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Department of Health and Human Services
Public Health Services

Grant Application

Follow instructions carefully.
Do not exceed 56-character length restrictions, including spaces.

LEAVE BLANK—FOR PHS USE ONLY.		
Type	Activity	Number
Review Group		Formerly
Council/Board (Month, Year)		Date Received

1. TITLE OF PROJECT
Functional Studies of Osteoblasts

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES
(If "Yes," state number and title)
Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR — **New Investigator** ☒ **No** ☐ **Yes**

3a. NAME *(Last, first, middle)*: Gay, Carol V.

3b. DEGREE(S): Ph.D.

3c. POSITION TITLE: Professor

3d. MAILING ADDRESS *(Street, city, state, zip code)*:
Pennsylvania State University
Dept. of Biochemistry & Mole. Biology
108 Althouse Laboratory
University Park, PA 16802

3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT: Biochemistry and Molecular Biology

3f. MAJOR SUBDIVISION: Eberly College of Science

3g. TELEPHONE AND FAX *(Area code, number and extension)*
TEL: (814) 865-6722 FAX: (814) 863-7024

E-MAIL ADDRESS: cvg1@psu.edu

4. HUMAN SUBJECTS RESEARCH
☒ No
☐ Yes

4a. Research Exempt ☐ No ☐ Yes
If "Yes," Exemption No. _____

4b. Human Subjects Assurance No.: M1145

4c. NIH-defined Phase III Clinical Trial ☐ No ☐ Yes

5. VERTEBRATE ANIMALS ☐ No ☒ Yes

5a. If "Yes," IACUC approval Date: 04/06/2001

5b. Animal welfare assurance no: A3141-01

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
07/01/02	06/30/06	175,000	246,750	700,000	987,000

9. APPLICANT ORGANIZATION
Name: The Pennsylvania State University
Address: Office of Sponsored Programs
110 Technology Center
University Park, PA 16802

Institutional Profile File Number (if known)

10. TYPE OF ORGANIZATION
Public: → ☐ Federal ☒ State ☐ Local
Private: → ☐ Private Nonprofit
For-profit: → ☐ General ☐ Small Business
☐ Woman-owned ☐ Socially and Economically Disadvantaged

11. ENTITY IDENTIFICATION NUMBER
1246000376A1
DUNS NO. *(if available)*
003403953
Congressional District 5th

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE
Name: Robert Killoren
Title: Assistant Vice President for Research
Address: Office of Sponsored Programs
110 Technology Center
University Park, PA 16802
Telephone (814) 865-1372 FAX (814) 865-3377
E-Mail osp@psu.edu

13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name: Robert Killoren
Title: Assistant Vice President for Research
Address: Office of Sponsored Programs
110 Technology Center
University Park, PA 16802
Telephone (814) 865-1372 FAX (814) 865-3377
E-Mail osp@psu.edu

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.

SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)* Carol V Gay — DATE 10/24/01

15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)* — DATE

DESCRIPTION: State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project. Describe concisely the research design and methods for achieving these goals. Avoid summaries of past accomplishments and the use of the first person. This abstract is meant to serve as a succinct and accurate description of the proposed work when separated from the application. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

During the tenure of this project we have shown that plasma membrane calcium ATPase (PMCA) and sodium-calcium exchanger (NCX) are deployed on opposing sides of the osteoblast. NCX, being on the matrix-facing cell surface, is advantageously positioned to direct Ca^{++} into site of mineralization. This past year we reported that specific inhibition of NCX blocks mineralization of extracellular matrix (Stains and Gay, J. Bone Mineral Res. 16:1434-1443, 2001). NCX has recently been found to exist as three distinct isoforms (NCX1, NCX2 and NCX3) and we have shown by RT-PCR that NCX1 and NCX3 are expressed in osteoblasts (Stains *et al.*, J. Cell. Biochem., in press). In Aim One of the present proposal we plan to determine which of the three NCX isoforms is critical for mineralization. Antisense oligonucleotides will be used to knockdown or ablate specific isoform expression. The hypothesis to be tested is that loss of functional NCX3 results in impaired mineralization, whereas ablation of NCX1 or NCX2 has little effect on mineralization. In Aim Two we focus on the distribution of the NCX isoforms and PMCA in relation to the development of osteoblast polarity. The hypothesis to be tested is that segregation of plasma membrane domains with respect to NCX and PMCA occurs when osteoblasts develop lateral contacts and become mature, polarized cells. Understanding the timing of expression of NCX isoforms in relation to expression of lateral adhesion devices (e.g. cadherin-11 and gap-junction protein, connexin-43), will provide insight into the cellular basis of mineralization. This will also provide a framework to assess how mineralization may be regulated. NCX is emerging as an important ion-translocating protein in osteoblasts. Derangements (*e.g.* mutations) in NCX can be predicted to result in an undermineralized skeleton. These studies will disclose novel mechanisms by which bone forming cells carry out mineralization of the extracellular matrix.

PERFORMANCE SITE(S) *(organization, city, state)*

The Pennsylvania State University
University Park, PA

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	Organization	Role on Project
Carol V. Gay, P.I.	Penn State University	Principal Investigator
Deborah S. Grove	Penn State University	Consultant
Roland M. Leach	Penn State University	Consultant
Donna M. Sosnoski	Penn State University	Sr. Project Associate

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes ☐ No

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.
Type density and size must conform to limits and specifications provided in the PHS 398 Instructions.

RESEARCH GRANT

TABLE OF CONTENTS

	Page Numbers
Face Page	1
Description, Performance Sites, and Personnel	2-
Table of Contents	3
Detailed Budget for Initial Budget Period	–
Budget for Entire Proposed Period of Support	4
Budgets Pertaining to Consortium/Contractual Arrangements	–
Biographical Sketch—Principal Investigator/Program Director *(Not to exceed four pages)*	5–7
Other Biographical Sketches *(Not to exceed four pages for each)*	8–13
Other Support	7
Resources	14
Research Plan	
Introduction to Revised Application *(Not to exceed 3 pages)*	–
Introduction to Supplemental Application *(Not to exceed one page)*	–
A. Specific Aims	15
B. Background and Significance	15–18
C. Preliminary Studies/Progress Report/ Phase I Progress Report (SBIR/STTR Phase II ONLY)	18–26
D. Research Design and Methods	26–38
E. Human Subjects	–
Protection of Human Subjects (Required if Item 4 on the Face Page is marked "Yes")	–
Inclusion of Women (Required if Item 4 on the Face Page is marked "Yes")	–
Inclusion of Minorities (Required if Item 4 on the Face Page is marked "Yes")	–
Inclusion of Children (Required if Item 4 on the Face Page is marked "Yes")	–
Data and Safety Monitoring Plan (Required if Item 4 on the Face Page is marked "Yes" ***and*** a Phase I, II, or III clinical trial is proposed	–
F. Vertebrate Animals	38
G. Literature Cited	39–44
H. Consortium/Contractual Arrangements	–
I. Consultants	44–46
J. Product Development Plan (SBIR/STTR Phase II and Fast-Track ONLY)	–
Checklist	–
Appendix *(Five collated sets. No page numbering necessary for Appendix.)*	47

(Items A-D: not to exceed 25 pages*)

* SBIR/STTR Phase I applications: Items A-D of the Research Plan are limited to 15 pages.

Check if Appendix is included: [X]

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited.

Number of publications and manuscripts accepted for publication *(not to exceed 10)* 8

Other items (list):

Number pages consecutively at the bottom throughout the application. Do *not* use suffixes such as 3a, 3b.

BUDGET JUSTIFICATION PAGE MODULAR RESEARCH GRANT APPLICATION				
Initial Budget Period $175,000	Second Year of Support $175,000	Third Year of Support $175,000	Fourth Year of Support $175,000	Fifth Year of Support
Total Direct Costs Requested for Entire Project Period				$700,000

Personnel

Dr. Carol V. Gay, Principal Investigator (25% effort) will oversee all phases of the study. She has been interested in the cellular basis of mineralization for many years and has, for the past 13 years, focused on mechanisms of calcium efflux from osteoblasts. She has experience with most types of microscopy and with evaluating three-dimensional structures. She will be responsible for coordinating publication efforts.

Virginia R. Gilman, Research Technician (100%) will be responsible for isolating and culturing primary osteoblasts and will perform all the immunocytochemical experiments and the changes in intracellular Ca^{++} using live cell analysis by confocal microscopy. She has been in Dr. Gay's laboratory for 15 years. She will share lab maintenance duties with Donna Sosnoski.

Donna M. Sosnoski, Senior Project Associate (100%) also brings cell culture expertise to the lab and will be responsible for maintaining the MC3T3E1 cell line. She has considerable experience with molecular technologies and will be responsible for carrying out the antisense knockdown studies and evaluating changes in expression of NCX, PMCA and lateral adhesive proteins with RT-PCR and immunoblotting.

