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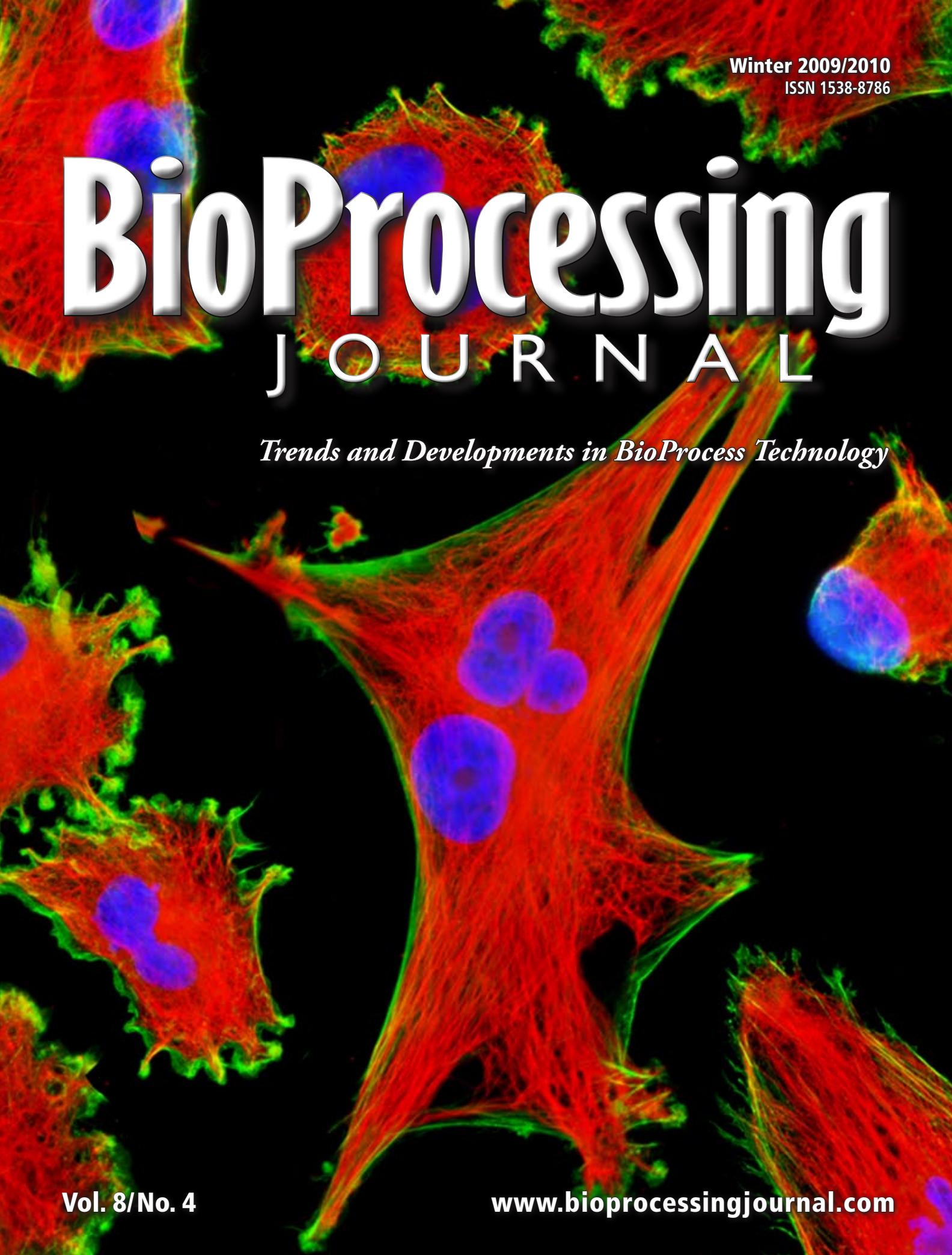

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FANG Vaccine: Autologous Tumor Cell Vaccine Genetically Modified to Express GM-CSF and Block Production of Furin

By PHILLIP B. MAPLES*, PADMASINI KUMAR, YANG YU, ZHAOHUI WANG, CHRIS M. JAY, BEENA O. PAPPEN, DONALD D. RAO, JOSEPH KUHN, JOHN NEMUNAITIS, and NEIL SENZER

Based on our previously published TAG vaccine design[1, 2] and the TAG vaccine clinical results to date (which demonstrate safety and evidence of efficacy—stabilizing disease plus one confirmed complete response; data submitted elsewhere), we have moved forward with a fundamentally new autologous tumor cell vaccine design incorporating a key technical enhancement through our proprietary bifunctional shRNA technology. The resulting FANG vaccine expresses both recombinant human GM-CSF protein and a furin bifunctional shRNA which blocks the expression of furin protein, and then in turn, significantly reduces the expression of both TGFβ1 and TGFβ2 in all primary human tumors tested to date.

TGFβ1 expression data generated from TAG vaccine manufacturing data (n = 33 vaccines, day 7 values, TGFβ1 assay post-vaccine manufacturing) clearly demonstrate that TAG does not interfere with TGFβ1 expression (Figure 1). The clinical significance of blocking TGFβ1 and TGFβ2 (as well as TGFβ3; data not shown) is that they are postulated to be significant negative immunomodulators expressed by the tumor. These TGFβ isoforms are ubiquitous and expressed in the majority of tumors.[3] Many tumors, including breast, colon, esophageal, gastric, hepatocellular, pancreatic, small cell lung cancer (SCLC), and non-SCLC produce high levels of one or more active TGFβ isoforms.[4-12] Furthermore, overexpression of TGFβ has been correlated with tumor progression and poor prognosis.[5, 6] Elevated TGFβ levels have also been linked with immunosuppression in both afferent and efferent limbs.[6,13-18] Additionally, TGFβ has antagonistic effects on natural killer (NK) cells as well as the induction and proliferation of lymphokine-activated killer (LAK) cells.[19-24]

The immune suppressor functions of TGFβ are therefore likely to play a major role in modulating the effectiveness of cancer cell vaccines. TGFβ inhibits GM-CSF induced maturation of bone marrow derived dendritic cells (DCs)[25] as well as expression of major histocompatibility complex (MHC) class II and co-stimulatory molecules.[26] It has been shown that antigen presentation by immature DCs results in T cell unresponsiveness.[27] TGFβ also inhibits activated macrophages[28] including their antigen presenting function.[29, 30] Hence, both the ubiquity of expression as well as the inhibitory effects of TGFβ on GM-CSF immunomodulatory function support the knockdown of all tumor TGFβ expression in this autologous cancer vaccine treatment approach.

