Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** Temple University of the Commonwealth System of Higher Education

2. **Reporting Period (start and end date of grant award period):** 01/01/2009 – 12/31/2012

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Germaine A Calicat

4. **Grant Contact Person's Telephone Number:** 215.204.7655

5. **Grant SAP Number:** 4100047651

6. **Project Number and Title of Research Project:** 17 – Control of Calcium Entry Mechanisms in Smooth Muscle

7. **Start and End Date of Research Project:** 9/15/2009 – 5/31/2010

8. **Name of Principal Investigator for the Research Project:** Donald L. Gill, Ph.D.

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $25,000

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Eunan Hendron	Graduate Student	17.75	$19,560

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Donald Gill	Professor and Chair	<1%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
None		

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes___X_____ No_____

If yes, please indicate the source and amount of other funds:

NIH R01 AI058173
Control of Calcium Entry Signals in B Cells 06/01/10 – 05/31/15
$247,500 – Current Direct Costs

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes_____ No_____X_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If

you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert “not funded” in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
	☐NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)		$	$

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes____X_____ No_____

If yes, please describe your plans:

New NIH grant for the study of calcium signaling in smooth muscle cells is planned to continue and expand the work in vascular smooth muscle

12. Future of Research Project. What are the future plans for this research project?

The future plans of the project are described in a new NIH grant application. The background and specific aims of this project are described here.

Calcium signals are vital in controlling a spectrum of cellular events. In smooth muscle cells (SMC), short term Ca2+ signals control contractile responses. Longer term Ca2+ signals regulate cell growth and proliferation through Ca2+-mediated transcriptional control. SMC can undergo an important phenotype transition from the contractile non-proliferative state to a non-contractile proliferative state. This mitogen-induced SMC growth transition is crucial for vascular regeneration and injury repair. However, uncontrolled SMC growth and migration of SMC is the basis for major vascular disorders including atherosclerosis, hypertension, and angioplasty-in-duced restenosis; it also underlies remodeling of airway SMC in asthma and angiogenesis in tumor development. SMC growth-transition is mediated by transcription factors, notably, NFAT, activated by spatially restricted and longer-acting Ca2+ signals generated through a dynamic inter-membrane signaling process induced by two STIM proteins, STIM1 and STIM2

The proposed studies focus on understanding operation of the two centrally important Ca2+ signal transducers, STIM1 and STIM2. The STIM proteins are finely-tuned endoplasmic reticulum (ER) Ca2+ sensors that detect slight changes Ca2+ stored within the ER lumen. As ER Ca2+ release signals lower luminal Ca2+, STIM proteins are triggered to self-associate and translocate into specialized ER-PM junctions within which the STIM proteins tether and gate a unique class of "store-operated" highly Ca2+ selective channels, known as Orai. STIM proteins also target "voltage-operated" L-type Ca2+ channels (LTCC) vital for controlling SMC contraction. STIM proteins exert reciprocal control over these two channel targets, activating Orai but deactivating LTCC, likely important to SMC growth phenotype change in which LTCC is lost and STIM-Orai signaling predominates.

The two STIM proteins, although similar in structure, vary considerably in their Ca2+ sensing properties, their molecular activation mechanisms, and their efficacy of coupling to target Ca2+ channels in the PM. While STIM1 has been intensively examined, a major challenge is to understand the role of the poorly-studied STIM2 protein: how differences in its structure, function and expression lead to powerful signaling and phenotypic changes. We take a genetic approach. While whole animal knockout of either STIM1 or STIM2 are lethal, we generated conditional SMC-targeted deletions of STIM1, STIM2, and STIM+STIM2 in mice. The SMC-specific STIM1-KO results in poorly-developed animals dying early, with major defects in the ability of SMC to undergo growth transition, in vivo and in vitro. The SMC conditional STIM1/STIM2 double knockout is perinatally lethal indicating important yet distinct roles of STIM1 and STIM2 in SMC function and growth transition.

We have two goals: first, to determine how STIM1 and STIM2 couple to target channels, and second, how their channel targeting and expression mediates changes in SMC growth transition. These two independent but inter-dependent goals are approached through two specific aims:

1. To understand the mechanisms and distinctions between STIM1 and STIM2 activation, and to define their molecular coupling to target Ca2+ channels. Using mutational modifications, high resolution Ca2+ and FRET imaging, and electrophysiology, we aim to define the molecular basis of sensing and target channel coupling for STIM1, defining mechanistically important distinctions in the poorly studied STIM2 protein. Building on new structural insights and major functional distinctions between STIM1 and STIM2, our studies focus on defining the interactions through which the two STIM proteins interact with and gate Orai and LTCC target channels.