Deborah S. Grove, Consultant, will assist us in the design and synthesis of primers for RT-PCR and with the sequence design of oligonucleotide for the antisense studies. Dr. Grove has directed the Nucleic Acid Facility since 1996. In addition to her formal training in biochemistry, she has acquired specific training at the Pittsburgh Supercomputing Center in sequence analysis and also on Internet Tools for Analysis of Biological Data at the National Center for Biotechnology Information (NCBI) at the NIH.

Roland M. Leach, Consultant, has collaborated with the P.I. for many years on their shared interest in mechanisms of calcification. We are grateful that he will continue to share his expertise at the biochemical and molecular level with us.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Carol V. Gay	Professor of Cell Biology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Univ. of Maine, Orono, ME	B.A.	1962	Zoology
Penn State Univ., University Park, PA	M.S.	1967	Biophysics
Penn State Univ., University Park, PA	Ph.D.	1972	Physiology/Biochem.
Penn State Univ., University Park, PA	Postdoc.	1973-75	Biophysics

A. Positions and Honors

1975-1988 Research & Senior Research Associate in Cell Biology, Penn State University (PSU)
1988-1991 Associate Professor, Depts. of Molecular/Cell Biology and Poultry Science, PSU
1991-present Professor, Dept. of Biochemistry and Molecular Biology and Physiology Program, PSU

1989-1995 Penn State Gerontology Center Advisory Board
1991-present Life Sciences Electron Microscopy Facility Advisory Board
1992-1995 University Scholars Program Advisory Board
1992-1995 NIDR Dentist-Scientist Award Prog. Advisor, Univ. of North Carolina, Chapel Hill, NC.
1998-present External Advisory Board, Center for Metabolic Bone Disease, Univ. of Alabama, Birmingham, AL

Honors

Phi Beta Kappa, Phi Kappa Phi, B.A. with High Distinction.
NIH Postdoctoral Fellowships, January, 1973-July, 1975.
Editorial Boards: Histochem. Cytochem., 1976-1992; J. Bone and Mineral Res., 1992-1997; J. Nutrition, 2000-
NIH Research Career Development Award, 1979-1984.
Oral Biology and Medicine Study Section, 1986-1991 and 1997-2001.
Overseas Fellow, Royal Society of Medicine, 1992.
NIDR Special Grants Review Committee, 1993-1995.

B. Selected peer-reviewed publications (Total = 102):

Yamamoto, T., Nagai, H. and Gay, C. V., An ultracytochemical study of cytochrome oxidase activity in rat metaphyseal bone cells. Acta Histochem. Cytochem. 24:411-419, 1991.
Gay, C. V., Avian osteoclasts, Calcified Tissue Int. 49:153-154, 1991.
Fukushima, O. and Gay, C. V., Ultrastructural localization of guanylate cyclase in bone cells. J. Histochem. Cytochem. 39:529-535, 1991.
Agarwala, N. and Gay, C. V., Specific binding of parathyroid hormone to living osteoclasts. J. Bone Min. Res., 7:531-539, 1992.
Gay, C. V., Kief, N. L. and Bekker, P. J., Effect of estrogen on acidification in osteoclasts. Biochem. Biophys. Res. Commun. 192:1251-1259, 1993.
Turner, R. T., Bell, N. H. and Gay, C. V., Evidence that estrogen binding sites are present in bone cells and mediate medullary bone formation in Japanese quail. Poultry Science, 72:728-740, 1993.
Hall, M. R., Kief, N. L., Gilman, V. R. and Gay, C. V., Surface binding and clearance of calcitonin by avian osteoclasts. Comp. Biochem. Physiol. 108A:59-63, 1994.
Brubaker, K. D. and Gay, C. V., Specific binding of estrogen to osteoclast surfaces. Biochem. Biophys. Res. Commun. 200:899-907, 1994.
Gay, C. V., Lloyd, Q. P. and Gilman, V. R., Characteristics of osteoblasts derived from avian long bone. In Vitro Cell. Dev. Biol. 30A:379-383, 1994.

Gay, C. V. and Lloyd, Q. P., Characterization of calcium efflux by osteoblasts derived from long bone periosteum. Comp. Biochem. Physiol., 111A:257-261, 1995.

Lloyd, Q. P., Kuhn, M. A. and Gay, C. V., Characterization of calcium translocation across the plasma membrane of primary osteoblasts using a lipophilic calcium-sensitive fluorescent dye, calcium-green C_{18}. J. Biol. Chem., 270:22445-22451, 1995.

Gay, C. V., Role of microscopy in elucidating the mechanism and regulation of the osteoclast resorptive apparatus. Micros. Res. Tech., 33:165-170, 1996.

Luan, Y., Praul, C. A., Gay, C. V. and Leach, R. M., Basic fibroblast growth factor: An autocrine growth factor for epiphyseal growth plate chondrocytes. J. Cellular Biochem. 62:372-382, 1996.

Wu, J., Pines, M., Gay, C. V., Hurwitz, S. and Leach, R. M., Immunolocalization of osteonectin in avian tibal dyschondroplastic cartilage. Dev. Dynamics 207:69-74, 1996.

May, L. G. and Gay, C. V., Parathyroid hormone uses both adenylate cyclase and protein kinase C to regulate acid production in osteoclasts. J. Cellular Biochem. 65:565-573, 1997.

Praul, C. A., Gay, C. V. and Leach, R. M., Chondrocytes of the tibial dyschondroplastic lesion are apoptotic. Int. J. Dev. Biol. 41:621-626, 1997.

Troyan, M. B., Gilman, V. R. and Gay, C. V., Mitochondrial membrane potential changes in osteoblasts treated with parathyroid hormone and estradiol. Exp. Cell Res. 233:274-280, 1997.

Hunter, S. J., Gay, C. V., Osdoby, P. A. and Peters, L. L., Spectrin localization in osteoclasts: immunocytochemistry, cloning, and partial sequencing. J. Cell. Biochem. 71:204-215, 1998.

Stains, J. P. and Gay, C. V., Asymmetric distribution of functional sodium-calcium exchanger in primary osteoblasts. J. Bone Miner. Res. 13:1862-1869, 1998.

Sugiyama, T., Kusuhara, S. and Gay, C. V., Parathyroid hormone and estrogen effects on adhesion of chicken medullary bone osteoclasts. In: Calcium Metabolism: Comparative Endocrinology. Danks, J., Dacke, C., Flik, G. and Gay. C. V. (eds). Bioscientifica Ltd., Bristol. 1999.

Brubaker, K. D. and Gay, C. V., Evidence for plasma membrane-mediated effects of estrogen. Calcif. Tissue Int. 64:459-462, 1999.

Brubaker, K. D. and Gay, C. V., Depolarization of osteoclast plasma membrane potential by 17β-estradiol. J. Bone Min. Res. 14:1861-1866, 1999.

Brubaker, K. D. and Gay, C. V., Evidence for plasma membrane mediated effects of estrogen. Calcif. Tiss. Int. 64:449-462, 1999.

Luan, Y., Praul, C. A., and Gay, C. V., Confocal imaging and timing of secretion of matrix proteins by osteoblasts derived from avian long bone. Comp. Biochem. Physiol. A, 126:213-221, 2000.

Armen, T. A. and Gay, C. V., Simultaneous detection and functional response of testosterone and estradiol receptors in osteoblast plasma membrane. J. Cell. Biochem., 79:620-627, 2000.

Gay, C. V. and Weber, J. A., Regulation of differentiated osteoclasts. Critical Reviews in Eukaryotic Gene Expression, 10:213-230, 2000.

Weber, J. A. and Gay, C. V., Expression of translation initiation factor IF2 is regulated during osteoblast differentiation. J. Cell. Biochem. 81:700-714, 2001.

Stains, J. P. and Gay, C. V., Inhibition of Na^+/Ca^{2+} exchange with KB-R7943 or bepridil diminishes mineral deposition by osteoblasts. J. Bone Miner. Res. 16:1434-1443, 2001.

Stains, J. P., Weber, J. A. and Gay, C. V., Expression of Na^+/Ca^{2+} exchanger isoforms (NCX1 and NCX3) and plasma membrane Ca^{2+} ATPase during osteoblast differentiation. J. Cell. Biochem., in press, 2001.

Shiels, M. J., Mastro, A. M. and Gay, C. V., The effect of donor age on the sensitivity of osteoblasts to the proliferative effects of TGF_β and $1,25(OH_2)\ D_3$. Life Sciences, in press.

Data Base Entries:

Weber, J. A., Stains, J. P. and Gay, C. V., Gallus gallus osteoblast regulatory factor 3A mRNA. May 18, 1999. Gene Bank Submission AF144636.

Weber, J. A., Stains, J. P. and Gay, C. V., Identification of novel cDNAs from cultured chick osteoblasts by similarity to the sodium calcium exchanger. May 18, 1999. Gene Bank Submission AF144637.