Vaccine Production Overview

The surgically excised tumor is placed in sterile saline containing gentamycin and packaged for transport to the manufacturing facility. The tumor is then mechanically and enzymatically dissociated into a single cell suspension. The cells are counted and then transfected with the FANG expression plasmid. The cells are incubated overnight to allow

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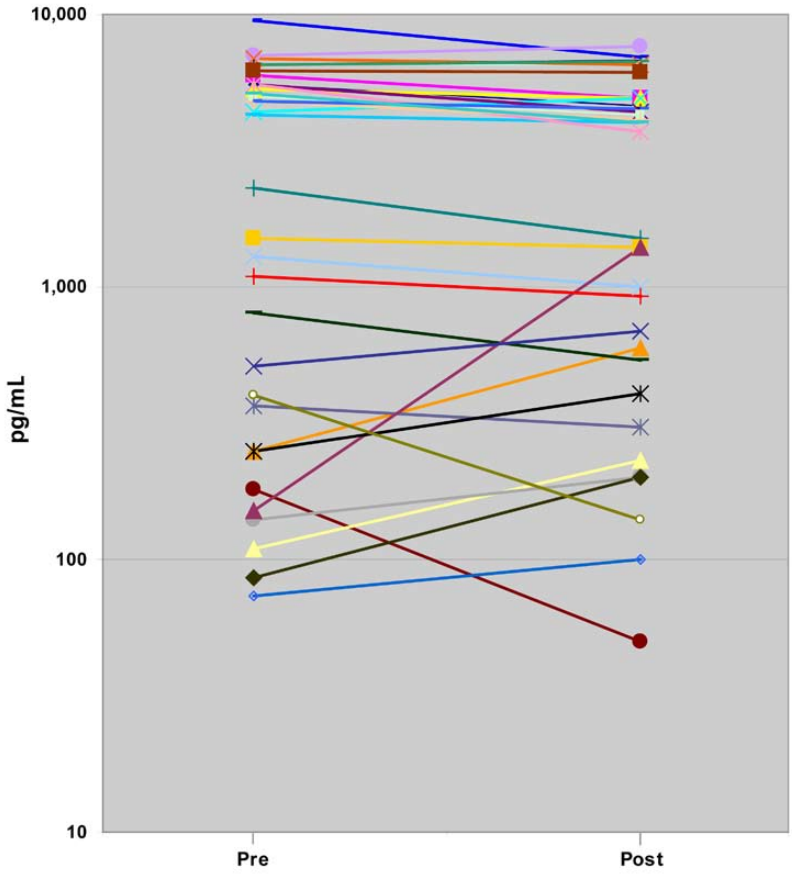


FIGURE 1.
TGFβ1 expression in pre- and post-TAG plasmid transfected autologous tumor cells.

	TGFβ1 Expression (Day 7)	Pre	Post
1	Lung	5,500	4,600
2	Lung	6,000	4,900
3	Lung	5,300	4,900
4	Lung	4,400	4,900
5	Lung	5,500	4,400
6	Lung	180	50
7	Lung	2,300	1,500
8	Lung	9,500	7,000
9	Breast	4,300	4,000
10	Breast	6,500	6,500
11	Breast	5,000	4,300
12	Breast	110	230
13	Melanoma	1,300	1,000
14	Melanoma	5,500	3,700
15	Melanoma	7,100	7,600
16	Melanoma	4,800	4,100
17	Colon	4,800	4,500
18	Colon	5,100	4,000
19	Colon	73	100
20	Colon	1,500	1,400
21	Rectal	250	600
22	Ovarian	6,900	6,500
23	Bladder	367	308
24	Bladder	140	200
25	Neuroendocrine	6,500	6,800
26	Adrenocortical	6,500	6,700
27	Adrenocortical	800	540
28	Gastric	86	200
29	Hepatocellular	6,200	6,100
30	Renal	150	1,400
31	Urachal	510	690
32	Prostate	249	404
33	Leiomyosarcoma	400	140
34	Hemangio pericytoma	1,100	925
All Tumor	Average	3,380	3,094
	Standard Deviation	2,843	2,568
	Median	4,350	3,850

transcription of bi-shRNA^{furin} and the GM-CSF mRNA. The following morning, the cells are harvested, enumerated, and then irradiated at 10,000 cGy in a standard blood bank gamma irradiator. The irradiated cell suspension is then washed, and depending on the total cells harvested, aliquotted and frozen at 1×10^7 or 2.5×10^7 cells per vial (dose cohort 1 or 2, respectively). The freeze media consists of 10% dimethyl sulfoxide ([DMSO], Cryoserv® USP; Bionichepharma USA, Lake Forest, Illinois), 1% human serum albumin ([HSA], Buminate; Baxter Healthcare) in Plasma-Lyte A, pH 7.4 (Baxter). After freezing, the vaccine doses are stored in the vapor phase of liquid nitrogen until all release testing is completed, all necessary approvals are obtained, and the patient is ready for treatment. Currently, each vaccine dose is released per patient/per scheduled injection.

Tumor Procurement and Transport

Tumor procurement takes place in an operating room or other similarly equipped clinical setting where an aseptic process can be conducted to remove tumor tissue or fluid containing tumor cells. Care is taken to confirm with the physician and staff that the patient identity is correct and that the procedure is yielding the appropriate tumor material for processing. All involved personnel understand the need for maintaining sterility of the target material by performing aseptic processing. Gradalis, Inc. personnel are trained to

perform the procurement and transport process. Prior to surgery, the patient has given protocol-specific informed consent (institutional review board [IRB]-approved).

Receipt of Tumor

The tumor shipment is received at the Gradalis, Inc. manufacturing facility within 48 hours of harvest. The outer package is inspected for damage and any signs of tampering. The shipper's information is compared with the expected shipping information. Any inconsistencies are noted and investigated prior to the tumor shipment being taken into the GMP manufacturing area proper.

Tumor Evaluation / Dissection / Preparation of Single Cell Suspension

Tissue processing is aseptically performed in an ISO Class 7 (Class 10,000) clean room under an ISO 5 (Class 100) certified biological safety cabinet (BSC). The tumor tissue is examined for nontumor and nonbiological elements (*e.g.*, fat, necrosis, staples, suture lines, etc.) which are trimmed away. The tissue is weighed and then cut into fine pieces (about 1 mm cubes). This is performed as quickly as possible. The tumor is then mechanically (gentleMACS Dissociator; Miltenyi Biotec, Gladbach, Germany) and enzymatically dissociated into a single cell suspension using GMP grade collagenase (Crescent Chemical Co., Islandia, New York) and DNase I (Pulmozyme; Genentech, South San Francisco, California). The tumor tissue dissociation is performed using gentleMACS C Tubes (the number of tubes depends on the volume of the tumor mass and media used). After dissociation is completed, the cells are washed, resuspended and enumerated (live and total cells) with trypan blue viability stain on a hemocytometer.

