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TITLE: Early Identification of Molecular Predictors of Heterotopic Ossification Following Extremity Blast Injury with a Biomarker Assay

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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> The <b>purpose</b> of this project is to identify predictive markers of heterotopic ossification in an established animal model that would forecast development of heterotopic ossification (HO) in humans soon after injury. Blast procedures have been completed on all 30 animals (Groups I & II) in the year 1 SOW and 45 animals (Groups III – V) in the year 2 SOW. All animals were biopsied and have been sacrificed according to protocol schedule. Groups I and II animals were also followed with scheduled routine radiographs to monitor progression of HO. Specimen samples were analyzed for gene and protein level expression with the Nesti partnering molecular biology lab. Early-appearing gene and protein biomarkers were identified by correlation between animals exhibiting radiographic evidence of HO.					
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## **I. INTRODUCTION:**

Heterotopic ossification (HO), characterized by the pathologic formation of mature bone in the soft tissues, is a frequent complication following high energy orthopaedic trauma. HO is prevalent in patients with severe extremity war-time wounds; specifically, its incidence is reported between 57-63% in patients that sustain a poly-trauma blast injury [1,2]. Complications related to HO in residual limbs following blast amputation include pain, overlying skin and muscle breakdown, poor fitting and functioning of prosthetic limbs, reoperation for amputation revision, and impaired limb function that delays or limits rehabilitation [3-7]. Current treatments to prevent HO are limited to mitigation rather than prevention. Furthermore, removal of heterotopic bone after it has formed can be difficult; this frequently requires resection of substantial amounts of soft tissue and risks injury to adjacent neurovascular structures that are often intimately associated with the ectopic bone. Hence, it is preferable to address the issue of HO before it begins. Prevention of HO in residual limbs is needed to offer amputation survivors the best possible quality of life and return to function. We have developed a validated blast amputation animal model and confirmed that it replicates the human condition with respect to formation of HO. The current studies are directed at identifying early-appearing biomarkers in the animal model that predict the occurrence of HO in our experimental animals and determine if a correlation exists to similarly predict the development of HO in the human condition. Patients exhibiting biomarkers predictive of exuberant HO formation can then be identified before the disease process begins and treated prophylactically.

## **II. BODY:**

**All 75 hind-limb blast amputation procedures under Specific Aims 1 & 2 in year 1 & 2 SOW (Groups I – V) have been completed, and all 150 specimens from both amputated and contralateral control limbs have been collected.** Group I and II animals (15 each) were followed with serial radiographs to monitor progression of HO and sacrificed at 24 weeks post-blast. Group I animals underwent bilateral muscle biopsy procedure at two weeks, while Group II animals underwent biopsy procedure at four weeks. Group III – V animals (15 each) were biopsied at 24 hours, 24 hours, and 72 hours, respectively, and sacrificed at the same time as biopsy procedures, as per protocol. All animals, except those in Group V, underwent standard wound care with bulb syringe irrigation prior to wound closure following blast amputation. Prior to wound closure, group V animals underwent pulsed lavage irrigation. The biopsy specimens were processed to collect total RNAs and protein lysates for both gene- and protein-level biomarkers.

*Results: HO progression has been assessed and graded between immediate post-blast and post-mortem radiographs on Group I & II animals. Radiographic HO data acquired from Group I & II animals and biomarker expression data are included in appendix a. Supporting Data.*

