

Development of Recombinant Transcription Factor Proteins and Antibodies for Application in Clinical Immunoassays

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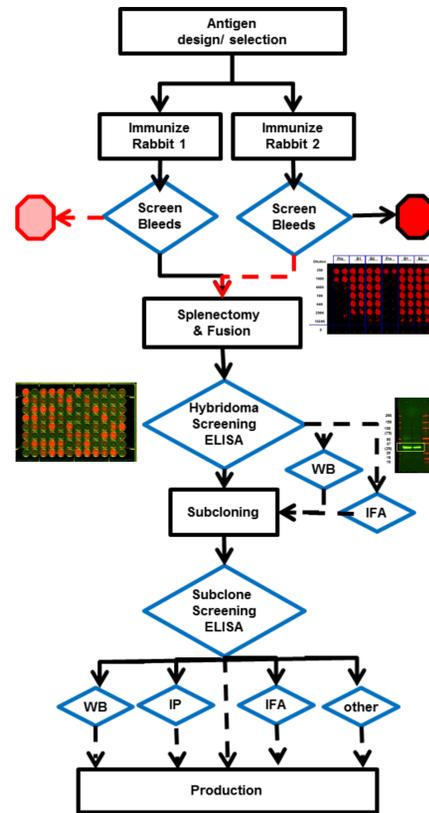
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Introduction

- Epithelial-mesenchymal transition (EMT)/stem cell transcription factors have been implicated in the differentiation of epithelial to mesenchymal cells during embryonic development.
- EMTs are determinants in the progression of carcinomas and their expression patterns can be altered by chemotherapy.
- A panel of experts in the Division of Cancer Treatment and Diagnosis (DCTD, NCI) and Whitehead Institute (MIT) have identified 12 key targets in EMT/stem cell differentiation for which there was an unmet need for high quality reagents. These include: Goosecoid (GSC), NANOG, SNAIL, SLUG, ZEB1, ZEB2, SOX9, LBX1, FOXO2, FOXQ1, CD133 and FOXO3.
- Rabbit monoclonal antibodies to these markers are required for development of pharmacodynamic assays to support clinical trials for new anticancer agents.
- We have developed a strategy to generate, characterize, and validate the EMT reagents starting with antigen design and selection to improve the specificity and minimize the cross-reactivity of the antibodies produced.
- Proof-of-assay analytical performance and stability require both a purified, characterized antibody and a calibrator molecule suitable for generation of an assay "standard" that may be used in several different contexts.
- In parallel, we have designed and produced the corresponding recombinant protein material for these targeted antibodies. Many of the proteins were expressed as specific domains tailored to provide maximum use, while avoiding cross-reactivity.
- Leading candidate hybridomas were tested in multiple immunoassays. Demonstration of fit-for-purpose of the antibodies included validation by ELISA, Western blot (WB), immunoprecipitation (IP) and immunofluorescence assay (IFA) in formalin-fixed, paraffin embedded (FFPE) tissues.

MAb Development



Protein Production Methods

Cloning and Expression for Proteins
Plasmid clones were obtained from DNASU plasmid repository (Arizona State University) or from Dr. Weinberg (MIT). PCR of each target gene was conducted using primers designed by Express Primer Tool (<http://tools.bio.net>) or JVA/Visp/ExpressPrimerTool and obtained from Integrated DNA Technologies (IDT). Soluble proteins were isolated by a previously described method (*Protein Expression and Purification*, 2002, 25:1-7). Large scale culture of the expression clones was conducted in 2 L plastic bottles using the method of C. Sanville Millard et al. (*Protein Expression and Purification*, 2003, 29:311-320).

Protein Purification
Expression and purification of soluble proteins followed a general set of methods described by the Structural Genomics Consortium et al. (*Nature Methods*, 2008, 5:135-146). The majority of proteins and protein domains expressed were insoluble and an inclusion body isolation method was used for purification. Pellets were resuspended in B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) and incubated for 30 min at room temperature, followed by a 10 min incubation on ice, shaking at 50 RPM. Diluted B-PER Reagent (1:10 in water) was added and the suspension sonicated for 3 min (5 sec on, 5 sec off) followed by a 10 min incubation at 4 C. Cell lysates were centrifuged at 4 C for 30 min at 34,540 x g and the supernatant was decanted. Cells were washed with 1:10 B-PER two more times. After the third centrifugation the supernatant was decanted, pellets were resuspended in 25 mL solubilizing buffer (8 M urea, 50 mM Tris pH 8.0). The resulting supernatant containing isolated inclusion bodies was tested for purity on SDS-PAGE. Size exclusion chromatography was used when further purification was required.

Antibody Development Methods

Immunogen Design: Regions of interest each protein target were selected based on a predicted minimal cross-reactivity with other targets; in particular, highly conserved DNA binding domains were avoided. Short sequences with surface exposure and/or high predicted antigenicity within the initial sequences were then used to generate peptide antigens. Antigens were conjugated to carrier proteins for immunization.