Weber, J. A., Stains, J. P. and Gay, C. V., Gallus gallus osteoblast 6D12C protein mRNA. May 18, 1999. Gene Bank Submission AF145780.

Weber, J. A., Stains, J. P. and Gay, C. V., Gallus gallus membrane protein mRNA. July 27, 1999. Gene Bank Submission AF172844.

Book:

Gay, C. V., Osteoclast ultrastructure and enzyme histochemistry: Functional implications. In: Rifkin, B. and Gay, C. V., eds., Biology and Physiology of the Osteoclasts. pp. 129-150. CRC Press, 1992.

C. Research Support.

GAY, C.V.

ONGOING

5 R01 DE09459-08 Gay (PI) 04/01/91 - 06/30/02 15%
NIH/NIDR $287,395
Functional Studies of Osteoblasts

The major goal is to determine calcification mechanisms used by osteoblasts. Renewal of this grant is the purpose of the present application.

PSU Seed Grant Mastro (PI) 07/01/01 - 06/30/02 0%
Penn State University Biotechnology Innovation Grant $48,472
Metastatic Breast Cancer-Osteoblast Interactions

The goal is to generate preliminary data for a larger grant.

COMPLETED

5 R01 AG13527-04 Gay (PI) 09/01/96 - 08/31/00 15%
NIH/AG $173,030
Evaluation of Bone Cell Function by Confocal Imaging

The goal was to determine changes in intracellular Ca^{++} homeostasis in aging osteoblasts.

DAMD17-00-1-0647 Gay (PI) 10/01/00 - 09/30/01 0%
U.S. Army Medical Research and Materiel Command $50,000
Directed Secretion by Bone Cells of a Factor that Attracts Breast Cancer Cells

This grant focused on the polarity of osteoblasts in relation to attracting breast cancer cells.

US-2627-95C Leach (PI) 09/01/96 - 08/30/99 10%
USDA/BARD $50,000
Developing Nutritional Protocols which Prevent Tibial Dyschondroplasia

The goal was to improve vascularization of growth plate cartilage.

Amount of each grant no longer requested

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format (on preceding page) for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Deborah Shuey Grove	Director of Research Projects, Nucleic Acid Facility

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Otterbein College	B.A.	1975	Biology
Ohio State University	Ph.D.	1980	Biochemistry

A. Positions and Honors

1980-1984	Postdoctoral Associate, Fox Chase Cancer Institute, Philadelphia PA
1984-1988	Postdoctoral Associate, The Pennsylvania State University
1988-1996	Research Associate, The Pennsylvania State University
1996-2000	Manager of the Nucleic Acid Facility, The Pennsylvania State University
2000-2001	Director of Research Projects, Nucleic Acid Facility, The Pennsylvania State University

Honors

1971-1975	The Walter Hughes Scholarship for the Study of Biology and Chemistry
July 1988 & Feb 1990	Research with Theilieria parva-infected bovine lymphocytes at the Institut fur Genetiks and Toxicologie, Kernsforschungcentrum, Karlsruhe, Germany (NATO Travel Award)

B. Publications

Kilker, R. D., D. K. Shuey and G. S. Serif. 1979. Isolation and Properties of Porcine Thyroid Fucokinase. Biochem,. Biophys. Acta 570:271-283.

Grove, D. S. and G. S. Serif. 1981. Porcine Thyroid Fucosidase. Biochem. Biophys. Acta 662:246-255.

Grove, D. S. and G. S. Serif. 1981 Radiometric Assay for Minute Quantities of L-fucose. Anal. Biochem. 111:122-125.

Rose, Z. B., D. S. Grove and S. N. Seal. 1986. Mechanism of Activation by Anions of Phosphoglycolate Phosphatases from Spinach and Human Red Blood Cells. J. Biol. Chem. 261:19006-11002.

Grove, D. S. and A. M. Mastro. 1987. Changes in Protein Kinase C. and cAMP-dependent Kinase in Lymphocytes after Treatment with 12-O-tetradecanoyl phorbol-13-acetate or Concanavalin A:Quantiiation of Activities with an *In Situ* Gel Assay. J. Cell Phys. 132:415-427.

Grove, D. S. and A. M. Mastro. 1988. Prevention of the TPA-mediated Downregulation of Protein Kinase C. Biochem. Biophys. Res. Comm. 51:94-99.

Grove, D. S. and A. M. Mastro. 1991. Differential Activation and Inhibition of Lymphocyte Proliferation by Phorbol Esters, Mezerein, Teleocidin, and Okadaic Acid. Cancer Research 51:82-88.

Grove, D. S. and A. M. Mastro. 1991. Differential Activation and Inhibition of Lymphocyte Proliferation by Modulators of Protein Kinase C: Diacylglyerols, "Rationally-designed Activators and Inhibitors of Protein Kinase C. Experimental Cell Research. 193:175-182.

Grove, D. S., B. Bour, B. Kacsoh, and A. M. Mastro. 1991. Effect of Neonatal Milk-Prolactin Deprivation on the Ontogeny of the Immune System of the Rat. Endocrine Regulation 2:111-119.

Mastro, A. M., C. G. Garlisi, D. S. Grove, C. E. Grier, III and S. A. Pishak. 1991. Suppression of Interleukin-2 Production in Primary T cells Through a Phorbol Ester Induced Mechanism. Lymphokine and Cytokine Research 10:153-164.

Grove, D. S., E. M. Stanek, and A. M. Mastro. 1992. Cytochalasan and 12-O-tetradecanoyl phorbol--13-acetate Induction of IL-2 Receptors in Lymphocytes. Experimental Cell Research. 202:303-309.

Gunes, H., D. S. Grove, B. Bour, S. M. Zawilla, and A. M. Mastro. 1993. The Expression of Forms of Prolactin Receptors in Splenocytes and Thymocytes of Neonatal Rats: The Effect of Milk Ingestion. Endocrine Regulation 27:193-201.

Bill, O., C. G. Garlisi, D. S. Grove, G. E. Holt, and A. M. Mastro. 1994. IL-2 mRNA Levels and Degradation Rates Change with Mode of Stimulation and Phorbol Ester Treatment of Lymphocytes. Cytokine 6:102-110.

Grove, D. S., S. A. Pishak, and A. M. Mastro. 1995. The Effect of a 10-Day Space Flight on the Function, Phenotype and Adhesion Molecule Expression of Splenocytes and Lymph Node Lymphocytes. Experimental Cell Research 219:102-109.

Bates, L. G., D. S. Grove and A. M. Mastro. 1995. Mechanisms of Activation and Suppression in Rat Nb2 Lymphoma Cells: A Model for Interactions between Prolactin and the Immune System. Experimental Cell Research 218:567-572.

Grove, D. S., C. V. Crowl, A. Gagajewski, C. S. Yang, G. R. Reddy, G. A. Hamilton and A. M. Mastro. 1996. Inhibition of Proliferation and of IL-2 Production and Utilization in Lymphocytes by S-Oxalylglutathione. Experimental Cell Research 225:162-170.

Grove, D. S., and A. M. Mastro. 1996. Modulation of Levels of a Negative Transcription Factor for IL-2 by 12-0-Tetradecanoyl Phorbol-1-3-Acetate and Okadaic Acid. Cytokine 8:809-816.

Grove, D. S., C. V. Crowl, G. A. Hamilton and A. M. Mastro. 1996. The S-Oxalin, N-Acetyl-S-oxalylcysteamine, Inhibits Lymphocyte Proliferation, IL-2 Production and Utilization. Biochem. Biophys. Res. Comm. 222:505-511.

Mastro, A. M., D. A. Schlosser, D. S. Grove, C. Lincoski, S. A. Pishak, S. Gordon and W. J. Kraemer. 1998. Lymphocyte Subpopulations in Lymphoid Organs of Rats after Acute Resistance Exercise. Medicine and Science in Sports and Exercise 31:74-81.

Mastro, A. M., D. A. Schlosser, D. S. Grove, C. Lincoski, S. A. Pishak, S. Gordon and W. J. Kraemer. 1998. Lymphocyte Subpopulations in Lymphoid Organs of Rats after Acute Resistance Exercise. Medicine and Science in Sports and Exercise 31:74-81.

Grove, D. S. 1999, Quantitative Real-time Polymerase Chain Reaction for the Core Facility Using TaqMan and the Perkin-Elmer/Applied Biosystems Division 7700 Sequence Detector. J. Biomol. Tech. 10:11-16.

