered and titrated. Note that iRGD facilitated phage accumulation into the tumor but not into the liver.

The phage pool after the 3rd *in vitro* panning was intravenously injected into mice bearing orthotopic MCF10CA1a human breast tumor xenografts. In some cases, the pool was injected together with synthetic iRGD peptide or 15 min after iRGD injection to facilitate extravasation of the phage pool within the breast tumor tissue. Co-injection of iRGD enhanced phage accumulation in the tumor by 4-6 fold, but minimally affected phage entry into the liver (Fig. 1B).

Phage particles recovered from the tumor tissue were then subjected to DNA sequencing using an Iron Torrent next generation sequencer (the work flow for the sequencing can be found in our Year 1 report). The phage pool recovered from the MCF10CA1a tumor in the mouse that received phage pool alone contained 511 phage clones expressing CISQERGESC (CIS: 0.6% of the recovered phage clones) and 202 phages expressing a relevant peptide CIFSGEGESC (CIF: 0.2%) (Table 1A). The phage pool from the iRGD/phage simultaneous injection screen contained 1782 phage clones expressing CQEKTKNRC (CQE: 1.8%) (Table 1B). The phage pool from the screen that involved phage injection after iRGD injection contained 2440 phages expressing CAVRQKGEC (CAV: 2.6%). The CAVRQKGEC phage was also present in the iRGD/phage simultaneous injection group (0.1%). Interestingly, phages expressing the iRGD sequence (CRGDKGPDC) were detected in the iRGD co-injection screens supporting our finding that iRGD is a CAF-targeting peptide (see Year 1 report).

A

Phage	Phage pool alone	Co-injection	Separate injection
CISQERGESC	511	0	0
CIFSGEGESC	202	0	0
CAVDGGSRC	48	0	0
CKRKDSRSC	31	0	0
CKTKDGRNC	11	0	0
CRLDKKGDC	11	0	0
LGRGGVAKL	11	0	0
CPPEARKRC	11	0	0
CQLGRSQKC	10	0	0
CKRTPDSKC	10	0	0
CTGTAHSKC	10	0	0
CNTGARSRC	10	0	0
CRTNGVKAC	9	0	0
CDLNTDSPC	9	0	0
CMGKGKKPC	9	0	0
CTEKMSQKC	9	0	0
CRSSAKL	9	0	0
[illegible]	8	0	0

B

Phage	Phage pool alone	Co-injection	Separate injection
CQEKTKNRC	0	1782	0
CAVRQKGEC	0	59	2440
CHKR	0	8	5
CAVD	0	8	5
CGAD	0	8	2
CPH	0	7	4
CRGDKGPDC	0	7	3
CNGREVSSC	0	7	0
CERP	0	7	0
CDLA	0	6	3
CKTPR	0	6	2
L	0	6	2
CPKL	0	6	0
CPKP	0	6	0
CSGSPQRKC	0	6	0
CAAS	0	5	4
CREPR	0	5	3
[illegible]	0	5	3

Table 1. Peptides recovered from the tumors analyzed by high throughput phage DNA sequencing technology. A, Peptides found in the "Phage pool alone" group are listed in a descending order of frequency. Note that CISQERGESC (CIS) and CIFSGEGESC (CIF) with relevant amino acid sequences were highly enriched in the pool. The total number of phage clones analyzed was 90347, and 57810 peptides were found. **B**, Peptides found in the "Co-injection (simultaneous injection of iRGD/phage pool)" group are listed in a descending order of frequency. CQEKTKNRC (CQE) and CAVRQKGEC (CAV) were highly enriched in the pool. Notably, CAV was the most enriched peptide in the "Separate injection (iRGD followed by phage pool injection)" group. In addition, CRGDKGPDC, the iRGD peptide sequence, was found in both groups. The total number of phage clones analyzed for the "Co-injection" group was 94674, and 70383 peptides were found. A total of 97638 phage clones were analyzed for the "Separate injection" group, and 73745 peptides were found.

Aim 2.

Fluorescein (FAM)-labeled CIS was synthesized in house as described earlier (Sugahara et al, 2009). Synthetic FAM-CIS injected intravenously into mice bearing orthotopic MCF10CA1a tumors specifically accumulated in the tumor tissue (Fig. 2A). FAM-CIS also homed to breast tumors in MMTV-PyMT transgenic mice (Fig. 2B). Co-injection of non-labeled iRGD synthetic peptide enhanced the tumor homing efficiency and specificity of FAM-CIS in MMTV-PyMT mice.