2. To understand the distinct roles of STIM1 and STIM2 in mediating mitogen-induced growth transition of SMC from the quiescent to proliferative phenotype. Using our SMC-targeted STIM1 and STIM2 null-background mice together with molecular probes to specifically modify STIM1- and STIM2-mediated target channel coupling, we aim to define the differential effectiveness of STIM1 and STIM2 in mediating SMC growth transition. Studies will determine how changes in STIM1 and STIM2 expression and distinctions in their activation and channel coupling, lead to distinct profiles of Ca2+ signals, NFAT activation, and altered growth transition.

Our studies apply: (a) new knowledge on the structure and channel-coupling of STIM proteins, (b) innovative STIM1 and STIM2 gene-deletion animal models, (c) innovative probes to assess STIM1 and STIM2 target channel coupling – to provide new understanding of the crucial role of STIM1 in controlling SMC growth transition. Our goals also address a fundamental paucity of information on understanding the mechanism, action and role of STIM2. Information from these studies is crucial to understanding how vascular SMC injury responses occur and how they may be controlled. Our model predicts that distinct patterns of STIM1 and STIM2 expression can be a determining factor in whether SMC undergo phenotype change. Hence the work has fundamental importance in understanding and preventing the SMC growth changes that underlie major vascular diseases including atherosclerosis, hypertension, and arterial restenosis, and SMC phenotype change that underlies lung responses in asthma and angiogenesis that supports growth of tumors.

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes___X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male			1	
Female				
Unknown				
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic				
Unknown			1	
Total			**1**	

	Undergraduate	Masters	Pre-doc	Post-doc
White			1	
Black				
Asian				
Other				
Unknown				
Total			**1**	

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes_____ No_____X_____

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes____X_____ No_____

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

The research project resulted in the hiring of two postdoctoral researchers and two graduate students who have led successful interaction research projects with others within Temple University School of Medicine working on the mechanisms of calcium signaling in disease states including vascular and immune diseases.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes____X_____ No_____

If yes, please describe the collaborations:

Collaborative partnership with Novartis Pharmaceuticals was initiated with the health research funds. This collaboration centers on understanding the pharmacological control of calcium entry channels in smooth muscle and immune cells.

16(B) Did the research project result in commercial development of any research products?

Yes_____ No_____X_____

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes_____ No_____X_____

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Our studies have addressed the challenge of understanding what signals cause vascular smooth muscle cell (VSMC) phenotype change. While the ability of VSMC to become proliferative and migrate into the intima is crucial to blood vessel repair and angiogenesis, the age- and damage-related changes in VSMC that give rise to major vascular diseases, presents a crucial area of investigation. The role of STIM and Orai in the process has become clear. However, Orai and STIM function in a dynamic and highly restricted microenvironment. Hence, the use of novel gene deletion models to selectively remove the proteins in vivo, together with new tools to measure the interactions between STIM and Orai proteins, provides new information to meet this challenge.

(a) New Concepts and Tools: One new concept that provides an important base for our future work, is our revelation that STIM proteins can control another major class of Ca^{2+} channels – the $Ca_V1.2$ (L-type) channel. This finding has special significance to SM cells which are both excitable and non-excitable. STIM proteins had been considered to be modifiers of TRPC channels but we and others have no evidence to support this. Indeed, our recent work revealed that all the molecular tools that so convincingly reveal the STIM-Orai and STIM-$Ca_V1.2$ connection, are without effect on TRPC channels. Our new tools include a spectrum of fluorescently-tagged derivatives of STIM, Orai and $Ca_V1.2$ proteins with which we can undertake high resolution real-time FRET imaging to assess their movement and interactions. Our development of a mouse model with targeted deletion of STIM proteins from smooth muscle, is a crucial new tool for the studies.

<u>(b) Generation of smooth muscle-specific STIM1 and STIM2 knock-out mouse models:</u> The homozygous STIM1-k/o mouse on the C57BL/6 background is embryonically lethal at E19, and the STIM2-k/o dies 1-4 weeks after birth of unknown causes. We used Cre recombinant technology to generate mice in which STIM proteins were selectively knocked out in smooth muscle. We obtained STIM1$^{flox/flox}$ and STIM1/STIM2$^{flox/flox}$ mice in which exon 2 encoding the EF-hand of the *Stim1* gene, and exon 3, encoding sequences C-terminal to the EF hand of the *Stim2* gene, were flanked by *lox*P recombination sites from Dr. Anjana Rao at Harvard University. A valuable mouse expressing Cre recombinase under control of the SM22α promoter (SM22α-CreKI) was recently developed. SM22α is a marker of smooth muscle cells (SMC) that is also transiently expressed embryonically in the heart (days E8.0-12.5). The SM22α-CreKI mice do not express Cre recombinase until approx E18, and then highly expresses in SMCs into adulthood. Our desire was to obtain mice in which STIM was selectively knocked out in SMC and after the vasculature had developed normally. We obtained SM22α-CreKI mice (129S6-Tagln$^{tm2(cre)Yec}$; stock #:6878) from Jackson Labs. We crossed the STIM1$^{flox/flox}$ and STIM1/STIM2$^{flox/flox}$ with SM22α-CreK to obtained SMC-targeted deletion of STIM expression. All the mice are exclusively on the C57/B6 genetic background, and experiments utilized control littermates. We have used knockouts heterozygous from Cre (STIM1$^{fl/fl}$-SM22α-Cre$^{+/-}$) and Cre-control mice (STIM1$^{wt/wt}$-SM22α-Cre$^{+/-}$). The phenotype of the STIM1$^{fl/fl}$-SM22α-Cre$^{+/-}$ (STIM1-k/o) compared with STIM1$^{wt/wt}$-SM22α-Cre$^{+/-}$ littermates (WT-Cre) are shown in Fig. 1. The STIM1-ko animals develop more slowly and are smaller in size than WT-Cre littermates (Fig. 1A). The knockouts die early for unknown reasons; 50% have died by 30 days (Fig.