Plasmid Design and Construction

The FANG plasmid (Figure 2a) used to transfect the autologous cells is derived from the TAG plasmid[2] by replacing the TGFβ2 antisense sequence with the bi-shRNA^{furin} DNA sequence. Otherwise, these two plasmids are identical (confirmed by DNA sequencing). The bi-shRNA^{furin} consists of two stem-loop structures with miR-30a backbone; the first stem-loop structure has complete complementary guiding strand and passenger strand, while the second stem-loop structure has three bp mismatches at positions 9, 10, and 11 of the passenger strand (Figure 2b). Our current strategy is to use a single targeted site for both cleavage and sequestration. By the use of a proprietary process, the encoding shRNA is able to accommodate mature shRNA loaded onto more than one type of RNA induced silencing complex (RISC).[31] Our reason for focusing on a single site is that multi-site targeting may increase the chance for a "seed sequence" induced off-target effect.[32] The two stem-loop double stranded DNA sequence was assembled with ten pieces of synthetic complementing and interconnecting oligonucleotides through DNA ligation. The completed 241 base pairs DNA with Bam HI sites at both ends was inserted into the Bam HI site of the TAG expression vector in place of the TGFβ2 antisense sequence. Orientation of the inserted DNA was screened by PCR primer pairs designed to screen for the shRNA insert and orientation.

The FANG construct has a single mammalian promoter (cytomegalovirus [CMV]) that drives the entire cassette, with an intervening 2A ribosomal skip peptide between the GM-CSF and the furin bifunctional shRNA transcript, followed by a rabbit poly-A tail. There is a stop codon at the end of the GM-CSF transcript. Insertion of picornaviral 2A sequences into mRNAs causes ribosomes to skip formation

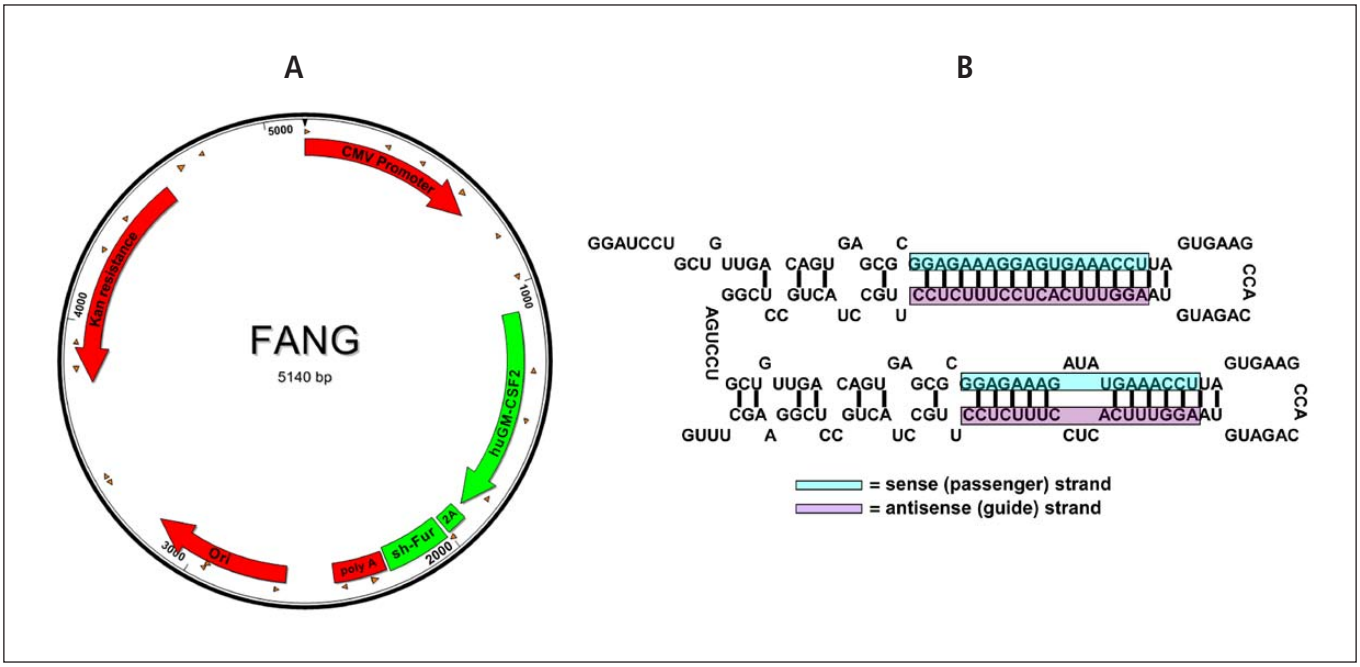
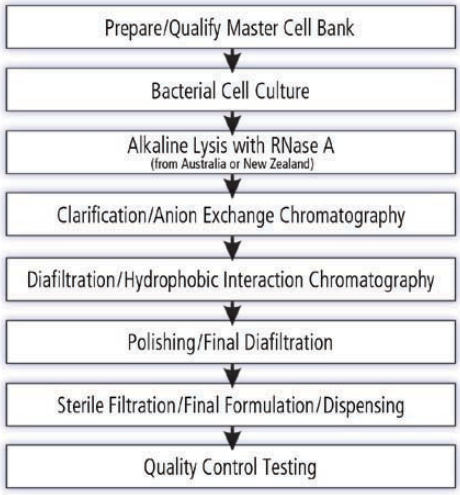


FIGURE 2. A) Schematic diagram of the FANG plasmid; and B) furin bifunctional shRNA.

of a peptide bond at the junction of the 2A and downstream sequences, leading to the production of two proteins from a single open reading frame.[33] We have found the 2A linker to be effective for generating approximately equal levels of GM-CSF and anti-TGFβ transcripts with the TAG vaccine, and elected to use the same design for FANG.