## **III. KEY RESEARCH ACCOMPLISHMENTS:**

- HO progression has been assessed and graded between immediate post-blast and post-mortem radiographs on Group I & II animals. Radiographic HO data acquired from Group I & II animals are included in **Figure 1**.
- Rat biopsy samples at 2 weeks, 4 weeks, 24h, and 72h post-injury were used for RNA biomarker screening.
  - The Wound healing and Osteogenesis pathway specific PCR Array, which contains 84 genes, was performed.
  - Data analysis was performed using the RT<sup>2</sup> Profiler PCR Array Data Analysis software (SABiosciences) and Venn Diagram analysis.
  - From these analyses, we found that many of genes in Wound healing pathway were related to fibrosis and inflammation (**Figure 2**) and as a result, we extended our analysis using the Fibrosis pathway specific PCR array.
  - We generated a list of genes with significantly altered expression (fold change  $\geq 2$ ) from each of these stages and applied it to a Venn diagram analysis.
  - We found that 47 genes overlapped among these four stages (**Figure 3A**). **Figure 3B** shows the list of 47 genes.
  - From these 47 genes, we categorized 3 patterns:
    - First, common genes (7: Bcl2, Cxcr4, Grem1, Itgav, Mmp14, Mmp2, and Tgfbr2) showed the increased gene expression pattern through the stages (**Figure 4**).
    - Second, Common genes (13: Ccl12, Ccl3, Hgf, Lox, Mmp3, Nfkb1, Plat, Serpinh1, Snai1, Stat6, Tgfb1, Thbs2, and Tnf) showed the same gene expression pattern between the early stages (24h and 72h) and late stages (2 weeks and 4 weeks) (**Figure 5**).

- Third, Common genes (13: Akt1, Ccr2, Eng, Il10, Ilk, Itga2, Itgb3, Itgb6, Plau, Serpine1, Smad2, Thbs1, and Timp1) showed the different gene expression pattern between the early stages (24h and 72h) and late stages (2 weeks and 4 weeks) (**Figure 6**).
- The first pattern may be used as prognostic markers of HO development.
- The second and third pattern may be used as a marker for early detection of HO.
- *Each of candidate genes should be understood in the context of HO and fibrosis and validated in vitro and in vivo animal and clinical human samples analyzed in the partnering PI laboratory of Dr Leon Nesti.*

#### **IV. REPORTABLE OUTCOMES:**

**Other Achievements:** The experience and training provided by this award directly contributed to the successful hiring of the past research resident to a position in the Orthopaedic residency at the Medical University of South Carolina.

#### **V. CONCLUSIONS:**

**Up-regulation of genes in the Sprague-Dawley rat contributing to fibrosis and inflammation have been correlated with the development of heterotopic ossification after traumatic blast amputation in an animal model. Correlation of similar gene expression in human specimens from the partnering PI lab may provide insights into mechanisms of HO that are operative following blast injury in humans. These observations may identify mechanisms that are potentially modifiable by therapeutic interventions designed to mitigate heterotopic ossification after blast injury.**

#### **VI. REQUEST FOR GRANT TRANSFER / NO COST EXTENSION:**

#### **VII. PERSONNEL RECEIVING SUPPORT FROM THE RESEARCH EFFORT**

Vincent D. Pellegrini, Jr, MD, PI  
 William Fourney, PhD – Collaborator  
 Ulrich Lieste, PhD - Collaborator  
 Zilan Lin, MD – Resident Research Technician  
 Yongren Wu, PhD – Post-doctoral scholar  
 Alexander Chiaramonti, MD – Resident Research Technician

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## IX. APPENDIX:

### a. Supporting Data:

Rat #	Biopsy Time	Post-op Radiographic Measurements (mm)		Postmortem Radiographic Measurements (mm)		%L	%W	HO Grade			HO Severity Score
		Length	Width	Length	Width			L	W	Overall	
1	2 weeks	8.85	10.9	11.3	12.1	27.7	11.0	moderate	mild	moderate	2
2	2 weeks	13.7	8.98	15	12.3	9.5	37.0	mild	moderate	moderate	2
3	2 weeks	11.6	16.8	13.1	17.8	12.9	6.0	mild	mild	mild	1
4	2 weeks	12.4	10.2	14.7	10.5	18.5	2.9	mild	mild	mild	1
5	2 weeks	15	6.66	12.7	7.38	-15.3	10.8	mild	mild	mild	1
6	2 weeks	15	11.9	12.6	7.94	-16.0	-33.3	mild	mild	mild	1
7	2 weeks	12.7	9.36	11.9	10.3	-6.3	10.0	mild	mild	mild	1
8	2 weeks	15.8	7.35	19.3	9.04	22.2	23.0	mild	mild	mild	1
9	2 weeks	8.87	10.8	10.9	12.1	22.9	12.0	mild	mild	mild	1
10	2 weeks	9.83	17.5	15.4	13	56.7	-25.7	severe	mild	severe	3
11	2 weeks	8.01	10.6	5.82	8.31	-27.3	-21.6	mild	mild	mild	1
12	2 weeks	9.89	7.64	8.31	9.88	-16.0	29.3	mild	moderate	moderate	2
13	2 weeks	9.36	10.7	14.8	9.82	58.1	-8.2	severe	mild	severe	3
14	2 weeks	14.9	9.04	15.9	8.63	6.7	-4.5	mild	mild	mild	1
15	2 weeks	10.1	10.7	12.7	10.8	25.7	0.9	moderate	mild	moderate	2
16	4 weeks	8.93	8.13	11.4	8.35	27.7	2.7	moderate	mild	moderate	2
17	4 weeks	15.8	11.1	17	11	7.6	-0.9	mild	mild	mild	1
18	4 weeks	11.5	12.6	8.41	12.7	-26.9	0.8	mild	mild	mild	1
19	4 weeks	7.39	8.29	5.81	11.8	-21.4	42.3	mild	moderate	moderate	2
20	4 weeks	13.5	10.3	15.2	11.6	12.6	12.6	mild	mild	mild	1
21	4 weeks	15.1	8.05	15.3	15.3	1.3	90.1	mild	severe	severe	3
22	4 weeks	16.7	8.29	19.7	16.2	18.0	95.4	mild	severe	severe	3
23	4 weeks	13.5	12.8	14.2	11.1	5.2	-13.3	mild	mild	mild	1
24	4 weeks	9.25	14.8	10.7	18.6	15.7	25.7	mild	moderate	moderate	2
25	4 weeks	19.7	10.6	16.8	16.2	-14.7	52.8	mild	severe	severe	3
26	4 weeks	15	7.81	17	9.68	13.3	23.9	mild	mild	mild	1
27	4 weeks	10.1	20	13.3	19.6	31.7	-2.0	moderate	mild	moderate	2
28	4 weeks	9.41	9.09	9.11	9.25	-3.2	1.8	mild	mild	mild	1
29	4 weeks	20	8.26			0.0	0.0	mild	mild	mild	1
30	4 weeks	15.8	7.19	13.9	15.7	-12.0	118.4	mild	severe	severe	3

Figure 1. HO radiographic data – Group I & I animals. (Provided by MUSC)