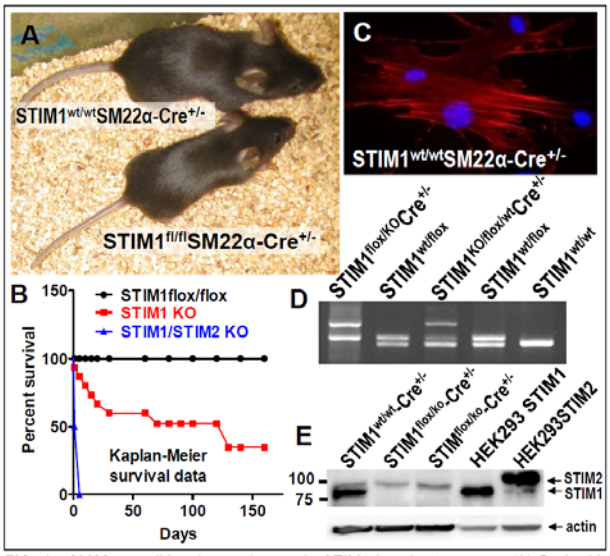

FIG. 1. SM22α-conditional smooth muscle STIM1 knockout mouse. (A) 5 wk old STIM1$^{fl/fl}$SM22α-Cre$^{+/-}$ and STIM1$^{wt/wt}$SM22α-Cre$^{+/-}$ littermates. (B) Kaplan-Meier survival curves for SM22α-conditional STIM1-ko, STIM1/STIM2-dko, and floxed STIM1 mouse. (C) VSMC from STIM1-ko mice have normal and typical SM-alpha-actin and DAPI immunostaining. (D) PCR genotyping of STIM1-ko; Cre is confirmed by genotyping with Cre-specific primers. (E) Western blot from primary VSCM showing compete absence of STIM1 in two STIM1-ko mice. Note: STIM2 expression is unchanged. Authentic HEK-expressed STIM1 and STIM2 are shown.

1B). The VSMC have normal appearance and expression of SM-specific alpha actin (Fig. 1C). Genotyping reveals the wild-type STIM1 allele (348 bp), the deleted allele (580 bp) and the floxed allele (399bp) (Fig. 1D). Western analysis reveals the complete absence of STIM1 in the STIM1-ko mice; importantly, there is no compensatory expression of STIM2 (Fig. 1E). Our STIM1-k/o driven by SM22α expression is largely specific to smooth muscle starting from embryonic day 16-18 in VSMC. Some expression is also observed in cardiomyoctyes and embryonically in somites (which give rise to skeletal muscle), but there is no later expression in skeletal muscle. The SM22α-CreKI mice have high specificity for SMC expression.

Validating the specificity of VSMC-Cre expression: To validate that the SM22 α-Cre recombinase activity is smooth muscle tissue specific, we use a double-fluorescent transgenic *Gt(ROSA) 26Sor*$^{tm4(ACTB\text{-}tdTomato,\text{-}EGFP)Luo}$/J reporter mouse (Jackson Labs). These mice possess loxP sites on either side of a membrane-targeted td-Tomato (mT) cassette and express red fluorescence in all tissues and cell types. When bred to the SM22α-Cre$^{+/+}$ mice, we obtain offspring having the mT cassette deleted in the Cre-expressing VSMC, allowing expression of the membrane-targeted EGFP (mG) cassette located downstream, revealed as green fluorescence in VSMC (Fig. 2A). Through crossings and back-crossings, we made the STIM1$^{fl/fl}$SM22α-Cre$^{+/-}$mouse carrying the mT/mG cassette as well as corresponding Cre control STIM1$^{wt/wt}$SM22α-Cre$^{+/-}$mouse also with the mT/mG. These mice permit direct visualization of VSMC in which STIM1 has or has not been knocked out, either within tissue or isolated from tissue (Fig. 2 B,C). The marker also provides us the means to trace lineage of isolated and cultured cells.