Manufacturing of Clinical Grade FANG Plasmid

The FANG vector was sent to Aldevron (Fargo, North Dakota) for GMP source manufacturing. There were no residual organic solvents in the plasmid product as they were not utilized in the purification process—only ethanol and isopropanol were used. The summary of the process was as follows:



An aliquot of the purified DNA was sent to SeqWright (Houston, Texas) for sequence analysis. The GM-CSF bi-shRNA^{furin} insert was sequenced and the consensus sequence perfectly matched the expected insert sequence. This GMP source plasmid DNA was used for all *in vitro* expression assays and electroporation into the autologous tumor cells.

The FANG vector was electroporated into the autologous tumor cells *ex vivo*. Therefore, only the cells present at the time of electroporation incorporated the transfected DNA. Electroporation conditions used for transfection were not optimized for maximal transfection efficiency, but instead ensured greater cell viability. This was because many different tumor types were being used for vaccine manufacturing.

The vector utilized is expected to remain extra-chromosomal. Amplification of the insert by PCR suggests that the vector is non-rearranged in the cells (data not shown). This is consistent with our prior vaccine experience. It is theoretically possible that the structure or expression of a gene near an integration site (if the vector is incorporated into a chromosome) may be affected by insertion of the added DNA. However, all cells used for vaccinations are irradiated with 10,000 cGy to block their growth potential. In addition to inhibiting cell proliferation, the 10,000 cGy irradiation further enhances immunogenicity by increasing MHCII expression.[34] The selection of this radiation dose is based on the desire to utilize the lowest possible radiation dose for the transfected cells to optimize the level and duration of furin shRNA transcription and GM-CSF protein production while maximizing the safety of vaccine cell injections at the same time. This same radiation dose has been used in prior approved clinical vaccine studies (*e.g.*, TAG[1] and Xeno TAG[2]).

Tumor Cell Transfection

Transfection is accomplished by electroporation of the tumor cells using an electroporator (Bio-Rad Laboratories, Hercules, California). A mixture of 50 µg of plasmid (50 µL) is combined with 2.0×10^7 cells (500 µL) in a sterile 0.4 cm gap cuvette. An exponential decay pulse waveform is applied using the following conditions: electrical current of 300 V, capacitance of 1000 uF and resistance set to infinity (determined by testing various voltages on similar cells for optimum viability of tumor cells and transfection of vector). Time constants are recorded for each electroporated aliquot of tumor cells. Cuvettes are visually inspected following electroporation for telltale signs that electroporation has been successful. Following electroporation, tumor cells are incubated overnight at 37°C. The cells are incubated to allow transcription of the bi-shRNA^{furin} and the GM-CSF mRNA.

The following morning, the tumor cells are harvested, washed, enumerated by hemocytometer, and then irradiated at 10,000 cGy in a standard blood bank gamma irradiator. Irradiation with 10,000 cGy arrests cell proliferation so that tumor cells cannot form any new tumors when vaccine is injected into the patient.

Preparation of the Final Product

Following irradiation, cells are washed and resuspended in 1% Buminate HSA (Baxter) in Plasma-Lyte A (Baxter), pH 7.4 at a cell concentration twice the final frozen concentration. Final cell concentrations are for two dose levels. Cohort 1 dosing is 1.0×10^7 and cohort 2 is 2.5×10^7 cells/injection. The goal is to make a minimum of five vaccine doses and the optimal result is to generate 12 vaccine doses at the higher level. The final vial for each dose of vaccine is a sterile 2.0 mL externally threaded screw cap cryovial (Nalgene, Rochester, New York). The freeze media consists of 10% Cryoserv and 1% Buminate in Plasma-Lyte A, pH 7.4. The cold cell suspension and freeze media are mixed in equal proportions and placed in the cold freezing container (Cryo 1°C "Mr. Frosty," Nalgene). The cells are gradually frozen to approximately -80°C. After freezing, the cells are stored in the vapor phase of liquid nitrogen until all release testing is completed, all necessary approvals are obtained, and the patient is ready for treatment (time from procurement to treatment is three weeks).

Before being injected into patients, a frozen vial (single dose) will be thawed at room temperature and processed in a biosafety hood. The cell suspension will be delivered in a capped 1 mL syringe. The prepared vaccine will be injected intradermally into the patient at a dose of 1.0×10^7 or 2.5×10^7 cells per injection.

Two full-scale preclinical manufacturing processes and eight clinical manufacturing processes have been performed.

Table 1 depicts the types of tumors processed (tumors 3–10 are the clinical vaccines).

The tumors processed range in size, as well as type, and the resulting viable cell yield varies greatly (Table 1). All vaccines are vialed at either 1.0 x 10^7 cells (dose cohort 1) or 2.5 x 10^7 cells (dose cohort 2) depending on the total viable cell count on day 2 of manufacturing. A minimum of five doses at the cohort 1 dose level is needed to consider the manufacturing process successful. Vaccines for patients who have undergone multiple separate tumor harvest procedures can be treated from multiple lots to qualify for the minimum clinical dose requirement. A maximum of 12 doses at cohort 2 dose level will be made available for patient treatment. Because tumor cell yield is highly variable due to tumor mass, cellularity, and processing compatibility, the minimum dose number and lower dose cohort (cohort 1) has been included (Table 1). There are data to suggest that even a few doses at a lower cell number may have some clinical benefit[35] (including our low dose TAG patient CR).

The protocol for setting up cultures pre- and post-transfection for autologous tumor cell vaccine testing for the expression of GM-CSF, TGFβ1 and TGFβ2 has been previously described.[1,2] Briefly, GM-CSF, TGFβ1, and TGFβ2 expression are determined by commercially available ELISA kits (R&D Systems, Minneapolis, Minnesota). The ELISA assays are performed according to the manufacturer's instructions. The pre-transfection sample (4.0 x 10^6 cells) is taken on day 1. On day 2, the post-transfection, post-irradiation, pre-freeze sample (64 x 10^6 cells) is taken. After each manufacturing day is completed, the sample is removed from the manufacturing facility so that the cell cultures can be set up to generate the ELISA data.

Ten vaccines (FANG-001 to -010) have been manufactured as part of the preclinical qualification process. These vaccines have been evaluated for GM-CSF, TGFβ1, and TGFβ2 mRNA and protein expression using post-transfection, post-irradiation samples compared with pre-transfection, pre-irradiation samples (per FDA review, TAG[1] vaccine, BB-IND 13650). In addition, furin protein detection was attempted by several methods. Furin mRNA was detected by reverse transcriptase (RT)-quantitative polymerase chain reaction (qPCR).

A summary of all ELISA data for each of the manufacturing processes (Table 2) indicates that the median level of GM-CSF

TABLE 1. Tumors processed for each FANG vaccine, tumor mass versus cell yield, and final product viability (day 2, pre-irradiation).