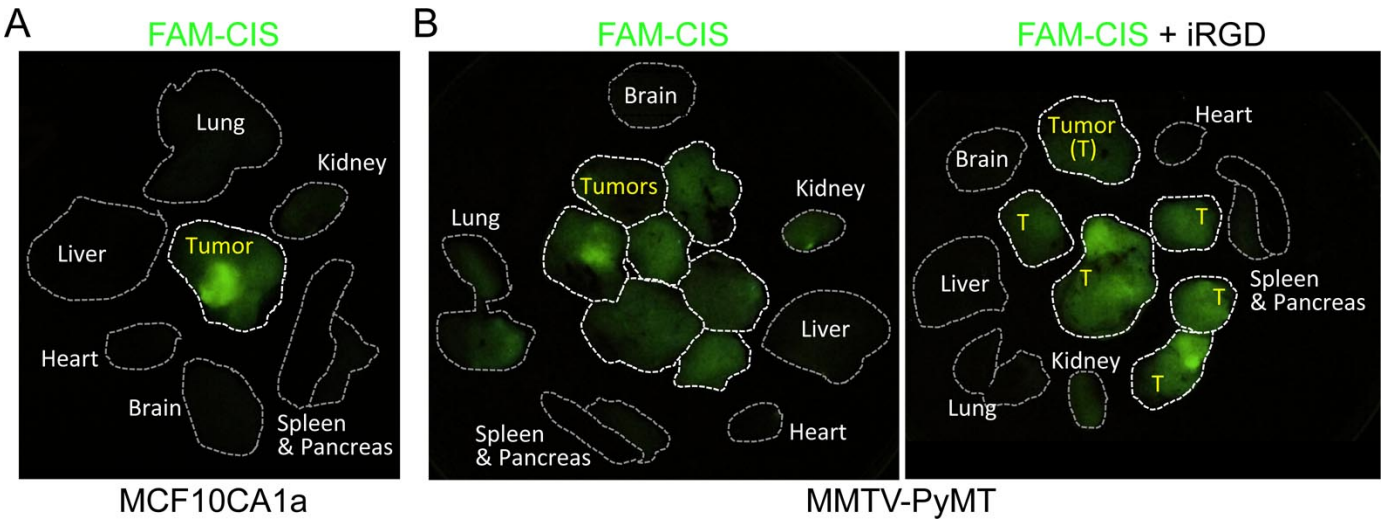

Figure 2. *In vivo* distribution of fluorescein-labeled synthetic CIS peptide (FAM-CIS). Approximately 150 mg of synthetic FAM-CIS peptide were intravenously injected into mice bearing orthotopic MCF10CA1a human breast cancer xenografts (**A**) and transgenic MMTV-PyMT mice bearing *de novo* breast tumors (**B**). In some cases, non-labeled synthetic iRGD peptide was co-injected with FAM-CIS. After 1 hr of peptide circulation, the mice were perfused through the heart with PBS, and tumors and tissues were collected for macroscopic imaging under a fluorescent light table. The dotted lines outline the tissues. Note the tumor-specific accumulation of FAM-CIS. iRGD appears to enhance the tumor homing efficiency and specificity of FAM-CIS.

Immunofluorescence revealed that FAM-CIS targets tumor blood vessels, and subsequently, the extravascular tumor stroma in both orthotopic MCF10CA1a xenograft and *de novo* MMTV-PyMT tumors (Fig. 3). This finding supports the possibility that CIS is a CAF-binding peptide. In addition to the vasculature and stroma, CIS accumulated into distinct cell populations (Fig. 3, arrows) within the breast tumor tissue.

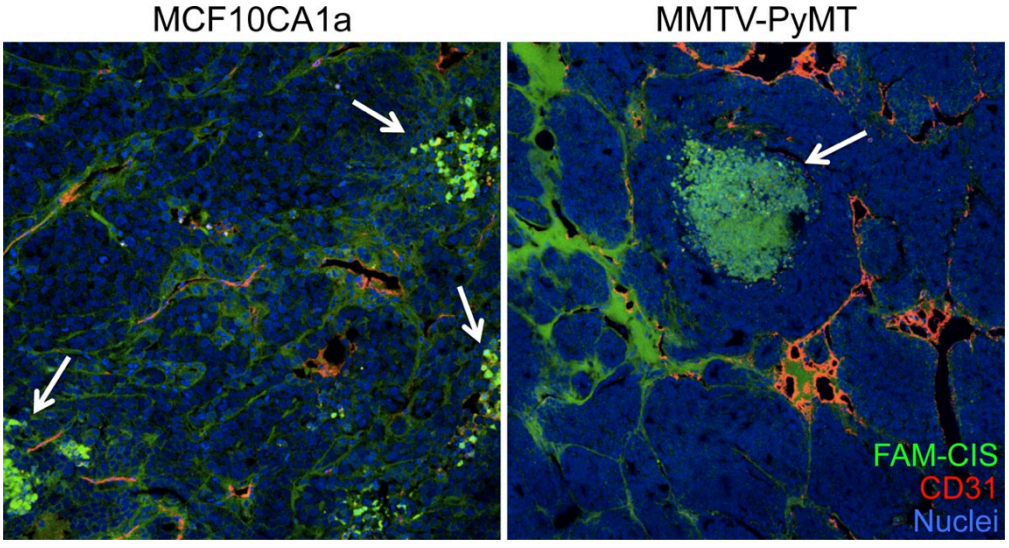

Figure 3. Intratumoral distribution of Intravenously injected FAM-CIS. Confocal images of tumors from mice intravenously injected with FAM-CIS (green) are shown. Red, CD31; blue, DAPI. Note that the CD31 positive tumor vessels and stromal network are highlighted by FAM-CIS. FAM-CIS also accumulated into distinct cell populations within the tumor tissue (arrows).