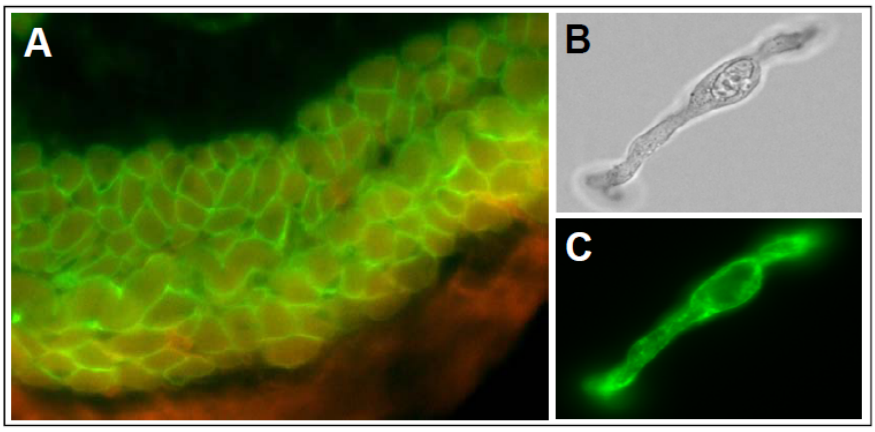

FIG. 2. VSMC-specific Cre-mediated EGFP fluorescence expressed in the SM22α-Cre$^{+/+}$ mT/mG mouse. (A) Fixed mouse aorta section showing EGFP-labeling in VSMC; mT labeling in surrounding non-VSM cells gives the red/yellow regions. (B) Isolated aortic VSMC (phase contrast). **C.** Cre-induced EGFP-label in same VSMC.

Validating STIM1 deletion through VSMC contractile Responses: In collaboration with Dr. Houser and Dr. Eguchi at Temple CVRC, we have undertaken detailed measurements on contractile responses (Fig. 3). Activation of α_1-adrenergic receptors with phenylephrine (PE) in the presence of verapamil to block $Ca_V1.2$ channels reveals an almost complete lack of response without STIM1 (Fig. 3A,B). Contraction induced by TG-induced store-depletion is also greatly reduced (Fig. 3C), whereas contraction in response to depolarization is unaffected (Fig. 3D). TG-induced Ca^{2+} store depletion-dependent Ca^{2+} entry was also largely absent, the residual likely reflecting the small amount of STIM2 (Fig. 3E). Remarkably, the STIM1/ STIM2$^{fl/fl}$-SM22α-Cre$^{+/-}$ mouse is lethal at or before birth (Fig. 1B). While not yet completed, the SM-conditional STIM2-KO mouse is being undertaken. Since we have the floxed STIM1, STIM2 and STIM1/STIM2 mice, we are also able to undertake in vitro Cre-induced elimination of STIM gene products.

(c) Development of a new model and concept of control of vascular growth by calcium signals: The STIM1$^{fl/fl}$SM22α-Cre$^{+/-}$ mice in which the STIM1 gene is selectively eliminated from smooth muscle provides an important model system to examine the functional role of STIM1 in the changes that occur in VSMC as they transition from the quiescent "contractile phenotype" to the actively dividing "synthetic" phenotype. This model states that STIM1-induced $Ca_V1.2$ channel inhibition and Orai channel activation are required for PDGF-induced phenotype transition in VSMC. The focus of our ztudies is on the switching process (yellow) which we approach under two specific aims. Under Aim 1 we examine how STIM1 causes changes in VSMC growth, examining the role of STIM1-induced reciprocal control of Orai and $Ca_V1.2$ channels in mediating phenotype change, determining whether STIM1 causes alteration in the expression of Orai and $Ca_V1.2$ channels, and assessing whether direct alteration of function of the STIM1-targeted channels is sufficient to trigger VSMC phenotype change. Our work shows the STIM1-mediated reciprocal control of Orai and $Ca_V1.2$ channels at the molecular level, and the design of a series of tagged-probes to investigate the molecular interactions between STIM1 and its channel targets. Using these probes, we are investigating whether the STIM1-induced reciprocal control of Orai and $Ca_V1.2$ channels is the mechanism ultimately determining NFAT activation and VSMC phenotype transition.