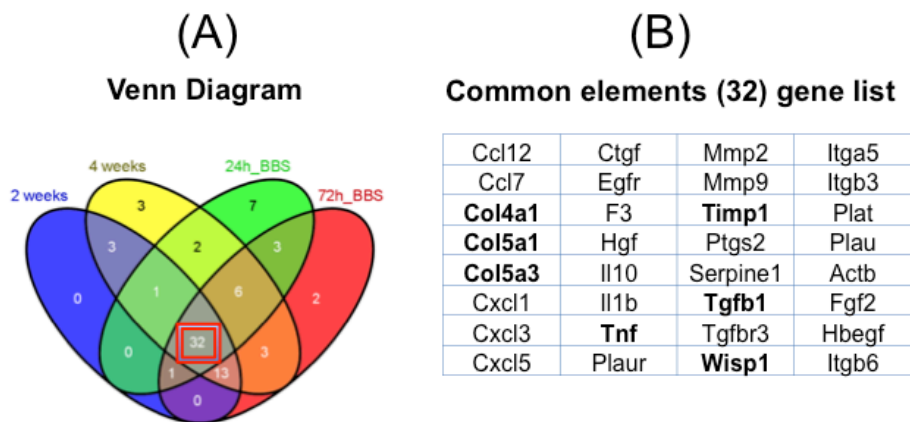
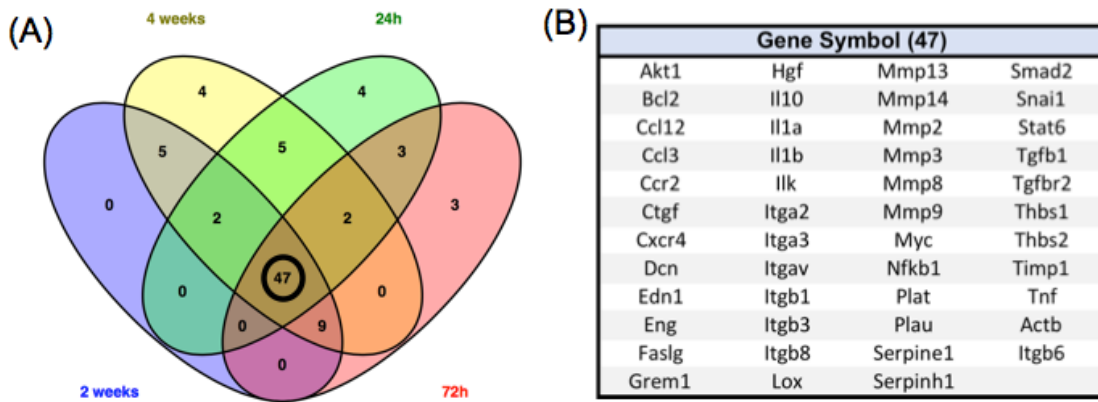


Figure 2. Wound Healing pathway specific PCR array. (A) Venn Diagram analysis. The gene lists were generated from different stages ( $FC \geq 2$ ,  $P \leq 0.05$ ) and applied to the Venny website (Oliveros, J.C. (2007) Venny. An interactive tool for comparing lists with Venn Diagrams. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>). 32 genes commonly appeared in 4 different stages. (B) A list of the common genes (32). The genes that were related to fibrosis and inflammation are in bold.



**Figure 3.** Fibrosis pathway specific PCR array Analysis. (A) Venn Diagram analysis. The gene lists were generated from different stages ( $FC \geq 2$ ) and applied to the Venny website (*Oliveros, J.C. (2007) VENNY. An interactive tool for comparing lists with Venn Diagrams. <http://bioinfogp.cnb.csic.es/tools/venny/index.html>*). 47 genes commonly appeared in four different stages. (B) List for common elements (47) genes.

Gene Symbol	24h		72h		2 weeks		4 weeks	
	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value
Bcl2	2.014	6.50E-02	3.314	3.12E-04	6.886	3.00E-05	7.872	4.00E-06
Cxcr4	4.133	3.50E-02	8.504	2.12E-02	12.808	8.00E-05	13.793	1.53E-03
Grem1	15.263	4.92E-02	28.753	1.54E-02	89.793	4.09E-02	126.962	2.72E-02
Itgav	3.134	1.28E-02	3.429	2.42E-04	5.457	6.99E-04	7.008	4.30E-05
Mmp14	6.296	1.03E-02	19.062	4.00E-06	23.138	1.11E-02	97.951	8.00E-05
Mmp2	5.527	7.29E-03	7.354	1.11E-04	15.286	9.75E-02	27.820	1.14E-03
Tgfb2	2.919	2.71E-02	6.183	8.70E-05	6.207	4.27E-03	10.259	3.40E-05

**Figure 4.** Common genes (7) showed the increased gene expression pattern through the stages with significant p-value (at least from three stages,  $P \leq 0.05$ ) and fold change ( $FC \geq 2$ ).