Co-injection of non-labeled iRGD peptide enhanced the extravasation of FAM-CIS into the tumor parenchyma, suggesting that a combination of CIS and iRGD can be a powerful tool to simultaneously target breast tumor parenchyma and stroma.

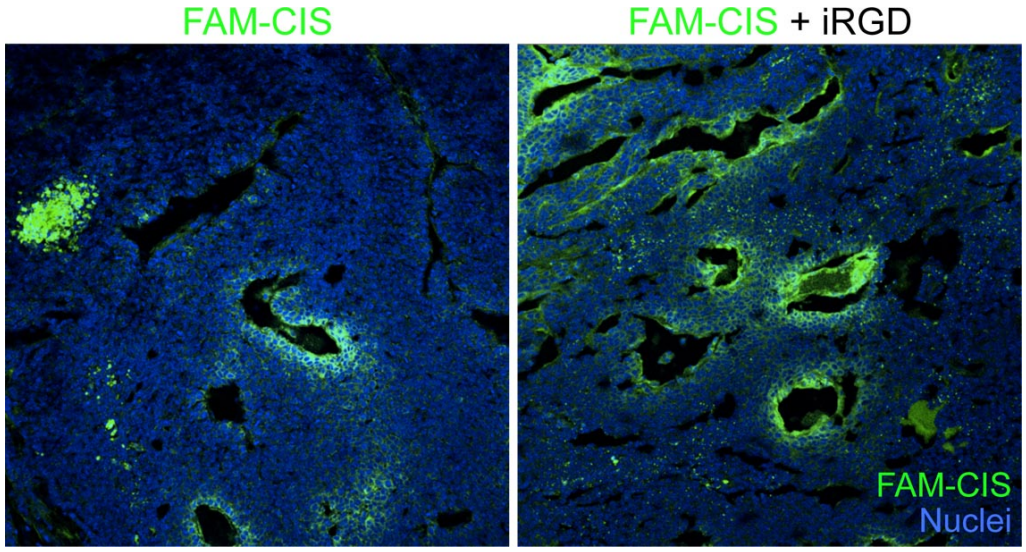

Figure 4. Effect of iRGD co-injection on the intratumoral distribution of FAM-CIS. Confocal images of *de novo* tumors from MMTV-PyMT transgenic mice that intravenously received FAM-CIS (green) with or without iRGD are shown. Blue, DAPI. Note the enhanced extravasation of FAM-CIS into the tumor parenchyma when iRGD was co-injected.

Further analysis of CIS regarding tumor-type specificity, *in vitro* and *ex vivo* CAF-binding, receptor identification, and tumor imaging/drug delivery capacity will be performed once additional funding is secured. FAM-labeled CIF, CQE, and CAV peptides are currently being synthesized. These peptides will also be analyzed to identify the most efficient CAF-targeting peptide.

Key Research Accomplishments:

- We have performed multiple phage screens in search for CAF-targeting peptides.
- We have demonstrated the utility of the next generation phage DNA sequencing technology in this context.
- We have identified at least 4 potentially novel CAF-targeting peptides.
- One of the peptides, CIS, was found to home to breast tumors in two different tumor mouse models.
- Intravenously injected CIS peptide efficiently accumulated into breast tumor stroma.
- iRGD co-injection enhances breast tumor targeting of CIS.

Reportable outcomes:

None.

Conclusions:

We have made progress in all of the tasks listed in our application. We have performed multiple tumor-penetrating phage screens (using iRGD co-injection) in which we have demonstrated the utility of our next generation phage DNA sequencing technology. One of the screens has yielded at least 4 potential CAF-targeting peptides. One of them, CIS appears to target breast tumors, especially tumor stroma, where CAFs mainly reside. The other 3 peptides are currently being synthesized.

In addition to the new screens reported this year, we have found that iRGD alone is a CAF-targeting peptide and that the iRGD receptor neuropilin-1 (NRP-1) is a potential CAF marker (Year 1). This finding is important not only for CAF-targeting, but also to further understand the origin and functions of CAFs in breast tumors. In addition, our results demonstrate that combination of iRGD with another potential CAF-targeting peptide, CIS, can be a powerful tool to target breast tumors. Additional funding will be secured for

further analysis of CIS and the other 3 peptides, and the potential role of iRGD in enhancing the effects of the peptides and understanding the biology of CAF relative to breast tumor progression.