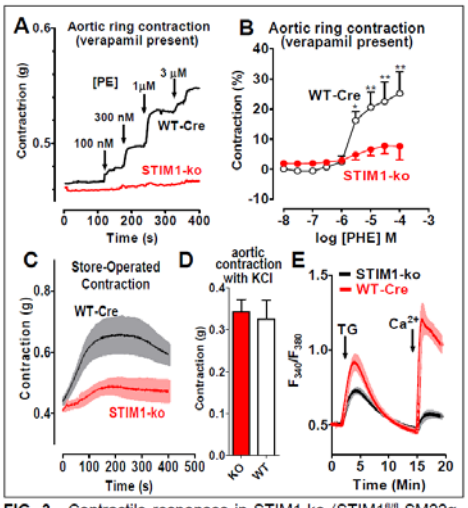

FIG. 3. Contractile responses in STIM1-ko (STIM1$^{fl/fl}$-SM22α-Cre$^{+/-}$) and WT-Cre control (STIM1$^{wt/wt}$-SM22α-Cre$^{+/-}$) litter-mates. (A,B) Responses to phenylephrine in the presence of 10 uM verapamil to block $Ca_V1.2$ channels. (C) Response to 2 uM thapsigargin-induced store-depletion. (D) No differences are seen in K^+-induced contraction. (E) Store-operated Ca^{2+} entry in response to TG is almost completely absent.

(d) Measurement of STIM1-dependent VSMC phenotype in STIM-KO and Cre-WT mouse systems. In conjunction with our collaborators, Dr. Satoru Eguchi and Dr. Michael Autieri (Dept. Physiology, Temple), we have been able to undertake the ligation injury model in mice inducing partial (30%) ligation of the left common carotid artery in mice resulting in the substantial increased growth of VSMC and formation of robust neointimal growth in which migrating VSMC can be clearly identified. Using the STIM1$^{wt/wt}$ SM22α-Cre$^{+/-}$ (mT/mG) Cre-expressing control mice carrying the EGFP reporter cassette, the neointimal migration of EGFP-fluorescent VSMC can be clearly observed 28 day after ligation (Fig. 4A). In sharp contrast, identical ligation on the STIM1$^{fl/fl}$ SM22α-Cre$^{+/-}$(mT/mG) mice in which STIM1 is knocked out in VSMC, results in no visible neointima formation an d VSMC migration is virtually abolished (Fig. 4B,C). VSMC responses in the left ligated carotid can be compared with the unligated right carotid as control. We undertake morphological and histological evaluation of the migration of VSMC into the carotid artery lumen tissue harvested at 24 hours, 7 and 14 days after ligation as described. Formalin-fixed ligated carotid from STIM1 KO and Cre-WT mice will be used and digitized images measured and averaged from at least 5 representative 5-μm-thick stained tissue sections imaged using Image Pro Plus (Media Cybernetics) accurately quantifying VSMC migration by comparing intimal, medial and luminal areas.

(e) In vitro analysis of STIM1-dependent phenotype change proliferation and migration in vitro: PDGF functions as a crucial activator of growth and migration of VSMC vascular smooth muscle cells. Our studies reveal that the proliferative response to serum of VSMC taken from STIM1$^{fl/fl}$SM22α-Cre$^{+/-}$ KO mice is diminished by almost 50% compared with STIM1$^{wt/wt}$ SM22α-Cre$^{+/-}$ wildtype VSMC (Fig. 5A). The response to low levels of PDGF is reduced by almost 80%. Migration assays on primary VSMC performed in Transwell migration chambers in which chemotaxis is induced with 20 nM PDGF, reveal that that migration is reduced by approximately 60% in VSMC from STIM1$^{fl/fl}$ SM22α-Cre$^{+/-}$ STIM1-KO as compared to the Cre-controlled STIM1$^{wt/wt}$SM22α-Cre$^{+/-}$ wildtype mouse-derived VSMC (Fig. 5 B). The in vitro growth and migration analyses are the basis for many of the new studies we are currently conducting