Dohi, K., A. M. Mastro, M. P. Miles, J. A. Bush, D. S. Grove, S. K. Leach, J. S. Volek, B. C. Nindl, J. O. Marx, L. A. Gotshalk, M. Putukian, W. J. Sebastianelli and W. J. Kraemer. Lymphocyte Proliferation in Response to Acute Heavy Resistance Exercise in Women: Influence of Muscle Strenghth and Total Work Eur. J. Applied Physiol (in press). 81.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Roland M. Leach, Jr.	Walther H. Ott Professor in Avian Biology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Maine, Orono, Maine	B.S.	1954	Agriculture
Purdue University, West Lafayette, Indiana	M.S.	1956	Nutrition/Biochemistry
Cornell University, Ithaca, New York	Ph.D.	1960	Nutrition/Biochemistry

A. Positions and Honors

1959-1968	Research Chemist Nutrition Research, U. S. Plant Soil and Nutrition Lab, Ithaca, New York
1960-1966	Assistant Professor, Cornell University
1966-1968	Associate Professor, Cornell University
1968-1973	Associate Professor of Poultry Science, Penn State University
1973 to present	Professor of Poultry Science, Penn State University
August 1, 2000 -	Walther H. Ott Professor in Avian Biology, PSU

Awards

1980 Recipient - Research Award, Poultry Science Association, sponsored by American Feed Manufacturers Association

1983 Recipient - Research Award, Gamma Sigma Delta

2000 – Distinguished Professor, Penn State University

B. Selected peer-reviewed publications (Total = 107)

Vasilatos-Younken, R. and R. M. Leach, Jr. 1986. Episodic patterns of growth hormone secretion and growth hormone status of normal and tibial dyschondroplastic chickens. Growth 50:84-94.

Gay, C. V. and R. M. Leach, Jr. 1986. Tritiated thymidine uptake in chondrocytes of chickens afflicted with tibial dyschondroplasia. Avian Diseases 29(4):1224-1229.

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Leach, R. M. and G. E. Rosselot. 1992. The use of avian epiphyseal chondrocytes for in vitro studies of skeletal metabolism. J. Nutr. 122:802-805.

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Liu, A. C.-H., B. S. Heinrichs, and R. M. Leach. 1994. Influence of manganese deficiency on the characteristics of proteoglycans of avian epiphyseal growth plate cartilage. Poultry Sci. 73:663-669.

Leach, R. M. and W. O. Twal. 1994. Autocrine, paracrine, and hormonal signals involved in growth plate chondrocyte differentiation. Poultry Sci. 73:883-888.

Twal, W. O., R. Vasilatos-Younken, C. V. Gay, and R. M. Leach, Jr. 1994. Isolation and localization of basic fibroblast growth factor-immunoreactive substance in the epiphyseal growth plate. J. Bone Miner. Res. 9(11):1737-1744.

Knopov, V., R. M. Leach, T. Barak-Shalom, S. Hurwitz, and M. Pines. 1995. Osteopontin gene expression and alkaline phosphatase activity in avian tibial dyschondroplasia. Bone 16:329S-334S.

Luan, Y.-J., C. A. Praul, C. V. Gay, and R. M. Leach, Jr. 1996. Basic fibroblast growth factor: An autocrine growth factor for epiphyseal growth plate chondrocytes. J. Cellular Biochemistry 62:372-382.

Twal, W. O., J. Wu, C. V. Gay, and R. M. Leach, Jr. 1996. Immunolocalization of basic fibroblastic growth factor in avian tibial dyschondroplastic cartilage. Poultry Sci. 75:130-134.

Wu, J., M. Pines, C. V. Gay, S. Hurwitz, and R. M. Leach, Jr. 1996. Immunolocalization of osteonectin in avian tibial dyschondroplastic cartilage. Dev. Dyn. 207:69-74.

Xu, T., R. M. Leach, Jr., B. Hollis, and J. H. Soares, Jr. 1997. Evidence of increased cholecalciferol requirement in chicks with tibial dyschondroplasia. Poultry Sci. 76:47-53.

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Leach, Jr., R. M., C. Sokol, and J. P. McMurtry. 1997. Immunolocalization of basic fibroblast growth factor in porcine epiphyseal growth plate. Domestic Animal Endocrinology 14:129-132.

Praul, C. A., C. V. Gay, and R. M. Leach, Jr. 1997. Chondrocytes of the tibial dyschondroplastic lesion are apoptotic. Int. J. Dev. Biol. 41:621-626.

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Reginato, A. M., R. I. Bashey, G. Rosselot, R. M. Leach, C. V. Gay, and S. A. Jimenez. 1998. Type X collagen biosynthesis and expression in avian tibial dyschondroplasia. Osteoarthritis and Cartilage 6:125-136.

Praul, C. A., K. D. Brubaker, R. M. Leach, and C. V. Gay. 1998. Detection of endogenous biotin-containing proteins in bone and cartilage cells with streptavidin systems. Biochemical and Biophysical Research Communications 247:312-314.

Ben-Bassat, S., O. Genina, I. Lavelin, R. M. Leach, and M. Pines. 1999. Parathyroid receptor gene expression by epiphyseal growth plates in rickets and tibial dyschondroplasia. Molecular Cellular Endocrinology 149:185-195.

Pines, M., N. Yarden, S. Ben-Bassat, I. Lavelin, and R. M. Leach. 1999. Regulation of the calcium-sensing and parathyroid hormone receptor genes in the chick. In: Calcium Metabolism: Comparative Endocrinology. pp. 67-73.

Praul, C. A., B. C. Ford, C. V. Gay, M. Pines, and R. M. Leach. 2000. Gene expression and tibial dyschondroplasia. Poultry Sci. 79:1009-1013.

Medill, N. J., C. A. Praul, B. C. Ford, and R. M. Leach. 2001. Parathyroid hormone-related peptide expression in the epiphyseal growth plate of the juvenile chicken: evidence for the origin of the parathyroid hormone-related peptide found in the epiphyseal growth plate. J. Cell. Biochem. 80:504-511.

Rousche, K. T., C. A. Praul, and R. M. Leach. 2001. The use of growth factors in the proliferation of avian articular chondrocytes in a serum-free culture system. Connect. Tissue Res. – Accepted.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
Donna M. Sosnoski	Senior Project Associate

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Albright College, Reading, PA	B.S., cum laude	1978	Biology

A. Positions

1985-1987	Cell Culture/Research Technician, Agritech Systems, Portland, ME
1986-1987	Junior Research Specialist, Howard Hughes Medical Institute, Dept. of Genetics, University of Pennsylvania, Philadelphia, PA
1987-1988	Research Technician I, Howard Hughes Medical Institute, Dept. of Genetics, University of Pennsylvania, Philadelphia, PA
1988-1993	Research Technician III, Howard Hughes Medical Institute, Dept. of Genetics, University of Pennsylvania, Philadelphia, PA
1993-1996	Associate Director, Vector Core, Institute for Human Gene Therapy, University of Pennsylvania, Philadelphia, PA
1996-1997	Principal Research Associate, Apollon, Inc., Malvern, PA
1997-1998	Research Scientist, Apollon, Inc., Malvern, PA
1998-2000	Project Assistant, Depts. of Biology and Anthropology, Penn State University, University Park, PA
2000-present	Senior Project Associate, Dept. of Biochemistry and Molecular Biology, Penn State University, University Park, PA

B. Publications:

Sosnoski DM, Emanuel BA, Hawkins A, van Tuinen P, Ledbetter DH, Nussbaum RL, Kao FT, Schwartz E, Phillips D, Bennett JS, Fitzgerald LA and Poncz M. Chromosomal localization of the genes for the vitronectin and fibronectin alpha subunits and for platelet glycoproteins IIa and IIIb. Jour Clin Invest 1988; 81:1993-1998.

Maddelena A, Sosnoski DM, Berry GT and Nussbaum RL. Mosaicism for an intragenic deletion in a boy with mild ornithine transcarbamylase deficiency. New Eng Jour Med 1988; 319:999-1003.

Reilly DS, Sosnoski DM and Nussbaum RL. Detection of translocation breakpoints by pulsed field gel analysis: practical considerations. Nucl Acids Res 1989; 17:5414.

Merry DE, Lesko JG, Sosnoski DM, Lewis RA, Lubinsky M, van den Engh G, Collins F and Nussbaum RL. Choroideremia and deafness with stapes fixation: a contiguous gene deletion syndrome in Xq21. Am Jour Hum Genet 1989; 45:530-540.

Lee JT, Murgia A, Sosnoski DM, Olivos IM and Nussbaum RL. Construction and characterization of a yeast artificial chromosome library for Xpter-Xq27.3: a systematic determination of co-cloning rate and X chromosome representation. Genomics 1992; 12:526-533.

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Yu MF, Ewaskiewicz JI, Adda S, Bailey K, Harris V, Sosnoski D, Tomasic M, Wilson J, and Kotlikoff MI. Gene transfer by adenovirus in smooth muscle cells. Respir Physiol 1996; 105(1-2):155-162.