1a.2. *Screens on cancer stem cells and tumor-associated macrophages*

Tumor stem cell screens

In Year 1, we reported the findings listed below.

- Acquired human breast cancer cell lines that are highly enriched in tumor stem cells for in vitro phage panning.
- Performed phage library screens with MDA-MB-468 cells in the presence of an excess of soluble RGD as an inhibitor to reduce the frequency of peptides containing the integrin-binding RGD motif in the output.
- Adapted ion torrent sequencing to T7 phage.
- Reported sequences of peptides with potential stem cell binding specificities.

In Year 2, we have used the screening method to isolate peptides that selectively recognize cancer stem cells as described below.

We used Aldehyde dehydrogenase 1 (ALDH1), a detoxifying enzyme responsible for the oxidation of intracellular aldehydes as a marker for cancer stem cells. This marker was used to select out stem cells using flow sorting. For our screening, naïve library was incubated with MDA 468 cells and ALDH+ cells were sorted out to recover phage bound to these cells. Recovered phage pool was injected with iRGD peptide in a MMTV-PyMT animal. Again, ALDH+ cell population, which was about 1% of the tumor was sorted out and the bound phage pool was recovered. For a subsequent round of selection, the phage recovered was again injected with iRGD and ALDH+ and ALDH- cell population was sorted from the tumor. The phage that homed to ALDH+ cells was significantly enriched as compared to ALDH- population (Figure 5).

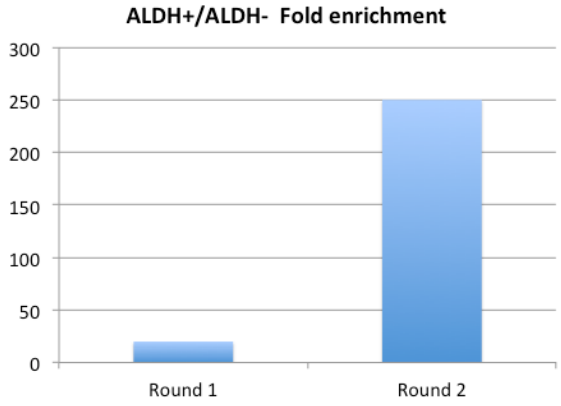

Figure 5. Fold enrichment with two rounds of in vivo phage screening in MMTV-PyMT tumors

We then carried out high throughput sequencing on this recovered pool of phage. Table 2 summarizes the top hits from the sequencing.

Table 2. Peptide sequences from *in vivo* tumor screen in ALDH+ cell population in PyMT-MMTV mice using a library enriched by panning on ALDH+ MDA-MB-468 cells.

HTS Round 1
Sorted ALDH + Cell Population

Phage	Repeats
CMDDLGKTC	21439
CQNTLASKC	13373
CKPHSQRNC	3110
CNVNNKPSC	2930
CQNTRTVKC	2726
CHGIAPTDC	2657
CGEITDNKC	2440
CAKGYKGSC	987
CALEKISDC	625
CTRKGVSEC	456
CLERELGAGC	294
CLSRKGSAC	106
CRIECTVAC	85
CRVASMMAC	77
CRPPRNNPC	75
CRIECRLGRC	64
CRIECRWGQLLSL	59
CLSLLEGAC	56
CKRIVRRNC	44
CRIECRWDSC	40
CDQNIKAHC	37
CVRLRMNKC	25
CRIECRLGRAKL	25
CHRTRGKC	23
CRNGKATLC	21
CKRIVRDC	21
CRSGNIGEC	20

We are currently testing the individual peptides that scored highly on our in vivo screening for binding to cancer stem cells.

Tumor macrophage (TAM) screens

In Year 1, we reported the following:

- Development of methods for TAM isolation from 4T1 mouse breast cancer tumors.
- Testing of our techniques with a previously identified peptide (LyP-1; Fogal et al., 2008) that strongly favors TAMs (; Hamzah et al., 2011). used the 4T1 breast cancer model to isolate TAMs for *ex vivo* enrichment of phage clones from libraries. We dissociate the tumor tissues to single cells by incubation

In Year 2, we have used the screening methods to isolate peptides that selectively recognize TAMs as described below.

We used CD11b as the marker for macrophages, and mouse orthotropic 4T1 breast tumor as the *in vivo* animal model. We initially isolated CD11b+ cells from 4T1 mouse breast tumor, and performed *in vitro* phage screen (10^10 pfu, 4°C incubation overnight with 10^5 cells) on these cells. However, we did not observe any enrichment over several rounds of biopanning (data not shown).

Identification of CRV peptide from in vitro phage screen

Since the *ex vivo* approaches did not yield any enrichment of phage binding, we switched to an established tumor-associated macrophage cell line, J774 A.1, as the cell model to perform *in vitro* phage screens.