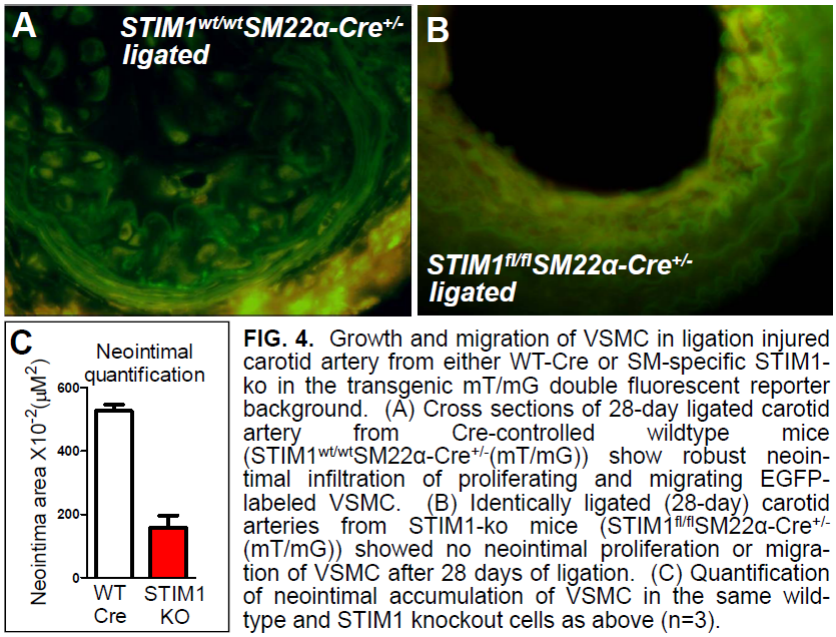

FIG. 4. Growth and migration of VSMC in ligation injured carotid artery from either WT-Cre or SM-specific STIM1-ko in the transgenic mT/mG double fluorescent reporter background. (A) Cross sections of 28-day ligated carotid artery from Cre-controlled wildtype mice (STIM1$^{wt/wt}$SM22α-Cre$^{+/-}$(mT/mG)) show robust neointimal infiltration of proliferating and migrating EGFP-labeled VSMC. (B) Identically ligated (28-day) carotid arteries from STIM1-ko mice (STIM1$^{fl/fl}$SM22α-Cre$^{+/-}$ (mT/mG)) showed no neointimal proliferation or migration of VSMC after 28 days of ligation. (C) Quantification of neointimal accumulation of VSMC in the same wild-type and STIM1 knockout cells as above (n=3).

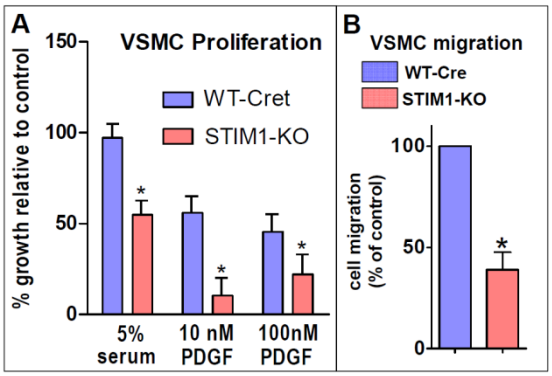

FIG. 5: (A) Proliferation of VSMC from WT-Cre and STIM1-KO mice. Proliferation was measured in response to 5% serum or 10 or 100 nM PDGF. (B) VSMC migration measured in Transwell migration chambers (18 hr, 37°C) induced by 20 nM PDGF. (*P=0.05 vs. WT-Cre, n=3)

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
___X__No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown
Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in

more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
___X__ No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed

acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. None				☐Submitted ☐Accepted ☐Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes____X_____ No_____

If yes, please describe your plans:

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

The work described has major implications for understanding the control of immunological diseases and also cardiovascular disease. The work has revealed fundamental changes in the control of calcium entry signals mediated by STIM and Orai proteins are show these proteins to be important targets for mediating control of immune and vascular disease.

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

As described above, the STIM and Orai proteins are crucial new targets against which control of immune and vascular diseases can be affected. The work has allowed the proteins to be assessed as powerful new targets for pharmacological alternation of major immunological and cardiovascular diseases.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes_____ No___X_____

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes_____ No_____X_____

If yes, please describe your plans:

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages. *For Nonformula grants only – include information for only those key investigators whose biosketches were not included in the original grant application.*

Donald L. Gill, PhD

A. Personal Statement

Our research is to understand the role of the Ca^{2+}-sensing STIM proteins, STIM1 and STIM2, in mediating Ca^{2+} signals which control proliferation and phenotype change of smooth muscle cells. Our aims are to understand the fundamental processes of Ca^{2+} entry control mediated by the Ca^{2+} sensing STIM proteins which modulate a crucial interplay between Ca^{2+} release and Ca^{2+} entry mechanisms. In particular we are interested to know how these signals are modified as the cells undergo phenotypic growth changes akin to the changes which occur in major vascular diseases including atherosclerosis, hypertension, and restenosis. My laboratory's research has major strengths in cellular signaling mechanisms, in particular, Ca^{2+} signaling and the control of function and growth of smooth muscle and immune cells. Over the last 5 years my lab has been dedicated to understanding some of the basic molecular mechanisms whereby STIM proteins effect control over Ca^{2+} entry channels, in particular, the Orai and L-type channels. Prior to this time, the lab had a strong interest in studying Ca^{2+} signaling mechanisms in smooth muscle and received funding for over 15 years from NIH and NSF to support this work. In recent years, we have focused our work on vascular smooth muscle and applied all the new information and approaches we have gained from studying STIM and Orai proteins. This is particularly important since my lab has become interested in how smooth muscle represents a fascinating hybrid between and excitable and non-excitable cells. We recently made the important discovery that STIM proteins expressed in vascular smooth muscle cells in addition to controlling the highly Ca^{2+} selective Orai channels, also effect direct control over the voltage-regulated L-type Ca^{2+} channels (*Wang et al, Science, 2010, 330, 105-109*). This was a major and novel finding and revealed that STIM proteins mediate reciprocal control on the Orai and L-type channels. Over this same 2-year period, my lab has also dedicated huge effort into developing new mouse models for studying the action of STIM proteins in vascular smooth muscle. We obtained the floxed STIM1, STIM2 and STIM1/STIM2 knockouts from Dr. Anjana Rao (at Harvard, now at Scripps). We have been able to cross the floxed mice with mice expressing Cre under the smooth muscle specific SMα22 promoter. In this way we have obtained conditional knockout mice in which STIM1 or STIM2 gene function is eliminated only within smooth muscle. These animals have proven to be exceedingly useful in order study the function of STIM-mediated Ca^{2+} signals and their role in vascular smooth muscle function and growth. We have also developed considerable expertise in examining the interactions between fluorescently tagged derivatives using Fluorescent Resonance Energy Transfer (FRET) technology. We have combined this approach with high resolution imaging capabilities. In addition, as Dept Chair, I have been building a group of individuals who bring much and varied expertise in cellular signaling mechanisms. These include Dr. Jonathan Soboloff, from Univ. Maryland, a long-time collaborator on calcium signaling, Dr. Madesh Muniswamy from U. Penn, who is an expert in confocal imaging and studying the interactions between cellular organelles, and also Dr. Brad Rothberg who is a renowned expert on channel structure and function, with expertise in electrophysiology and structural relationships among membrane proteins. Our new work on calcium signaling in VSMC has important significance to transcriptional control and how the generation of calcium signals is involved in changes in gene expression. Calcium signals are a fundamental means of integrating cellular signals and as such their study has broad translational significance.