Tumor Processed	Vaccine ID	Diagnosis	Tissue Weight (grams)	Cell # Per Dose	Number of Vials	% Viability
1	FANG-001	Melanoma	12.72	1.0 x 10^7	40	78
2	FANG-002	Melanoma	27.41	1.0 x 10^7	28	90
3	FANG-003	Gall Bladder Cancer	6.04	2.5 x 10^7	9	94
4	FANG-004	NSCLC	41.08	2.5 x 10^7	11	89
5	FANG-005	Melanoma	6.96	2.5 x 10^7	8	94
6	FANG-006	Melanoma	12.48	1.0 x 10^7	8	91
7	FANG-007	Colon Cancer	10.90	2.5 x 10^7	15	96
8	FANG-008	Colorectal Cancer	9.80	2.5 x 10^7	13	95
9	FANG-009	Breast Cancer	6.80	1.0 x 10^7	6	95
10	FANG-010	Colorectal Cancer	13.00	2.5 x 10^7	12	93

TABLE 2. FANG vaccines 001–010, TGFβ1, TGFβ2, and GM-CSF expression in the 14 day post-manufacturing expression assay.

Time Point (Day)	TGFβ1 pg/mL Pre			TGFβ1 pg/mL Post			TGFβ2 pg/mL Pre			TGFβ2 pg/mL Post			GM-CSF pg/mL Pre			GM-CSF pg/mL Post		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
0	625	678	416	105	202	7	70	116	25	9	22	0	2	2	2	157	277	29
1	1154	1266	760	93	187	11	138	139	113	9	19	0	3	4	3	359	469	281
2	998	1014	620	180	446	0	199	107	197	12	21	4	3	3	3	407	418	310
3	1832	3221	879	173	394	4	247	156	229	12	16	8	3	4	2	580	531	475
4	1241	1115	1039	211	421	20	293	189	257	9	12	4	4	6	3	657	550	602
7	1729	1735	778	264	723	3	292	150	235	14	16	8	5	9	3	683	681	471
10	1367	994	1629	243	530	21	335	135	310	23	21	28	5	8	4	745	546	673
14	1108	892	887	281	661	19	308	158	229	17	23	12	18	24	4	821	631	645
Overall	**1251**	**1544**	**778**	**191**	**455**	**13**	**232**	**164**	**225**	**13**	**19**	**5**	**5**	**10**	**3**	**543**	**540**	**400**

expression is about 400 pg/mL, and the average is 543 pg/mL. Further, the level of GM-CSF tends to increase with time. In all manufactured products, GM-CSF expression is observed although the level of expression is variable between manufacturing processes (tumor types). In addition to documented variability in the level of GM-CSF expression between manufacturing processes, the levels of expression achieved with the FANG vaccine are deemed clinically relevant as: 1) use of a plasmid rather than a viral vector obviates the obfuscating effects of elicited anti-viral neutralizing antibodies; 2) use of a plasmid likewise prevents the development of elicited antibodies interfering with long-term gene expression; and 3) concurrent suppression of furin, TGFβ1, and TGFβ2 will minimize tumor-associated inhibition of GM-CSF induced dendritic cell maturation.[25]

The electroporation of FANG plasmid into patient tumor cells (the cGMP vaccine manufacturing process) demonstrated GM-CSF protein production and concomitantly, TGFβ1 and TGFβ2 knockdown as predicted. Figure 3 depicts day 7 assay data of a FANG-transfected NSCLC tumor's expression profile (FANG-004) versus tissue from the same tumor processed by the cGMP TAG vaccine method (denoted TAG-004). There are similar reductions in TGFβ2 and similar increases in GM-CSF expression. However, while TGFβ1 expression is completely inhibited by FANG, it is unaffected by TAG as the TGFβ2 antisense cannot block TGFβ1 expression.

The day 4 expression profiles of the ten tumors processed to date are depicted in Figure 4 and Table 2. Note that the y-axis scales are different for all three cytokines. These data are representative of the 14 day assay (remainder of data not shown). These data in Table 2 indicate that the GM-CSF expression is consistent with the TAG vaccine as is the TGFβ2 knockdown. In contrast, FANG vaccines have reduced the TGFβ1 expression more than five-fold (refer to TAG vaccine values, Figure 1). The minimum detection limit for each corresponding Quantikine kit (R&D Systems) is: 1) TGFβ1, approximately 4.6 pg/mL; 2) TGFβ2, approximately 7 pg/mL; and 3) GM-CSF, approximately 3 pg/mL.

Furin Protein Detection

We have attempted to detect endogenous furin protein in cell lines via Western blot and flow cytometry. Five different antibodies (from three different vendors) were screened for Western blot and one pre-labeled antibody was screened for flow cytometry. All experiments yielded negative results (data not shown).

As an alternative to furin protein detection, we also screened samples for furin enzyme activity. Using a fluorometric-based assay, cell lines were screened for the conversion of substrate (Pyr-Arg-Thr-Lys-Arg-AMC) by

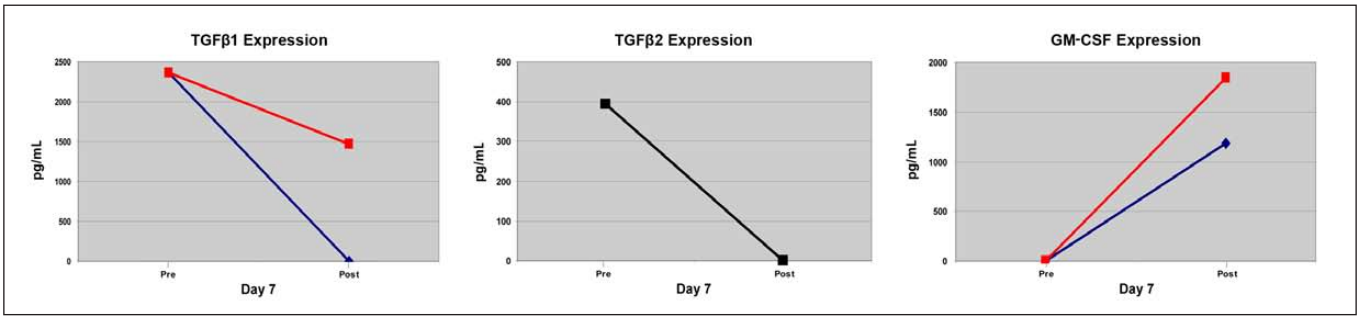


FIGURE 3. Side-by-side comparison of cGMP FANG-004 and TAG-004 manufactured consecutively, same patient tumor. Values represent ELISA determinations of cytokine production at day 7 post-transfection in harvested autologous cancer cells transfected with either FANG (blue) or TAG plasmid (red). The TGFβ2 post transfection values are the same for TAG and FANG, hence the black line.