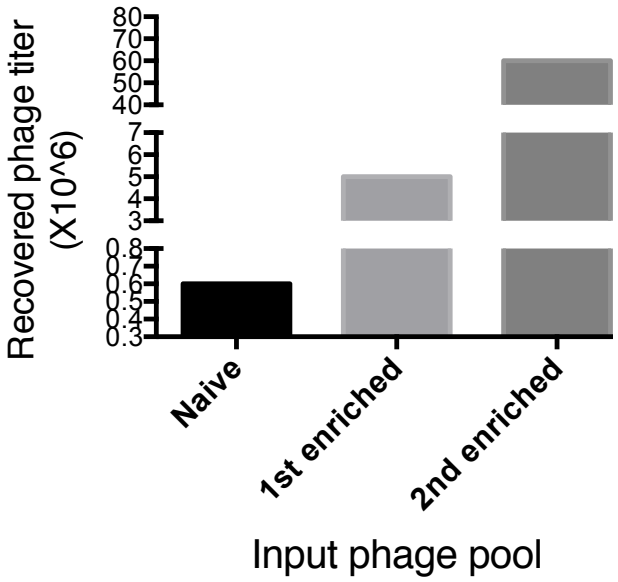

Figure 6. **Quantification of phage enrichment.** Protocol: 10^5 J774 A.1cells were incubated overnight at +4 °C with 10^9 pfu of phage library (starting with naïve CX7C library) After four rounds of washing, phage clones associated with cells were amplified for the next round.
To quantify the enrichment over several rounds of biopanning, we incubated 10^5 J774 A.1 cells with 10^9 pfu of input phage pool from each round. Phage titer recovered was plotted at the y-axis. There was over 100-fold enrichment after three rounds of biopanning.

High throughput sequencing of to the phage pool after 3rd round of biopanning revealed a few highly enriched peptide sequences (Table 3). A RVLRS motif and closely related motifs were enriched in the pool, indicating that this motif binds to tumor macrophages. Therefore, we synthesized the peptide CRVLRSGSC ("CRV" peptide hereafter) and investigated its *in vivo* behavior.

Table 3. Peptide sequences from J774 A.1 cell screen

RVLRSGS	63428
GG**RVLRS**	62028
SVAYD	10187
RSGLRSS	9519
G**RLLRS**G	9124
G**RMLRS**G	8922
GGASIT	8677
SVGRSMRS	7916
GRVLRSS	6774
SGRVLRS	5682
RRVLRGG	5492
SSVD	4756
RTTRSKA	4556

CRV recognizes the macrophages and inflammatory sites within tumors

a) *In vivo* distribution:

The tumor samples were then sectioned and examined microscopically. The results show that CRV can

preferentially homes to the tumors, extravasates outsideblood vessels, and co-localizes with markers for macrophages and lymphatic vessels in tumors (Figures 7-11). A likely explanation for the co-localization with tumor lymphatics is that bone-marrow derived macrophage/myeloid cells incorporate into the endothelium of lymphatic vessels in the process of lymphangiogenesis (Fogal et al., 2008). The CRV peptide contains a CendR motif (Teesalu et al., 2009; Sugahara et al., 2009), which is probably responsible for the extravasation and tumor penetration.

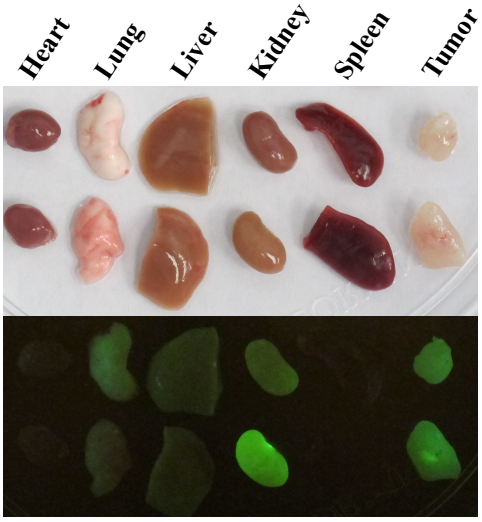

Figure 7 CRV homing in 4T1 mice. Tumor mice (n=2) were intravenously injected with100 µg of CRV peptide labeled with a green fluorophor (FAM). The peptide was allowed to circulate for 1 hour, and the mice were perfused through the left ventricle of the heart to eliminate any remaining peptide in the blood. Major organs were excised and examined under a fluorescent light. The tumors are positive for peptide fluorescence. Some fluorescence is seen in a lung from one mouse and in the kidneys. Peptides are eliminated through the kidneys, which makes them positive.