B. Positions and Honors

Positions and Employment

1975-1979	MRC Research Scholar, University of London, Dept. Molecular Biophysics, Middlesex Hospital Medical School, London England (Mentor: Roger P. Ekins)
1979-1982:	Fogarty International Visiting Fellow, NIH, Section of Membrane Regulation, Lab. Molecular & Cellular Biology, NIADDK, NIH, Bethesda MD 20892 (Mentor: Martin Rodbell)
1982-1987	Assistant Professor, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201
1987-1990	Associate Professor, Department of Biological Chemistry, University of Maryland School of Medicine, Baltimore, MD 21201
1990-2007	Professor, Department of Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD 21201
2007-present:	Professor and Chair, Department of Biochemistry, Temple University School of Medicine, Philadelphia, PA 19140
2008-present:	Professor, Cardiovascular Research Center, Temple University School of Medicine, Philadelphia, PA 19140

Awards and Honors

Fogarty International Visiting Fellow, NIH1979-1982
Cell Biology Advisory Panel, NSF, 1986-1990
Chairman and Founder, Gordon Conference on Calcium Signaling, 1993
Cell Biology Study Section, NIH (ad hoc), 1995 - 2004
Editorial Board, Journal of Biological Chemistry, 1990-1996
Visiting Professor, Department of Biological Chemistry, Johns Hopkins School of Medicine, 1997-1998
Plenary Lecturer, Calcium Signaling Gordon Conference, 2001
Chairman, Appointments Promotion and Tenure Committee, Univ. Maryland Sch. Med., 2001-2002
Plenary Speaker, 8th Symposium of the European Calcium Society, 2004
Chairman, Science STKE E-Conference on Defining Calcium Entry Signals, 2004
Chair, FASEB Conference on Calcium and Cell Function, 2008
Member of the Editorial Board, Science Signaling, 2011
Editorial Board, Journal of Biological Chemistry, 2006-2011
Board of Reviewing Editors, Science Signaling, 2011

C. Selected Peer-reviewed Publications (SI Science Citation Record: 7,711 citations (Jan 2013) - average of 56.70 citations/article (136 articles). H-index = 47

1. Patterson, R.L., van Rossum, D.B., and Gill, D.L. (1999) "Store-operated Ca^{2+} entry: evidence for a secretion-like coupling mechanism" ***Cell*** 98, 487-499
2. Ma, H-T., Patterson, R.L., van Rossum, D.B., Birnbaumer, L., Mikoshiba, K., and Gill, D.L. (2000) "Requirement of the inositol trisphosphate receptor for activation of store-operated Ca^{2+} channels" ***Science*** 287, 1647-1651
3. Patterson, R.L., van Rossum, D.B., Ford, D.L., Hurt, K.J., Bae, S.S., Suh-P-G., Kurosaki, T., Snyder, S.H., and Gill, D.L. (2002) "Phospholipase C-γ is required for agonist induced