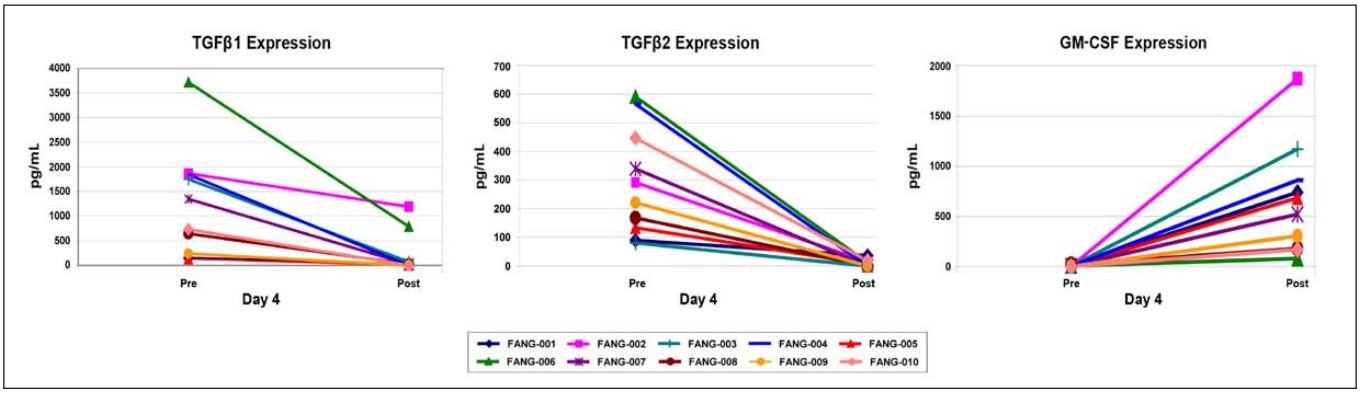


FIGURE 4. Summary of TGFβ1, TGFβ2, and GM-CSF protein production pre- and post-FANG plasmid transfection. ELISA values from day 4 of the 14 day determinations of cytokine production in manufactured autologous cancer cells. Data represents autologous vaccines independently generated from ten patients who underwent FANG processing (FANG-001 through -010).

furin to release the fluorophore 7-amino-4-methylcoumarin (AMC). However, the detected signal of released AMC was too low to accurately demonstrate significant knockdown of furin enzyme activity (data not shown). A second barrier to the assay is that the substrate is cleaved by all serine proteases in the subtilisin-like prohormone convertase (PC) family. Therefore, similar proteases that are not targeted by our FANG shRNA product will remain active and cleave the fluorogenic substrate in the assay, thus further reducing the capability to detect furin knockdown.

RT-qPCR Detection of GM-CSF, Furin, TGFß1, and TGFß2 mRNAs

RT-qPCR analysis was performed on ten FANG vaccine samples (FANG-003 did not have adequate mRNA for analysis). Samples were cultured pre- and post-electroporation for up to 14 days. Total RNA was extracted from each sample at various time points and converted into cDNA via reverse transcription, and qPCR was performed to assess the amount of template present in each sample at each time point. Furin, TGFβ1, and TGFβ2 qPCR samples were normalized to endogenous glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to produce a relative cycle threshold (Ct) value. GM-CSF was quantified against an external standard curve to produce an absolute Ct value, relative to the standard curve. The GM-CSF mRNA detection is shown in Figure 5. Post-transfection, GM-CSF mRNA is detected in all vaccines but the values are variable depending on mRNA quality—a persistent issue. The data in Table 3 illustrates representative data from two FANG vaccines (Figures 6 and 7). All samples were calculated as normalized pre-electroporation Ct values minus normalized post-electroporation Ct values (pre-post) to calculate the delta Ct (Δ Ct). A calculated Δ Ct < 0.00 represents a decrease in template DNA and a calculated Δ Ct > 0.00 represents an increase in template DNA. The Δ Ct value is used to estimate the percent change in expression (% expression). Values less than 100% represent a decrease in DNA (from pre to post) and values greater than 100% represent an increase in DNA (from pre to post). The nature of shRNA/siRNA silencing can optimally reduce the template DNA by 90%, which is equivalent to a Δ Ct = -3.3 (a Δ Ct = -1.0 is equivalent to a 50% knockdown). Therefore, Figures 6 and 7 and Table 3 demonstrate that the FANG plasmid DNA is able to reduce endogenous furin mRNA from 80% to 26% (average = 48%) and the downstream targets TGFβ1 and TGFβ2 are reduced from 98% to 30% (average = 75%). The mechanisms of action of the furin bifunctional shRNA are to block furin protein production at the post-transcriptional and translational levels. The reduced levels of furin protein also impact (by feedback regulation) the expression of TGFβ1 and TGFβ2 mRNA, the conversion of the proform of TGFβ1 and TGFβ2 protein into the mature (active) form of their respective proteins[36] and, by interfering with the TGFβ → furin amplification loop, further dampen the expression of furin itself.[37] The reduced expression of TGFβ1 protein also decreases TGFβ1 gene expression by modulating an autoinductive feedback.[38] This is postulated to be true for TGFβ2 as well.