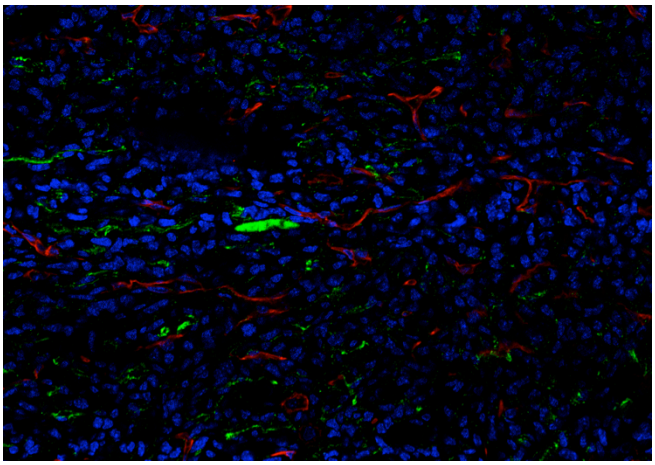

Figure 8. Fluorescent microscopy of tumor sections showing lack of co-localization of CRV peptide with a blood vessel marker. The peptide has clearly entered into the the extravascular compartment of the tumor. Green, CRV peptide; red, anti-mouse CD31.

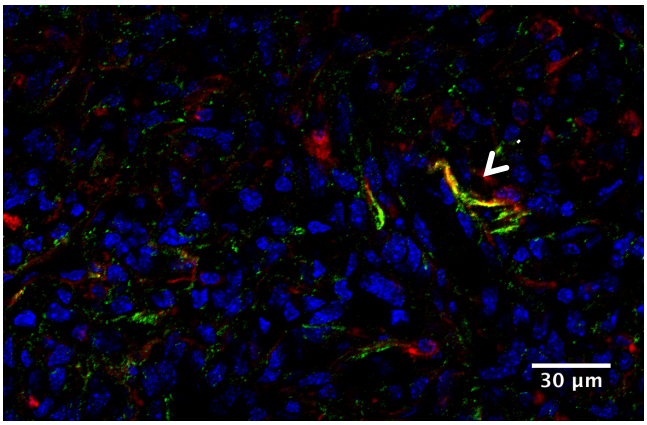

Figure 9. Partial co-localization of CRV with the macrophage marker F4/80. Green, CRV peptide; red, anti-mouse F4/80. A point of co-localization is indicated with an arrow.

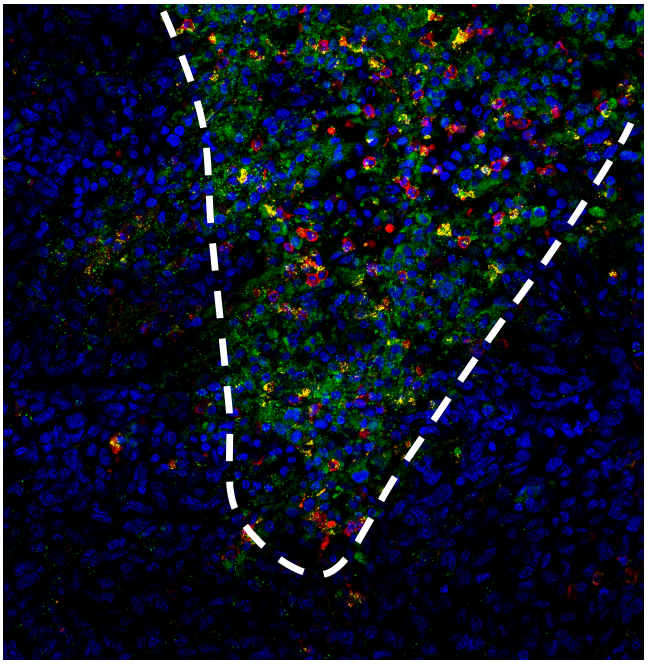

Figure 10. CRV localizes in a CD45 a leukocyte-enriched tumor region outlined with anti_CD45 Green, CRV peptide; red, anti-mouse CD45. The boundary of CD45-enriched region is marked with a dashed line.

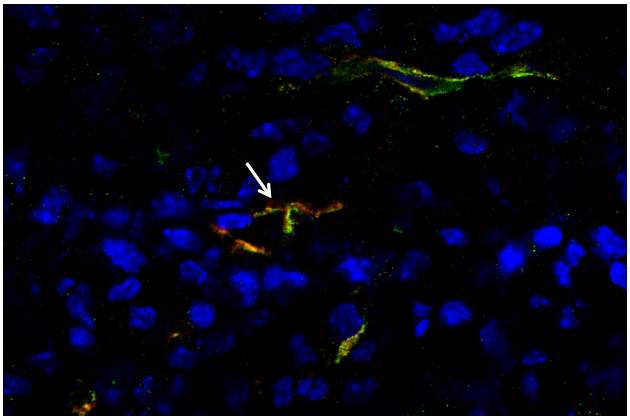

Figure 11. CRV co-localizes with PROX-1, a lymphatic vessel marker. Green, CRV peptide; red, anti-mouse PROX-1). A point of co-localization is indicated with an arrow).