Pachuk CJ, Ciccarelli RB, Samuel M, Bayer ME, Troutman RD, Zurawski DV, Schauer JI, Higgins TJ, Weiner DB, Sosnoski DM, Zurawski VR, Satishchandran C. Characterization of a new class of DNA delivery complexes formed by the local anesthetic bupivacaine. Biochem Biophys Acta. 2000; 1468(1-2):20-30.

Akey JM, Sosnoski D, Parra E, Dios S, Hiester K, Su B, Bonilla C; Jin L, Shriver MD. Melting curve analysis of SNPs (McSNP): a gel-free and inexpensive approach to SNP genotyping. Biotechniques 2001; 30(2):358-36.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory: Dr. Gay's laboratory is in the Frear Building (North Wing, 4th Floor) at the University Park Campus. It consists of an 840 ft^2 general lab, a microscopy lab (265 ft^2), and a 212 ft^2 tissue culture lab.

Clinical:

Animal: Animals will be housed in a ground floor facility in S. Frear. The animal facility has separate rooms for surgery, recovery, animal procedures and housing. The care for animals is provided by the University Laboratory Animal Resources Program and is AAALAC accredited. This institution has an Animal Welfare Assurance on file with the Office of Protection and Research Risks.

Computer: Dell Dimension V433C, Pentium II processor (433 MHz) and Dell Dimension L600CX, Celeron processor (600 MHz) with data analysis, graphics, word processing and spread sheet capabilities. Also, it interfaces with PSU mainframe, internet and PSU library.

Office: Dr. Gay's office (212 ft^2) is adjacent to the labs.

Other: Centrifuges, autoclaves, -80°C freezer, darkrooms, walk in cold room, scintillation and gamma counters, Molecular Dynamics Densitometer (Model SI), Strategene Eagle Eye II Still Video System. Access to Molecular Dynamics Phosphor Imager in adjacent building and to Molecular Dynamics Storm System (8 min walk). Dr. Gay is a member of the Life Sciences Consortium that provides shared technology facilities at cost including a Nucleic Acid Facility, Center for Computational Biology, a Hybridoma and Cell Culture Laboratory, Electron Microscopy Center, and the Center for Quantitative Cell Analysis. The last named center has a benchtop Coulter flow cytometer (Coulter XL-MCL) and a Coulter flow cytometric cell sorter (Beckman-Coulter Elite ESP). It is run by a trained operator. Several isoelectric focusing apparati are available in the Biochemistry and Molecular Biology Department.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

Bio-Rad MRC 1024 confocal microscope with triple excitation (488, 568 and 647 nm) supported by a Compaq Prosignia 300 computer; Leitz microscope with photometer, darkfield, phase contrast, regular and fluorescence optics; Leitz Vario-Orthomat 35 mm camera; Hund Wetzlar inverted microscope; Reichert-Jung Supercut microtome; LKB Ultratome III ultramicrotome; Isomet Bone Saw; Osmometer; RC5C Sorvall Centrifuge; Orion ion analyzer; three incubators for eukaryotic cells; laminar flow hood; Perkin Elmer HTS 7000 plate reader (absorbance and fluorescence), and Hewlett-Packard High Resolution Scanner (Scanjet 6300C) both supported by a Dell Computer; Beckman DU-64 Spectrophotometer; Hitachi F-2000 fluorescence spectrophotometer supported by a Gateway 2000 computer; Centricon 3-microconcentrator; VarioMACs magnetic beads separation system; Bio-rad Mini-Protean®II Electrophoresis system; Ericomp Delta Thermacycler I; VWR Hybridization Oven.

RESEARCH PLAN

A. SPECIFIC AIMS

Three results from our earlier studies provide the impetus for this proposal, namely 1) that a Na-Ca exchanger (NCX) is asymmetrically positioned in osteoblasts, *i.e.* it is on the secretory (apical) and lateral sides of the cell, 2) that inhibition of sodium-calcium exchange blocks mineralization of extracellular matrix, and 3) that the NCX isoform, NCX3, predominates over the ubiquitous NCX1 in osteoblasts. We propose two specific aims to further elucidate the role(s) of sodium-calcium exchanger in osteoblasts:

Specific Aim 1: To examine NCX isoform function in osteoblasts by selective reduction of expression of NCX1, NCX2 and NCX3 using antisense technology. The major goal of this aim is to ascertain which of the NCX isoforms is responsible for supplying Ca^{++} to sites of mineralization.

Specific Aim 2: To determine the distribution of NCX isoforms and other critical proteins in osteoblasts in relation to the development of osteoblast polarity. The goal is to determine if the sorting of NCX isoforms into specific membrane domains occurs in register with the occurrence of lateral adherence and to determine if preventing lateral adherence will alter the sorting.

B. BACKGROUND AND SIGNIFICANCE

Osteoblasts, the cells responsible for bone formation, synthesize and secrete a complex extracellular matrix that has the capacity to nucleate hydroxyapatite crystal formation when sufficient amounts of calcium and phosphate are supplied (reviews: Boskey, 1998; Rodan, 1998). However, despite the great strides made at the physico-chemical level, the mechanism of mineralization at the physiological level has yet to be adequately defined. One aspect of mineralization that is poorly understood is how Ca^{++} is translocated from the circulation into the bone matrix. Our data support a model in which Ca^{++} is actively supplied to bone fluid by osteoblasts. Simply stated, the model predicts that Ca^{++} enters the osteoblast from interstitial fluid by a Ca^{++} influx mechanism, such as stretch-activated calcium channels known to exist in osteoblasts (review: Duncan and Turner, 1995). Ca^{++} then associates with calcium binding proteins and is shuttled through the cytoplasm to the matrix-facing, secretory side of the cell. In this manner, the Ca^{++} shuttling compartment and the Ca^{++} signaling compartments are maintained as distinct entities. Finally, Ca^{++} is passed off to a Ca^{++} efflux mechanism for delivery into the bone fluid. As Ca^{++} levels in bone fluid become elevated, nucleation of calcium phosphate crystals occurs. The formation of calcium phosphate crystals in bone matrix serves as a sink, maintaining the gradient for continued Ca^{++} efflux.

In this proposal, we focus on the mechanism of Ca^{++} efflux from osteoblasts into bone matrix. There are two known mechanisms of energy-driven Ca^{++} efflux from cells: the plasma membrane Ca^{++}-ATPase (PMCA) and Na^{+}/Ca^{++} exchange (NCX) (reviews: Carafoli, 1987;

Carafoli, 1994; Guerini, 1998). Both proteins have been identified and localized in osteoblasts; PMCA by Shen *et al.*, 1983; Akisaka, Yamamoto and Gay, 1988; Kumar *et al.*, 1993 and NCX by Krieger and Tashjian, 1980; Krieger, 1992; Stains and Gay, 1998. PMCA has been suggested to play a key role in bone mineralization (review: Abramowitz and Suki, 1996) and offers the attractive advantage of being highly specific for Ca^{++}. However, PMCA has been shown by cytochemical means to be present primarily on the surface of the osteoblast most distal to the bone matrix, while being absent from the matrix-facing membrane of osteoblasts (Akisaka *et al.*, 1988; Watson *et al.*, 1989). The absence of PMCA on the mineralizing face of the osteoblast suggests that a direct role in the mineralization process is not likely. The other Ca^{++} efflux mechanism, bidirectional Na^{+}-gradient-driven sodium-calcium exchange, lacks the high Ca^{++} affinity of PMCA driven efflux, but compensates with its high capacity (review: Philipson and Nicoll, 1993). Recently, we have shown by immunofluorescence and confocal microscopy that NCX is deployed primarily along the plasma membrane of the matrix-facing side of osteoblasts (Stains and Gay, 1998 {*PR-5, see Progress Report}). This asymmetric distribution of NCX suggests that sodium-calcium exchange is involved in the delivery of Ca^{++} into the bone fluid and supports mass mineralization of bone matrix.

We have examined the role of NCX in mineralization through the use of the relatively specific inhibitors of NCX, bepridil and KB-R7943 (2-[2-[4-(4-nitrobenzyloxy) phenyl]ethyl]isothiourea methanesulfonate), as described in greater detail in Section C (Stains and Gay, 2001 {*PR-10}). Bepridil is an effective blocker of Na^{+}-dependent Ca^{++} efflux, having an IC_{50} of 30 μM in excitable cells (Garcia *et al.*, 1988). KB-R7943 is a more potent inhibitor of NCX. Under physiological conditions in which sodium calcium exchange is reversible, KB-R7943 blocks Ca^{++} exchange at an IC_{50} of 1.0 μM (Kimura *et al.*, 1999). In our study, primary osteoblast cultures which had become confluent were treated with bepridil (3.0 μM, 0.3 μM) or KB-R7943 (1.0 μM, 0.1 μM) for up to 14 days, and the effects of inhibitors on cell viability, cell differentiation, and mineralization were assessed. Both inhibitors caused a reduction in accumulation of mineral in the bone matrix in a dose-dependent manner. While our study makes it clear that sodium-calcium exchange is important in mineralization, there is no information on which of the three isoforms of NCX (NCX1, NCX2, NCX3) may be involved. Also, the timing of expression of the NCX proteins and their deployment into the plasma membrane has not been determined. These basic properties need to be defined in order to begin assessing how calcification of extracellular matrix may be controlled.