ELISA: Plates were coated overnight at 4 C with 100 ng antigen/well in 0.1 M carbonate buffer, pH 9.6. Plates were blocked with LI-COR Odyssey Blocking buffer (LI-COR, Cat#: 927-40010) and then sample (rabbit serum, hybridoma supernatant or purified MAb) was added and incubated at 24 C for 2 h. Goat anti-rabbit IR-680 was used as the secondary antibody and plates were read at 700 nm on a LI-COR Odyssey scanner.

Western Blot Analysis: Tumor cell lysates or recombinant proteins were run on 4-12% SDS PAGE gels in 1x MOPS buffer using the Novex system at 150V and transferred to nitrocellulose membrane using the iBlot System. Blots were blocked with LI-COR Blocking buffer and incubated overnight with hybridoma supernatants (1:30). LI-COR goat anti-rabbit IR-680 antibody was used for detection and read at 700 nm on a LI-COR Odyssey scanner.

IFA: FFPE cell pellets were generated according to the methods described at <http://molcrd.cancer.gov/info/assays/immunohistochemistry.aspx>. Human tissues (pancreas, colon, skin, seminoma) were purchased from Pantomics, Inc (CA). Slides were stained with the rabbit MAbs and DAPI and anti-rabbit—AF488 was used as the secondary.

Immunoprecipitation-Mass Spectrometry Analysis (IP-MS): Monoclonal antibodies (MAb) were first diluted in 1XPBS and antigens were diluted in 1XPBS/BOG. MAbs were bound to magnetic beads (DynaBeads Protein A, Invitrogen) and then antigen solution was added and incubated for 1 h at 4 C. The beads were washed to eliminate salts and detergent that could affect the mass spectrometry analysis. Antigens were then eluted from the beads by addition of 20 mL 0.2% Trifluoroacetic acid solution in HPLC-grade water and spotted directly onto the surface of the MALDI target and mixed on the plate with the appropriate matrix solution. For peptide analysis, α-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix of choice and for full length protein analysis, sinipinic acid (SA) was used. The plate was dried at room temperature and then transferred into the mass spectrometer. Peptide analysis was carried on a Bruker Ultraflex III MALDI-TOF/TOF in reflectron mode and data analysis was performed with FlexAnalysis software. Protein analysis was performed on an Applied Biosystems Voyager-DE Pro time-of-flight mass spectrometer. The instrument was operated in linear mode under positive ion conditions. Voyager was equipped with a Covaxi HM-1 high mass detector. Data analysis was carried out using "Data Explorer" software resident on the Voyager mass spectrometer.

Antigenic Peptides were Designed to Avoid Conserved DNA Binding Region



MHHHHHSSGVDLGTENLQFSNMFASMFSDNLAARPKDCKSVLPAHSAAPVV
FPALHGDSLYGASGGASDYGAFYPRPVAPGGAGLPAVSGSRGLYNNYFYGLHVQ
AAPVGPACCGAVPPLGAQQCSCVPTPPGVEGPGSVLVSPVPHQMLPYMNVGTLRSRT
LQLLNQLHCRRKRRHSTTETDEQLALENLFGQETKYPDVTREQLARKYHLREKVEV
WFKNRRRAKWRROKRSSESESENAEKWNTSSKASPEKREEGKSLDSDS

The above sequence and image represent the recombinant GSC protein used as an immunogen and for screening. The recombinant protein includes a 24 amino acid tag for purification (black underlined sequence). The homeodomain of GSC (underlined red sequence) was avoided during the design of peptides for antibody production. The final immunogens for GSC MAbs were full length recombinant protein or a mixture of the 2 peptides: PEP1 (aa 2-18) and PEP2 (aa 74-93).

Production of Recombinant Transcription Factor Proteins

Gene	Domain	Expressed as	Purification Strategy	Molecular Weight (kDa)	Yield (mg/L)
GSC	full	inclusion bodies	Inclusion body purification	30.9	5
NANOG	full	inclusion bodies	Inclusion body purification and IMAC (Ni)	37.4	3.5
SLUG	full	inclusion bodies	Inclusion body purification	32.7	2
SNAIL	full	inclusion bodies	Inclusion body purification	31.8	15
SOX09	375-509	inclusion bodies	Inclusion body purification and IMAC (Ni)	18.1	8
SOX09	1-150	soluble	IMAC (Ni)	17.0	2.5
ZEB1	583-1003	soluble	IMAC (Ni)	46.6	4.5
ZEB1	300-510	soluble	IMAC (Ni)	22.7	0.75
ZEB2	181-707	inclusion bodies	Inclusion body purification	61.7	10
ZEB2	647-1214	soluble	IMAC (Ni)	64.6	5
LBX1	full	inclusion bodies	Inclusion body purification	33.0	2.5
CD133	180-400	inclusion bodies	Inclusion body purification	27.4	50
CD133	515-745	inclusion bodies	Inclusion body purification and IMAC (Ni)	28.9	7
FOXO3	355-673	soluble	IMAC (Ni)	34.2	1.5
FOXO2	1-70	inclusion bodies	Inclusion body purification and IMAC (Ni)	10.3	6
FOXO2	200-300	soluble	IMAC (Ni)	10.3	1
FOXO1	1-110	soluble	IMAC (Ni)	10.1	2