Identification of a receptor for the CRV peptide

We used affinity chromatography to identify a receptor for CRV, followed by identification of specifically eluted protein band by mass spectrometry (Figure 12). The top protein band was identified as HSPA8 (heat shock protein 8). Staining of tumor sections with anti-HSPA8 revealed co-localization of HSPA8 with CRV peptide (Figure 13), supporting the notion that HSPA8 may be the receptor for CRV.

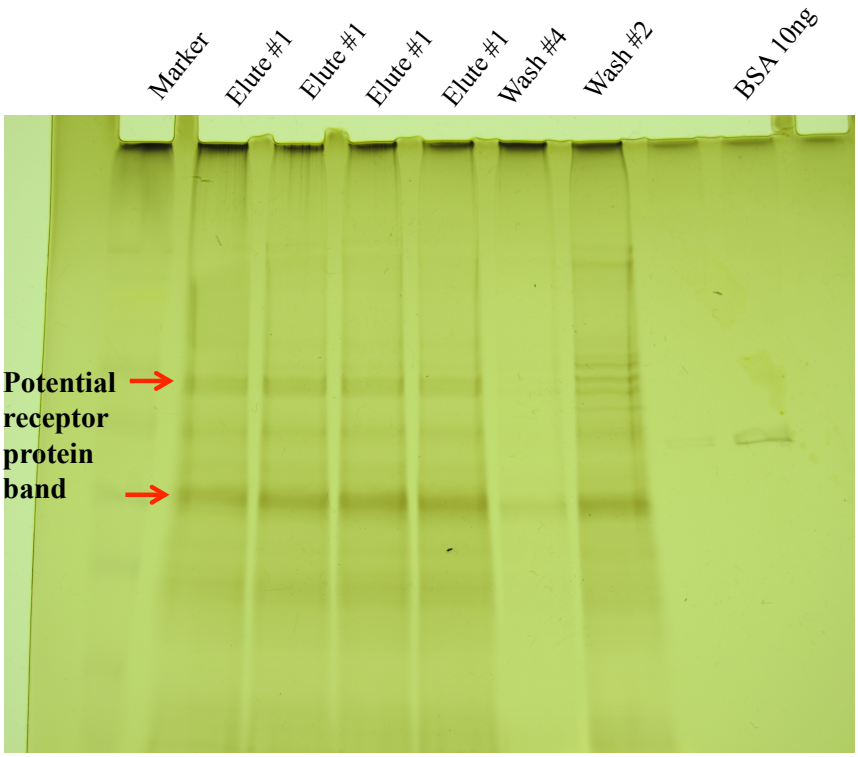

Figure 12. SDS-PAGE of fractions eluted from a CRV affinity matrix. To isolate a CRV receptor, we incubated biotinylated CRV peptide with a 4T1 tumor lysate. After immobilizing the biotin-peptide onto streptavidin magnetic beads, we eluted the putative receptor by incubating the beads with an excess of non-biotinylated CRV peptide. The eluted and wash fractions were separated by SDS-PAGE and the gel was silver-stained to detect protein bands. Two protein bands were selectively eluted by the free CRV peptide (arrows).

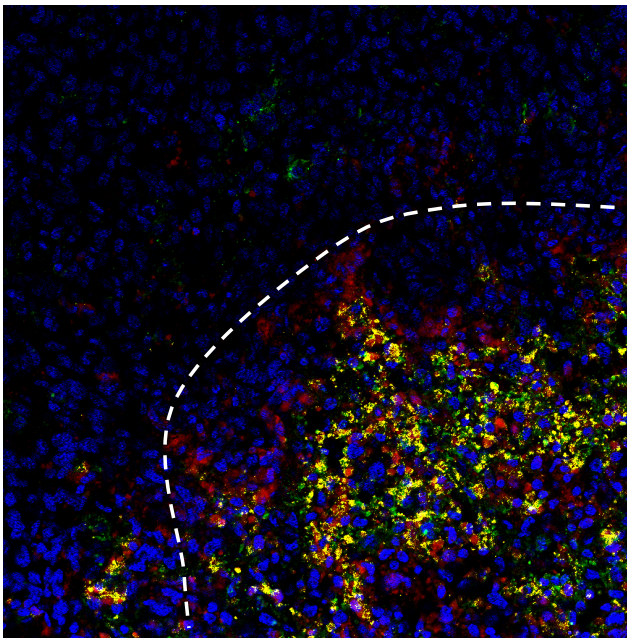

Figure 13. CRV localizes in HSPA8-enriched Region in tumor sections. Green, CRV peptide; red, anti-mouse HSPA8; the boundary of a HSPA8-enriched region is outlined with a dashed line)

CRV does not target a non-malignant site of chronic inflammation

To determine whether CRV is tumor-specific or recognizes general inflammatory events, we injected the peptide into atherosclerotic mice with abundant plaque in the aorta. Another peptide that recognizes tumor macrophages (LyP-1; Fogal et al., 2008; Hamzah et al., 2011) was injected as a positive control. As shown in Figure 14, CRV does not accumulate in plaque, suggesting that it is tumor-specific.