The three known isoforms of NCX are expressed by different genes; however, all share a striking degree of sequence homology and the functional properties are highly similar (Quednau *et al.*, 1997; Philipson and Nicoll, 2000). NCX1 is present in all tissues examined to date and is considered to be ubiquitous (Kofuji *et al.*, 1992; Lee *et al.*, 1994). NCX2 and NCX3 have been identified in brain and skeletal muscle and at notably lower levels than NCX1 (Li *et al.*, 1994; Nicoll *et al.*, 1996). We present evidence in the progress report that shows, for the first time, the presence of NCX3 in osteoblasts; interestingly, it is present in substantially greater abundance than NCX1 (Stains *et al.*, in press {*PR-11}).

All three isoforms of NCX are similar, based on sequence comparisons and hydropathy plots. Each has 9 transmembrane domains, 2 domains partially lodged in the membrane, and a

large cytoplasmic loop located between membrane-spanning segments 5 and 6. The loop contains a Ca^{++} binding site and a Ca^{++} regulatory domain. The C-terminus of the loop contains a variable region of 110 amino acids which is specified by 6 exons in the genomic structure (Kofuji *et al.*, 1994). Alternative exon splicing in the variable region generates tissue specific variants and results in a variety of ways sodium-calcium exchange is regulated. There are 32 known alternative splice variants for NCX1, only one form of NCX2 and 3 variants of NCX3. (Reviews: Quednau *et al.*, 1997; Philipson and Nicoll, 2000).

Most information on sodium-calcium exchange has been gained from studies of NCX1 in cardiac tissue. There are a variety of factors that influence the rate of Na-Ca exchange (Shigekawa and Iwamoto, 2001). Activators of the process include Ca^{++}, phosphorylation of the exchanger, PIP2, and PKC activators. Inhibitors include Na^{+}, protons, La^{3+}, Cd^{++}, and the exchange inhibitor peptide XIP. A means of regulating tissue distribution of NCX is its association with the cytoskeleton. Many of these factors are likely to regulate NCX2 and NCX3 also, since phosphorylation sites and ion binding sites are located in the cytoplasmic loop distant from the variable region.

Many membrane bound proteins have a close association with the cytoskeleton (Mills *et al.*, 1994; Verrey *et al.*, 1995) and this is also true for the Na-Ca exchanger (Condrescu *et al.*, 1995). Ankyrins, which are proteins that function as adapters between the cytoskeleton and membrane spanning proteins, have been shown to co-localize with NCX1 in ventricular myocytes (Chen *et al.*, 1997). The distribution pattern changes with development (Chen *et al.*, 1997). In the present proposal, we plan to study the development of polarity of osteoblasts and lateral adherence with respect to NCX distribution.

NCX antagonists (e.g. bepridil, KB-R7943, ameloride analogues, and high Na^{+}) inhibit all three NCX proteins (and their multiple splice variants) and therefore they are not useful for elucidating unique roles of NCX1, NCX2 or NCX3 (Garcia *et al.*, 1990; Smith *et al.*, 1991; Watano *et al.*, 1996). Removal of Na^{+} can inhibit NCX-mediated Ca^{++} efflux, but this approach also results in changes in $[Na^{+}]_i$ and other ion imbalances (Smith *et al.*, 1991). Antisense technology offers an attractive approach to knockout or knockdown specific isoforms to elucidate physiological roles of ion transporters in intact cells.

Antisense oligonucleotides to NCX (and appropriate controls) have been introduced into a number of cell types including arterial myocytes (Slodzinski *et al.*, 1995; 1998), pancreatic β-cells (Van Eylen *et al.*, 1998), astrocytes (Takuma *et al.*, 1996), renal distal tubule (White et al., 1998) and cardiac myocytes (Lipp *et al.*, 1995) and used to address function. Based on these successes, we propose to determine the roles of NCX isoforms in osteoblasts in Specific Aim I by specifically blocking function with antisense oligonucleotide administration. The focus of Aim 2 will be on the time frame in which NCX is sorted into specific membrane domains in relation to lateral cell-cell contact and development of osteoblast polarity.

Significance. In the last few years, it has become clear that bone formation is carried out by a "syncytium" of osteoblasts which act synchronously as a unit (Ziambaras *et al.*, 1998; Lecanda *et al.*, 2000). Several molecular devices contribute to the unified function. Osteoblasts adhere

laterally through Ca^{++}-dependent adhesive proteins including N-cadherin (Cheng et al., 2000) and cadherin-11, also called OB-cadherin (Kawaguchi *et al.*, 2001). In both of those studies, depletion of cadherin, N- or OB-, resulted in substantial decreases in bone volume and density. Osteoblasts also interact through a variety of integrins (Helfrich and Horton, 1999) and are able to share ions, cyclic AMP and other small molecular signals which can pass from cell to cell through gap junctions (Civitelli *et al.*, 1993; Donahue *et al.*, 1995). Therefore, it is becoming clear that a layer of laterally connected osteoblasts can act as a unified structure. Our study is designed to learn how this structure directs calcium ions into the collagenous extracellular matrix produced by osteoblasts.

The focus of this application, on defining the role of sodium-calcium exchange in supplying calcium ions to sites of mineralization, will help elucidate the process of bone formation and will set the stage for determining how calcification is regulated at the cellular level. As we show in the Progress Report, specific NCX inhibition results in substantially reduced mineralization of extracellular matrix. This result did not appear to a consequence of cytotoxic effects. It is conceivable that mutations in NCX, especially NCX3, in osteoblasts would result in under-mineralized bone. It is possible that the NCX isoforms will have different roles in osteoblasts; we predict that NCX3 fosters mineralization and that NCX1 is involved in intracellular Ca^{++} homeostasis. Therefore, characterizing these Ca^{++}-translocating proteins in osteoblasts is a worthwhile endeavor. The studies proposed are novel: to our knowledge, no other group is examining the expression, distribution and function of NCX in osteoblasts. Additional background specific to each aim is presented in the rationale section of each aim.

C. PROGRESS REPORT (07/01/95 to 10/15/01)

The objective of the previous application was to identify calcium efflux mechanisms in osteoblasts and to ascertain if osteoblasts direct Ca^{++} into sites of mineralization. Specifically, we focused on:

1. Further characterization of primary chicken osteoblasts derived from long bone periostea, especially with respect to their ability to synthesize a calcified matrix.

2. Identifying the distribution and roles of sodium-calcium exchange (NCX) protein relative to plasma membrane calcium ATPase (PMCA) in osteoblasts.

3. Correlating the function of Na/K-ATPase and NCX in osteoblasts.

Progress towards meeting the Specific Aims:

We were able to meet the goals almost completely and made a number of unexpected discoveries. Publications from this project, cited as PR-1 to PR-17, are listed at the end of this section. An asterisk indicates those provided in the appendix.

1. Characterizing a primary osteoblast culture system. In 1992, we began developing a primary osteoblast culture system using chicken tibial periosteal cells as a source of osteoblasts and have described that system in several publications. Osteoblasts are obtained from long bone

periosteal surfaces of chickens in their rapid growth phase at 2-3 weeks of age. These cells quickly reach confluence (by day 6) when seeded at a density of ~10^4 cells/cm^2. Both alkaline phosphatase and Type I collagen are abundantly expressed (Gay *et al.*, 1994). The primary chicken osteoblasts respond to PTH, as detected by a shape change (*PR-2) and reduced mitochondrial potential (*PR-4). Responses to estrogen (*PR-4, *PR-8) and to androgen (*PR-8) were observed. Responses to the steroids occurred in seconds and utilized the Ca^{++} signaling pathway, an indication that the responses observed are non-genomic. By 12 days of culture, mineralized nodules were evident (*PR-7) and by 28 days extensive extracellular mineralization had occurred (*PR-10). Confocal microscopy imaging revealed that osteocalcin, bone sialoprotein (BSP), osteopontin (OPN) and osteonectin were expressed by day 4 of culture and were restricted to the cell interior. By day 8, BSP and osteopontin had been secreted and became attached to collagen fibrils in the matrix. [Confocal microscopy provided clear images of fibers in matrix to which BSP and OPN had attached (Fig. 1 of *PR-7).] Osteocalcin and osteonectin were secreted by day 12, as indicated by decreased protein in the cytosol; however, these two peptides did not adhere to the collagen fibers in the matrix (*PR-7) as expected. Collagen type I message was expressed constantly over a 12 day culture period and PTHrP receptor was increasingly expressed (*PR-7).