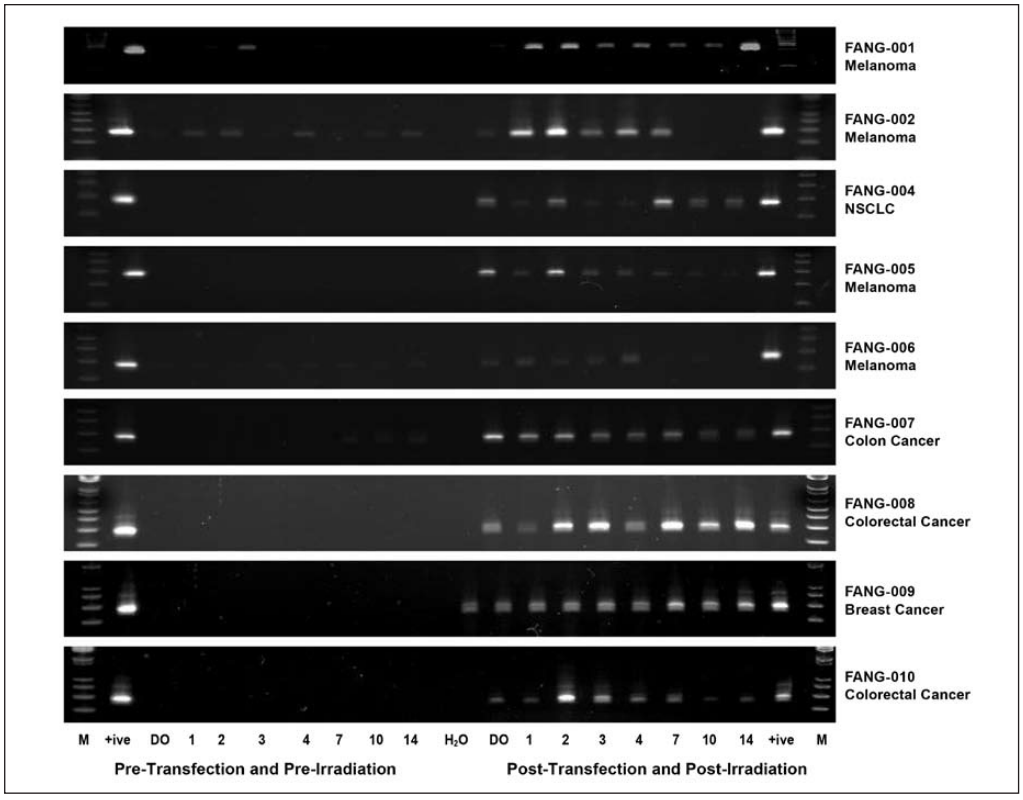


FIGURE 5. GM-CSF mRNA by RT-qPCR in pre- and post-FANG transfected/irradiated tumor cells. The absence of bands in some of the lanes is due to degraded RNA. The extra band in FANG-009 is day 0 sample loaded twice.

TABLE 3. RT-qPCR analysis of FANG vaccines (pre- versus post-electroporation).												
Time Point (Day)	FANG-008						FANG-009					
	Furin		TGFβ1		TGFβ2		Furin		TGFβ1		TGFβ2	
	Δ Ct	% Expression	Δ Ct	% Expression	Δ Ct	% Expression	Δ Ct	% Expression	Δ Ct	% Expression	Δ Ct	% Expression
0	-1.52	35	-0.09	94	0.00	N/A*	-0.66	63	-0.54	69	-0.53	69
1	-1.50	35	-0.10	93	0.00	N/A*	-0.69	62	-0.49	71	-0.47	72
2	-1.48	36	-0.08	95	0.00	N/A*	-0.34	79	-0.42	75	-0.45	73
4	-1.50	35	-0.05	97	0.00	N/A*	-0.31	80	-0.31	81	-0.52	70
7	-1.22	43	-0.08	95	0.00	N/A*	-1.93	26	-0.04	98	-1.70	31
10	-1.41	38	-0.11	93	0.00	N/A*	0.00	N/A*	-1.29	41	-1.74	30

Δ Ct = 0.00 % expression baseline = 100% *N/A = not applicable because template was below detection limits

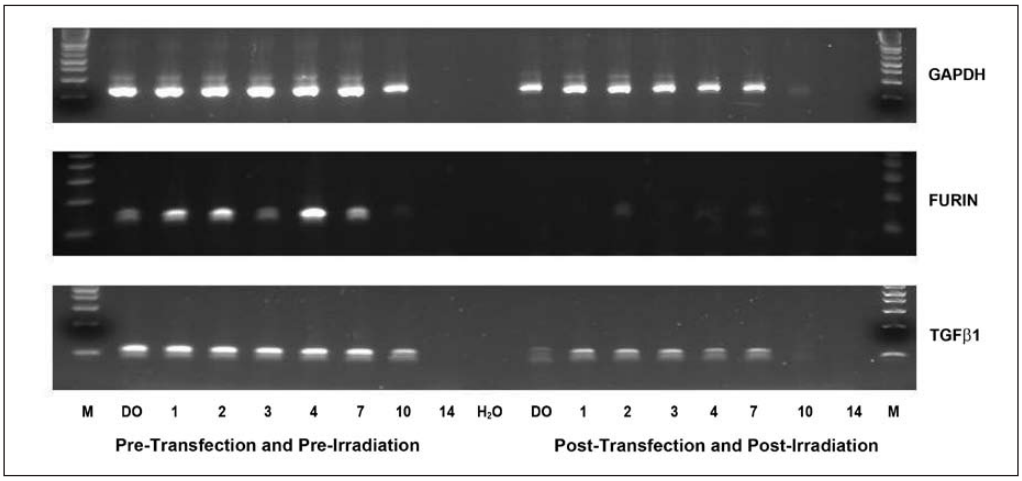


FIGURE 6. FANG-008 vaccine cells pre-transfection and post-transfection/irradiation mRNA by PCR. Did not detect signal in pre- and post- samples for TGFβ2.

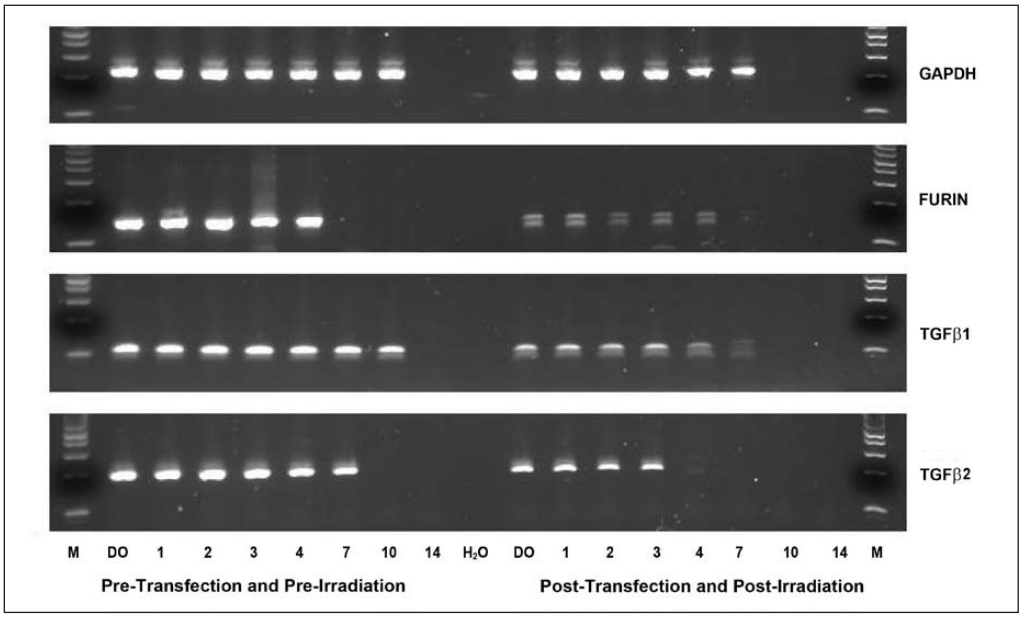


FIGURE 7. FANG-009 vaccine cells pre-transfection and post-transfection/irradiation mRNA by PCR.