Gene Symbol	24h		72h		2 weeks		4 weeks	
	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value
Ccl12	51.173	2.78E-02	91.460	5.50E-05	29.370	7.79E-02	67.236	7.06E-03
Ccl3	195.431	2.14E-01	60.676	9.29E-04	397.456	3.21E-03	282.620	4.42E-04
Hgf	3.279	1.51E-02	14.182	4.38E-04	14.037	1.61E-04	20.143	9.22E-04
Lox	21.549	2.89E-04	27.376	2.94E-04	11.133	6.33E-03	23.355	2.04E-04
Mmp3	146.574	2.34E-01	5.860	4.98E-02	1194.924	5.03E-03	455.079	5.02E-04
Nfkb1	3.093	4.88E-02	4.695	1.20E-03	3.050	6.60E-02	5.162	6.80E-04
Plat	6.281	9.11E-03	6.545	1.26E-02	5.254	1.63E-02	10.693	1.98E-03
Serpinh1	3.582	1.70E-02	11.073	1.07E-02	4.270	5.89E-02	10.077	6.04E-03
Snai1	17.689	2.57E-03	19.809	1.10E-05	6.131	5.32E-02	17.265	5.20E-03
Stat6	8.140	1.68E-03	9.408	1.00E-06	6.575	8.34E-03	10.234	1.38E-04
Tgfb1	10.354	3.08E-04	38.460	2.84E-04	31.176	5.52E-04	43.768	1.48E-04
Thbs2	12.423	4.56E-02	43.203	0.00E+00	30.660	1.68E-01	70.204	3.13E-04
Tnf	21.861	7.06E-03	21.732	7.00E-06	22.749	1.00E-06	19.584	1.89E-02

**Figure 5.** Common genes (13) showed the same gene expression pattern between the early stages (24h and 72h) and late stages (2 weeks and 4 weeks) with significant p-value (at least from three stages,  $P \leq 0.05$ ) and fold change ( $FC \geq 2$ ).

Gene Symbol	24h		72h		2 weeks		4 weeks	
	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value	Fold Regulation	p-value
Akt1	4.313	1.20E-01	4.107	2.54E-02	2.946	3.76E-02	4.649	6.39E-04
Ccr2	7.214	4.92E-02	18.349	5.25E-03	19.935	2.07E-04	17.751	1.23E-03
Eng	10.150	1.39E-02	6.689	7.45E-03	3.342	3.04E-02	8.054	2.33E-04
Il10	56.495	3.26E-03	50.854	3.61E-04	31.282	6.80E-03	46.560	1.78E-04
Ilk	2.297	4.55E-03	2.201	9.42E-03	2.402	1.16E-02	3.173	9.45E-03
Itga2	4.387	6.72E-02	3.615	5.09E-03	2.923	1.44E-02	4.752	1.97E-03
Itgb3	15.987	3.02E-02	12.569	2.52E-02	20.768	1.37E-02	45.752	2.55E-04
Itgb6	-7.294	1.11E-02	-4.657	1.36E-02	-2.189	5.61E-02	-2.949	2.57E-02
Plau	5.082	2.63E-02	4.831	1.30E-05	3.399	2.34E-03	6.018	1.63E-04
Serpine1	236.609	2.54E-02	40.772	1.17E-02	22.938	5.47E-02	28.870	2.32E-02
Smad2	2.542	2.31E-03	2.265	6.42E-04	2.287	1.61E-02	3.456	9.30E-05
Thbs1	212.522	1.52E-01	50.852	7.99E-03	10.892	3.50E-05	32.504	4.38E-03
Timp1	71.977	6.69E-03	26.177	4.40E-05	18.370	6.00E-06	20.014	1.68E-04

**Figure 6.** Common genes (13) showed the different gene expression pattern between the early stages (24h and 72h) and late stages (2 weeks and 4 weeks) with significant p-value (at least from three stages,  $P \leq 0.05$ ) and fold change ( $FC \geq 2$ ).