Overall, the chicken-derived primary osteoblast cultures appear to be authentic osteoblasts that function normally. They offer the advantages of being easily obtained in abundance, quickly becoming confluent, and not losing or acquiring abnormal features as is sometimes found in cell lines.

2. Distribution and function of Na-Ca Exchanger (NCX) in osteoblasts. Additional characteristics of the primary osteoblast cultures studied included identifying the presence and polarized distribution of NCX in relation to plasma membrane Ca-ATPase (PMCA). Previously we reported that PMCA is present on the marrow-facing side of the osteoblast (Akisaka, Yamamoto, Gay, 1988) and that the direction of pumping of Ca^{++} is into the marrow space (PR-1). In this progress report we report that NCX is deployed along the secretory surface of osteoblasts, juxtaposed to the matrix (*PR-5, *PR-11), as shown in Fig. 1. The antibody employed was developed in rabbits to recognize NCX in canine cardiac sarcolemma (rabbit anti-Na^+Ca^{++} exchanger protein, Research Diagnostics, Inc.). We found good cross-reactivity with chicken cardiac myocytes and osteoblasts. This polyclonal antibody recognizes NCX1, NCX2 and NCX3 through conserved regions common to all three isoforms (and their splice varients). Dual labeling of PMCA and NCX in whole mount osteoblasts was achieved using antibodies to each protein sequentially. A confocal optical section through the free surface of cultured cells stained almost exclusively for PMCA (red stain) whereas, near the matrix secreting side of the cells adherent to fibronectin or collagen I coated coverslips, NCX (green) was abundant (Fig. 1). A more thorough description may be found in *PR-11 in the appended material.

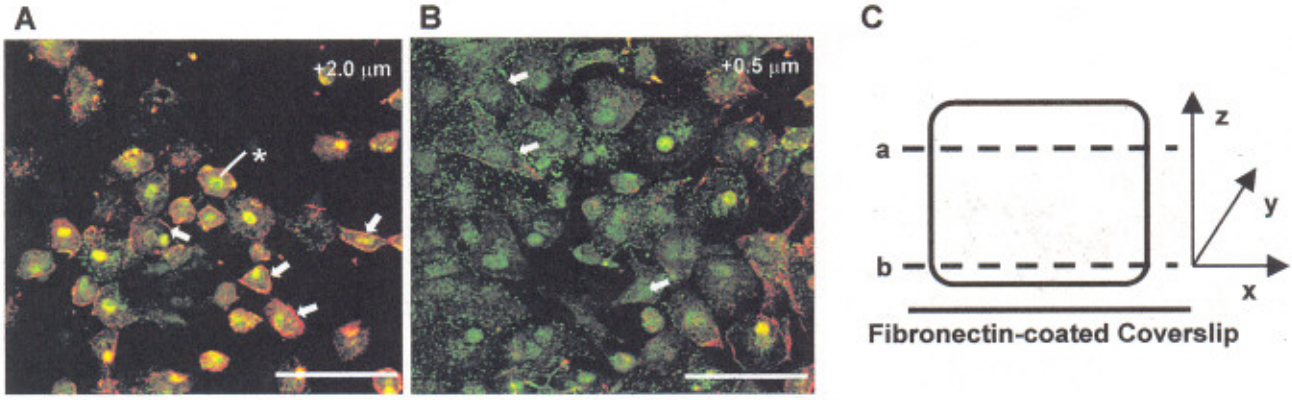

Fig. 1. Panels A and B are two optical sections, or Z-planes (a,b in Panel C) from the same set of cells to show dual immunostaining using confocal microscopy. Osteoblasts, cultured for 16 days, were fixed in 4% paraformaldehyde, then stained with anti-NCX followed by anti-PMCA. The secondary antibody for NCX was tagged with BODIPY (green); PMCA was tagged with Cy3 (red). Panel A was near the top surface of the cells. In Panel A, most of the fluorescence of cell perimeters (arrows) is red (PMCA). Panel B shows green stained NCX near the cell surface attached to the fibronectin coated coverslip. Non-specific nuclear staining (asterisk) also appeared in control slides. Scale bar=25 µM.

We used Bio-Rad time-course software (TCSM) integrated with the confocal microscope to demonstrate that the cultured osteoblasts can exchange Na^+ for Ca^{++} and that the process is dependent on Na/K-ATPase activity, *i.e.* it is affected by ouabain, a specific inhibitor of Na/K-ATPase (*PR-5). This result, shown in Fig. 2 and Table I, was obtained by loading the cells with Calcium Green-1 (Molecular Probes, Eugene, OR) and forcing the exchange process to operate in reverse, *i.e.* Ca^{++} entered the cell in exchange for internal Na^+. When Na/K-ATPase is inhibited with ouabain, cytosolic levels of Na^+ are higher (due to normal gradient-driven diffusion) and therefore, more reverse exchange can occur.

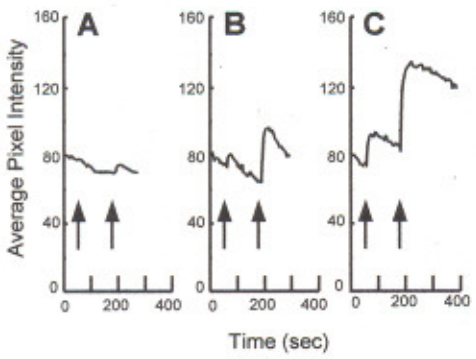

Fig. 2. Na^+-dependent Ca^{++} uptake by cultured osteoblasts. Plots represent typical changes in pixel intensity of the calcium sensitive dye Calcium Green-1. Arrows indicate the time point at which a bolus of 1.5 mM $CaCl_2$ was added to the live cell observation chamber. ***(A)*** *The average change in pixel intensity of cells maintained in a high-sodium buffer, 140 mM NaCl to block exchange.* ***(B)*** *The average change in pixel intensity of cells treated in low-sodium buffer, 5 mM NaCl.* ***(C)*** *The average change in pixel intensity for cells treated in buffer containing 5 mM NaCl and 0.3 mM ouabain, an inhibitor of Na/K ATPase. See Table I for average changes for all experiments.*

Table 1. Numerical Analysis of Na^+-Dependent Ca^{++} Uptake

			1st bolus of $CaCl_2$		2nd bolus of $CaCl_2$	
	Treatment	n	*ΔPI±SD*	*% change*	*ΔPI±SD*	*% change*
A.	+Na	44	-0.6±1.9	-0.8%	+3.2±0.81	+4.5%
B.	-Na	46	+8.3±2.2	+11.8%	+26.6±6.1	+30.8%
C.	-Na + Ouabain	36	+16.3±5.2	+22.6%	+49.3±7.9	+46.2%

Changes in relative pixel intensities (ΔPI) of cells treated (A) in high external sodium (+Na), (B) low external sodium (-Na), or (C) low sodium plus ouabain (-Na + ouabain) were calculated at points of addition of $CaCl_2$ (point prior to addition versus maximum change). The changes are expressed as standard deviation from the mean (+/- sd). Percent change in relative pixel intensity was calculated to highlight the magnitude of the responses. The total number of individual cells tested from four independent cell isolations is denoted by *n*. (From *PR-5).

Collectively, these studies show that NCX is functional and appropriately positioned to provide Ca^{++} to sites of mineralization.

3. Inhibition of NCX blocks mineralization. Next, we examined matrix mineralization over a culture time of 28 days during which time extensive amounts of matrix were deposited and became mineralized when Ca^{++} and phosphate ions (from β-glycerophosphate) were provided. We found that specific inhibitors of NCX, bepridil and KBR7943, blocked mineral desposition by 50% and 100%, respectively (*PR-10), as shown in Fig. 3.

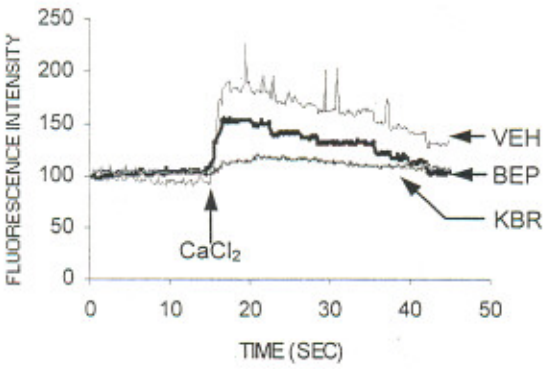

Fig. 3. Inhibition of Na^+-dependent Ca^{2+} uptake by osteoblasts treated with bepridil or KB-R7943. Osteoblasts cultured for 12 days were loaded with 2.0 μM Calcium Green-1 in a low sodium buffer (5 mM NaCl) and then examined for their ability to take up external Ca^{2+} added in the presence or absence of NCX inhibitors (3.0 μM bepridil or 1.0 μM KB-R7943). The plot represents the change in relative fluorescence of the Ca^{2+}-sensitive dye Calcium Green-1. The arrow indicates the point of addition of a bolus of $CaCl_2$ (final concentration, 1.5 mM).