Ca^{2+} entry" ***Cell*** 111, 529-541.
4. Soboloff, J., He, L-H., Xu, W., Dziadek, M.A., and Gill, D.L. (2006) "STIM2 is an inhibitor of STIM1-mediated store-operated Ca^{2+} entry" **Curr. Biol.** 16,1465-70.
5. Hewavitharana, T., Deng, X., Soboloff, J., and Gill, D,L. (2008) "Location and function of STIM1 in the activation of Ca^{2+} entry signals" **J. Biol. Chem.** 283, 26252-26262
6. Deng, X., Wang, Y., Zhou, Y., Soboloff, J., and Gill, D.L. (2009) "STIM and Orai - dynamic intermembrane coupling to control cellular calcium signals" **J. Biol. Chem.** 284, 22501-22505
7. Zhou, Y., Mancarella, S., Wang., Y., Yue, C., Ritchie, M.F., Gill, D.L., and Soboloff, J. (2009) "Short N-terminal domains of STIM1 and STIM2 control the activation kinetics of Orai1 channels" **J. Biol. Chem.** 284, 19164-19168
8. Mancarella, S., Wang, Y., and Gill, D.L. (2009) "Calcium signals: STIM dynamics mediate spatially unique oscillations" **Curr. Biol.** 19, 950-952.
9. Wang, Y., Deng, X., Zhou, Y., Hendron, E., Ritchie, M.F., Tang, X.D., Kurosaki, T., Mori, Y., Soboloff, J., and Gill, D.L. (2009) "STIM Protein Coupling in the Activation of Orai Channels" **Proc. Natl. Acad. Sci. USA,** 106,7391-7396
10. Hawkins, B.J., Irrinki, K.M., Lien, Y., Wang, Y., Bhanumathy, C.D., Subbiah, R., Mallilankaraman, K., Richie, M.F., Soboloff, J., Joseph, S.K., Gill, D.L., and Madesh, M. (2010) "S-glutathionylation activates STIM1 and alters mitochondrial homeostasis" **J. Cell Biol.** 190, 391-405
11. Wang, Y., Deng, X., and Gill, D.L. (2010) "Calcium Signaling by STIM and Orai: Intimate Coupling Details Revealed" **Science Signaling** 3, 1-4
12. Wang, Y., Deng, X., Mancarella, S., Hendron, E., Eguchi, S., Soboloff, J., Tang, X.D., and Gill, D.L. (2010) "The calcium store-sensor, STIM1, reciprocally controls Orai and CaV1.2 channels" **Science** 330, 105-109
13. Mancarella, S., Wang, Y., Deng, X., Landesberg, G., Scalia, R., Panettieri, R.A., Mallilankaraman, K., Tang, X.D., Madesh, M., and Gill, D.L. (2011) "Hypoxia-induced acidosis uncouples the STIM-Orai calcium signaling complex" **J. Biol. Chem.** 286, 44788-44798
14. Soboloff, J., Madesh, M., and Gill, D.L. (2011) "Sensing cellular stress through STIM proteins" **Nature Chem. Biol.** 7, 488-492
15. Li, C., Li, J., Cai, X., Sun, H., Jiao, J., Bai, T., Zhou, X.W., Chen, X., Gill, D.L., and Tang, X.D. (2011) "Protein kinase D3 is a pivotal activator of pathological cardiac hypertrophy by selectively upregulating the expression of hypertrophic transcription factors. **J. Biol. Chem.** 286, 40782-40791
16. Robinson, L.J., Mancarella, S., Tourkova, I.L., Zacherl, J.R., Barnett, J.B., Gill, D.L., Soboloff, J., and Blair, H.C. (2012) "Gene disruption of the calcium channel Orai1 results in inhibition of osteoclast and osteoblast differentiation and impairs skeletal development requirement for the calcium release-activated calcium channel Orai1 for normal rates of bone synthesis and degradation. **Lab. Invest.** 92, 1071-1083
17. Soboloff, J., Muniswamy, M., Rothberg, B.S., and Gill, D.L. (2012) STIM proteins: dynamic calcium signal transducers. **Nature Reviews Mol. Cell Biol.** 13, 549-565
18. Mancarella,S., Potireddy, S, Wang, Y., Gaob, H., Gandhirajan, R., Autieri, M., Scalia, R., Cheng, Z., Wang, H., Madesh, M., Houser, S.R. and Gill, D.L. (2012) "Targeted STIM Deletion Impairs calcium homeostasis, NFAT activation, and growth of smooth muscle". **FASEB J.** 27, 893-906

19. Gandhirajan, R.K., Meng, S., Chandramoorthy, H.C., Mallilankaraman, K., Mancarella, S., Gao, H., Razmpour, R., Yang, X.F., Houser, S.R., Chen, J., Koch, W.J., Wang, H., Soboloff, J., Gill, D.L., and Madesh, M. (2013) "Blockade of NOX2 and STIM1 signaling limits lipopolysaccharide-induced vascular inflammation". **J. Clin. Invest.** [EPub, Jan 25 2013]
20. Rothberg, B.S., Wang, Y., and Gill (2013) "Orai Channel Pore Properties and Gating by STIM: Implications from the Orai Crystal Structure" ***Science Signaling*** 6, 9-13