TABLE 4. Final product release specifications and characterization.		
Release Test	**Test Method**	**Specification**
Cell number	Hemocytometer	Dose cohort 1 or 2
Cell viability	Trypan blue dye exclusion	≥ 70% viable
Endotoxin	GLP kinetic chromogenic LAL	≤ 5 EU/mL (dose)
Sterility	21 CFR 610.12	No growth
Mycoplasma	GLP rapid PCR	No *mycoplasma* DNA detected
GM-CSF	ELISA	≥ 30 pg/10^6 cells/mL

Stability of the Final Product

The preclinical manufacturing runs (FANG-001 and FANG-002) were stored in the vapor phase of liquid nitrogen. After three months, one aliquot of each was thawed and assessed for cell number, viability, GM-CSF, TGFβ1, and TGFβ2 by ELISA and RT-qPCR. Cell recovery was essentially 100%. The post-thaw viable cell recovery was 88.8% and 93.6% of input, respectively. The pre-freeze viability of FANG-001 and -002 were 78% and 90%, respectively. The post-thaw viabilities were 80% and 95%, respectively. Total cell numbers declined over 48 hours in culture, post-thaw. After 48 hours, total cell numbers for FANG-001 was 90% of input and FANG-002 was 83% of input. Viable cell numbers similarly decreased with FANG-001 viable cell number at 84% of input after 48 hours, and FANG-002 at 77% of input. TGFβ1 and TGFβ2 protein expression remained suppressed over 48 hours of culture at levels consistent with pre-freeze, post-irradiation data. GM-CSF protein increased over 48 hours of culture which was consistent with pre-freeze, post-irradiation data, and GM-CSF mRNA was detectable by RT-qPCR (data not shown).

These initial stability results indicate that significant cell recovery is possible after three months frozen storage and that GM-CSF protein expression (by ELISA and RT-qPCR) and TGFβ1 and TGFβ2 knockdown (by ELISA) persist and are readily detectable.

The ongoing stability plan is formulated around the results of the preliminary test data. Cell count and viability determinations, along with the ELISA and RT-qPCR procedures, are a part of the stability assessment. In addition to these tests, each sample will be tested for sterility by USP sterility test (21 CFR 610.12).[39] Besides, FANG-001 and FANG-002 stability study samples are generated from vaccines manufactured for patients who will not receive vaccine or that have come off study (become ineligible). These stability samples are generated by the same manufacturing process as any other clinical vaccine. All study samples are stored in the vapor phase of liquid nitrogen and are otherwise handled as any clinical vaccine. The stability program analyzes samples at 6 (if possible), 12, 18, 24, 36, 48, and 60 months.

Product Characterization and Release Specifications

The final product release specifications are listed in Table 4. The specifications and corresponding assays used are listed in the following paragraphs.

Potency

Cell number is used as the primary indicator of potency. Based on previous vaccine trials, cell dosage is a significant factor in determining response to vaccine treatment. GM-CSF protein is another indicator of the vaccine's biological activity.

Identity and Specificity

Cell viability is used as the primary indicator of identity and specificity. The cells liberated from the tumor tissue are distinct in size and morphology and easily distinguishable throughout the manufacturing process. Because many tumor types are being processed for vaccines, no common marker is readily available at this time to denote identity or specificity more conclusively.

Endotoxin

The level of endotoxin present in the final product is determined by the Limulus amebocyte lysate (LAL) kinetic chromogenic method, according to the USP Chapter <85> Bacterial Endotoxins test.

Sterility

Sterility is confirmed by USP Sterility 14 day test as detailed in 21 CFR 610.12.[39]

Mycoplasma

Mycoplasma detection is performed by Touchdown PCR (TD-PCR) GLP rapid assay (WuXi AppTec, Philadelphia, Pennsylvania) for release. The limit of detection is ten copies.

For Information Only (FIO) Testing

We collect additional samples for TGFβ1 and TGFβ2 ELISA and for GM-CSF, TGFβ1, TGFβ2, and furin RT-qPCR assays, as well as for additional assays. These test results will allow Gradalis, Inc. to better define potency, identity, and specificity aspects of the vaccines.

Conclusion

Gene modified cell-based cancer vaccines have demonstrated durable responses in selected patients. We have developed the FANG expression vector which we believe, when transfected into tumor cells, will evoke an enhanced immune recognition/stimulation versus our previous TAG vaccine vector. The FANG nonviral vector system expresses both GM-CSF and a proprietary bifunctional shRNA to furin. Preclinical data demonstrates that blocking furin protein expression in turn blocked the activation of both TGFβ1 and TGFβ2. In contrast, our TAG vector expressed both GM-CSF and a TGFβ2 antisense. Data from our TAG Phase I autologous vaccine clinical trial and others indicate that TGFβ1 overexpression is present in a wide range of cancers. In fact, our data suggest that TGFβ1 tends to be about five- to ten-fold higher than TGFβ2 expression in the more than 30 tumors we have analyzed in the study. So while the TAG vector blocked TGFβ2 expression, there was no effect on TGFβ1 expression. The FANG expression vector is identical to the TAG expression vector except that the TGFβ2 antisense coding sequence has been replaced with the shRNA^{furin} sequence. We have generated two nonclinical and eight clinical vaccines under cGMP as part of our IND submission data. All vaccine manufacturing processes have met final product specifications (no contamination or failure to meet final dose or quality requirements). GM-CSF expression was consistent with the TAG vaccine values as was the TGFβ2 knockdown. In contrast, FANG vaccines have reduced the TGFβ1 expression more than five-fold. The outcome of the clinical studies will determine whether this added reduction has a significant added clinical impact. Gradalis has received IND approval from FDA (BB-IND 14205), and the FANG Phase I clinical trial is now open.

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NOTES

1. Cryoserv is a trademark of Bionichepharma USA.
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