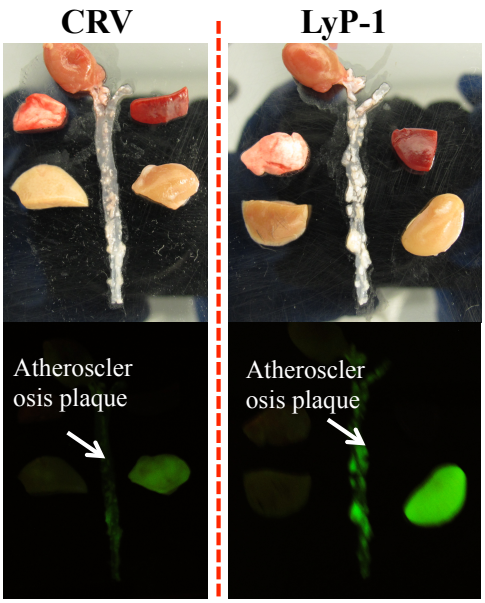

Figure 14. CRV does not recognize atherosclerotic plaques. we injected 100 µg FAM-labeled CRV peptide into mice with atherosclerosis generated with ApoE null phenotype and high-fat diet, and imaged the aorta (shown in the middle split open and attached to the heart) and major tissues. CRV does not accumulate in the plaques, whereas LyP-1, a peptide known to recognize plaque (Hamzah et aL., 2011), does highlight the aorta.

1c. *Optimize and validate the experimental approach and custom-made bioinformatics software for high throughput phage sequencing*

The methods for high-throughput phage sequencing and the bio-informatics for data handling were successfully developed in Year 1.

Aim 2. ***To validate the homing specificities of individual phage and synthetic peptides from Task 1 in ex vivo and in vivo tests***

The results pertaining to Aim 2 have been described under each target cell type above.

3. KEY RESEARCH ACCOMPLISHMENTS

During Years 1 and 2, we have:

1.

- Established tools and key technologies required for the proposed phage display project. These include mouse tumor models, human tumor explant model, immortalized human breast CAFs, cell lines enriched in breast cancer stem cells, CAF and TAM isolation techniques, and high throughput phage DNA sequencing.
- Demonstrated that phage co-administered with iRGD accumulates into tumors in vivo, and penetrates into into human breast tumor explants.
- Established an *in vivo* perfusion technique for focusing phage library screening to extravascular tumor tissue.
- Performed *in vitro*, *ex vivo*, and *in vivo* phage display screens using the new methods.
- Discovered that iRGD efficiently targets breast CAFs, and the iRGD receptor, NRP-1, is a potential CAF marker in breast tumors.

2.

- Identified 4 potentially novel CAF-targeting peptides and partially characterized one of .

- Shown that interavenously injected CIS specifically homes to breast tumors in two different tumor mouse models.
- Shown that intravenously injected CIS accumulates in tumor stroma of breast cancer.
- Shown that iRGD co-injection enhances breast tumor targeting of CIS.
- Identified candidate peptides for breast cancer stem cells.
- Identified a peptide motif for the recognition of tumor macrophages.
- Shown that a peptide carrying the macrophage recognition motif (CRV peptide) accumulates in tumor macrophages and tumor lymphatics.
- Identified a potential receptor for the CRV peptide.
- Generated data suggesting that CRV has a different, more restricted specificity than a previously identified tumor macrophage-recognizing peptide.

4. Reportable Outcomes

Sanford-Burnham Institute has filed/will file patent applications on the peptides described in this report, and on their receptors, when known. No publicaitons

5. Conclusions

We have carried out all of the phage library screens for cell types present in breast cancer tumors proposed in the original application. The result has been a collection of potential homing peptides for tumor fibroblasts, tumor stem cells and tumor macrophages. The fibroblast peptides include a previously identified tumor-penetrating peptide, iRGD. This observation led to the important conclusion that iRGD can use the tumor stroma as a conduit through extravascular tumor tissue, even though the stroma ordinarily constitutes an important barrier to drug entry. One new peptide recognizing tumor fibroblast was also validated and partially characterized. The set of peptides from the screens for breast cancer stem cell recognition still require validation. A new recognition motif for tumor macrophages was identifies validated and characterized, including identification of a putative receptor for the peptide. Further work on some of these peptides is going on under other support, and support for work on the rest is being sought. We fully expect to bring all of this work to a point where it can be published and that way made available to the research community.

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