- Recombinant Protein Uses:**
- Immunogens
 - Antibody screening and validation
 - Assay calibrators
 - Assay validation (blocking)

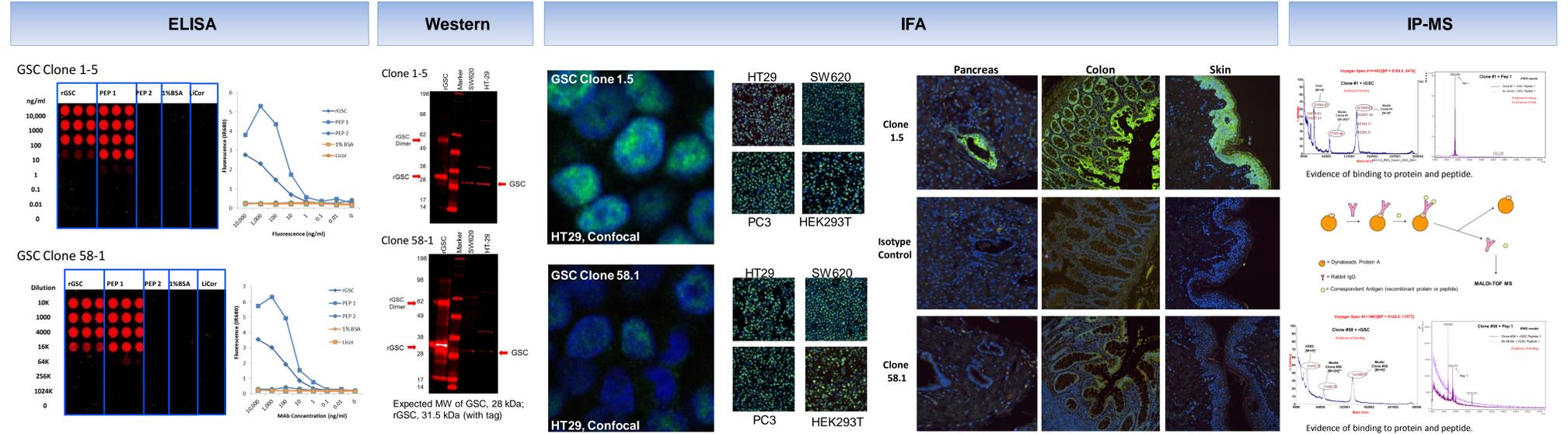
Immunogens for MAb Development

Target	Full length	Immunogen	Amino acid sequence of Immunogen(s)	Status
GSC	257	rProtein	1-257 - -	Characterization
		Peptides	2-18 74-93 - -	Production
NANOG	305	Peptides	A B C	Characterization
SNAIL	264	Peptides	22-39 90-109 137-151	Characterization
SLUG	268	Peptides	29-41 90-104 101-116	Characterization
SOX9	509	Peptides	1-19 48-66 437-455	Production
ZEB1	1124	Peptides	458-477 696-720 736-756	Production
ZEB2	1214	Peptides	436-454 523-543 785-803	Screening
LBX1	281	Peptides	1-22 88-106 - -	Screening
CD133	865	Peptides	A B - -	Production
FOXO3	673	Peptides	43-61 87-95 655-673	Screening
FOXO2	501	Peptides	15-33 45-63 253-273	Screening
FOXO1	403	Peptides	1-22 53-70 386-403	Screening

Conclusions

- The Pharmacodynamics and Therapeutic Functional Working Group (NCI/DCTD and CPTC) in consultation with Dr. Weinberg (MIT) identified a need for specific reagents for EMT/stem cell targets to support research and assay development
- Recombinant proteins have been made for 12 EMT/stem cell proteins (either as full length or domains)
- Antibodies are being developed against these same 12 targets
 - Production of purified MAb to GSC, NANOG, SNAIL and SLUG have been completed; planned completion for the remaining MAb will be in 2013
 - MAbs to GSC and NANOG have been demonstrated to work by ELISA, WB, IFA and IP-MS assays.
 - Additional characterization is in progress

Example A: Characterization of Purified Anti-Goosecoid (GSC) MAbs for use in Immunoassays



Example B: Characterization of Purified Anti-NANOG MAbs for use in Immunoassays