It is possible that osteoblast development could have been impaired when intracellular Ca^{++} homeostasis was disturbed by NCX inhibitors. To address this concern, we examined several cell and molecular parameters and found that cell viability, alkaline phosphatase activity and expression of matrix protein (osteopontin, bone sialoprotein and osteocalcin) mRNA were not impaired by the inhibitors (*PR-10). Confocal imaging revealed that type I collagen, bone sialoprotein and osteopontin were present in the matrix, i.e. had been secreted (*PR-10).

4. Probing chicken osteoblast cDNA using NCX cDNA sequences. We screened chicken osteoblast cDNA using two canine NCX-1 cDNA fragments obtained from Dr. Kenneth Philipson as probes to identify the NCX gene in our system, initially using 8 day cell cultures. An ~5.6 kb message that had high homology to NCX was obtained; however the gene product turned out to be a translation initiation factor (IF) that was apparently more abundantly expressed than NCX message. We also obtained three other unique sequences and submitted all four to GenBank (PR-14 to PR-17). One is a 1358 base fragment that is homologous to regulatory factor 3A (PR-14); the second has 1965 bases homologous to translation initiation factor IF2 (PR-15) and we have characterized and published this (*PR-9); the third has 1188 bases homologous to 6D12C protein, a non-specific ion transporter (PR-16); the fourth has 2975 bases homologous to transmembrane domains of the Na-Ca exchanger (PR-17). In pursuing this further, we found that the translation initiation factor was homologous to other translation initiation factors, including human IF2. Noteworthy in this study is that both the chicken (cIF2) and human (hIF2) forms were abundantly expressed in both chicken primary osteoblasts and a human osteoblast cell line (hFOB), respectively, at 8 days of culture, but at lower levels both earlier and later during osteoblast development (Fig. 4). For cultured chicken osteoblasts, 8 days of culture is a time when translation and synthesis of many proteins occurs, including alkaline phosphatase (*PR-7), plasma membrane calcium ATPase (*PR-11), and several matrix proteins (*PR-7).

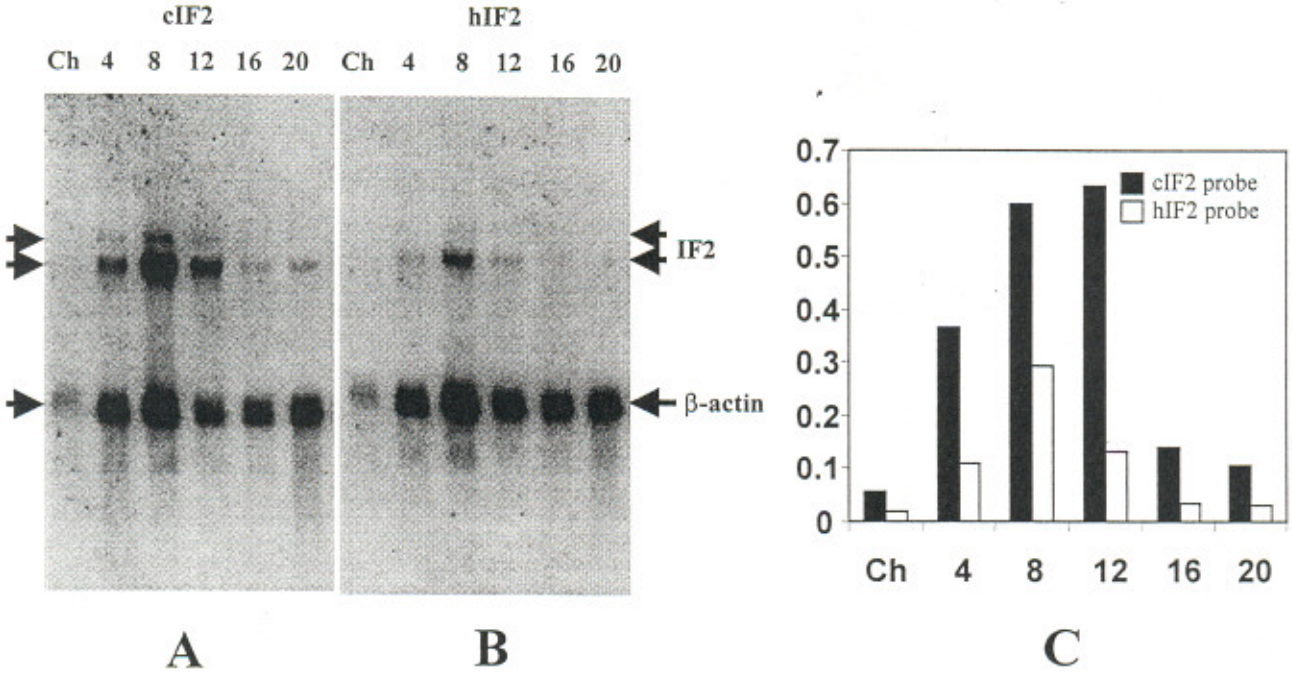

Fig. 4. (See legend on next page.)

Fig. 4. Levels of cIF2 expresson change during osteoblast differentiation in culture. ***A:*** *Northern blot analysis of A^+ RNA isolated from chondrocytes (Ch), and chicken osteoblasts cultured for 4, 8, 12, 16, and 20 days. The probes used are the 1.5 kb fragment of cIF2 and the mouse β-actin probe. The amount of RNA loaded in each lane was: Ch, 8 μg; osteoblasts at 4 days, 4 μg; 8 days, 1 μg; 12 days, 3 μg; 16 days, 2 μg; and 20 days, 3 μg.* ***B:*** *The same blot shown in A, stripped and reprobed with a 2.6 kb fragment of hIF2 and the mouse β-actin probe.* ***C:*** *Graphical representation of the ratio of IF2 (detected with either the cIF2 probe or the hIF2 probe) to β-actin for the blots shown in A and B. The black bars represent the cIF2 probe to β-actin ratios while the white bars represent the hIF2 probe to β-actin ratios. From *PR-9.*

Because NCX1 message was lower than other homologous sequences, we began to study another NCX isoform, NCX3. Using primers designed from chicken NCX1 and NCX3 sequences (GenBank: AJ012579 and AJ012580, respectively), we assessed expression of NCX1 and NCX3 messages over a 20 day time course for cultured chicken osteoblasts using RT-PCR (Stains *et al.*, in press {*PR-11}). Relative quantitative RT-PCR of chicken heart, brain and 8 day cultured osteoblasts revealed substantial levels of NCX1 mRNA in heart and high levels of NCX3 mRNA in brain and osteoblasts (Fig. 5).

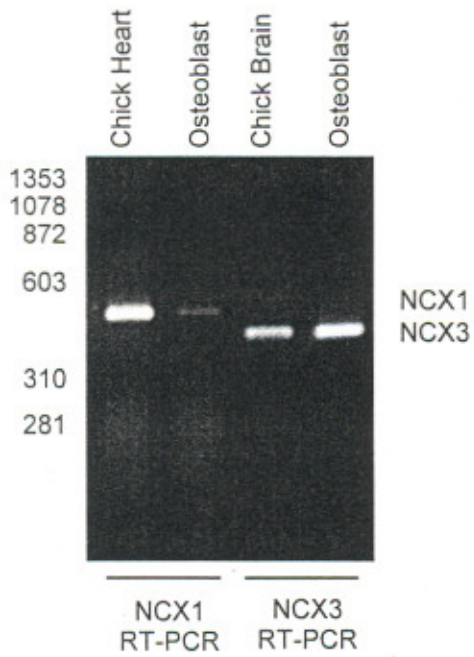

Fig. 5. Detection of NCX1 and NCX3 by RT-PCR in chicken tissues. RNA isolated from 8 day cultured osteoblasts, chicken heart, or chicken brain was reverse transcribed. RT-PCR products generated using isoform specific primers for chicken NCX1 and NCX3 were electrophoresed on a 2.0% agarose gel and stained with ethidium bromide. NCX1 expression was weakly detected as a 483 bp band by RT-PCR, while NCX3 was readily detected as a 367 bp product. Chicken heart and chicken brain cDNA were used as positive controls for NCX1 and NCX3, respectively. All reactions were performed under identical conditions over 28 rounds of amplification. Molecular weights (in bp) are indicated to the left.

Immunoblotting revealed that NCX protein is expressed at nearly constant levels (Fig. 6A), although the relative contributions of the three NCX isoforms was not assessed since the antibody available recognized all three isoforms. NCX-3 message was expressed at a constant level with the exception of the 8 day culture in which expression was greater (Fig. 6B); NCX-1 message was expressed at a constant level from 4 to 20 days (Fig. 6C).

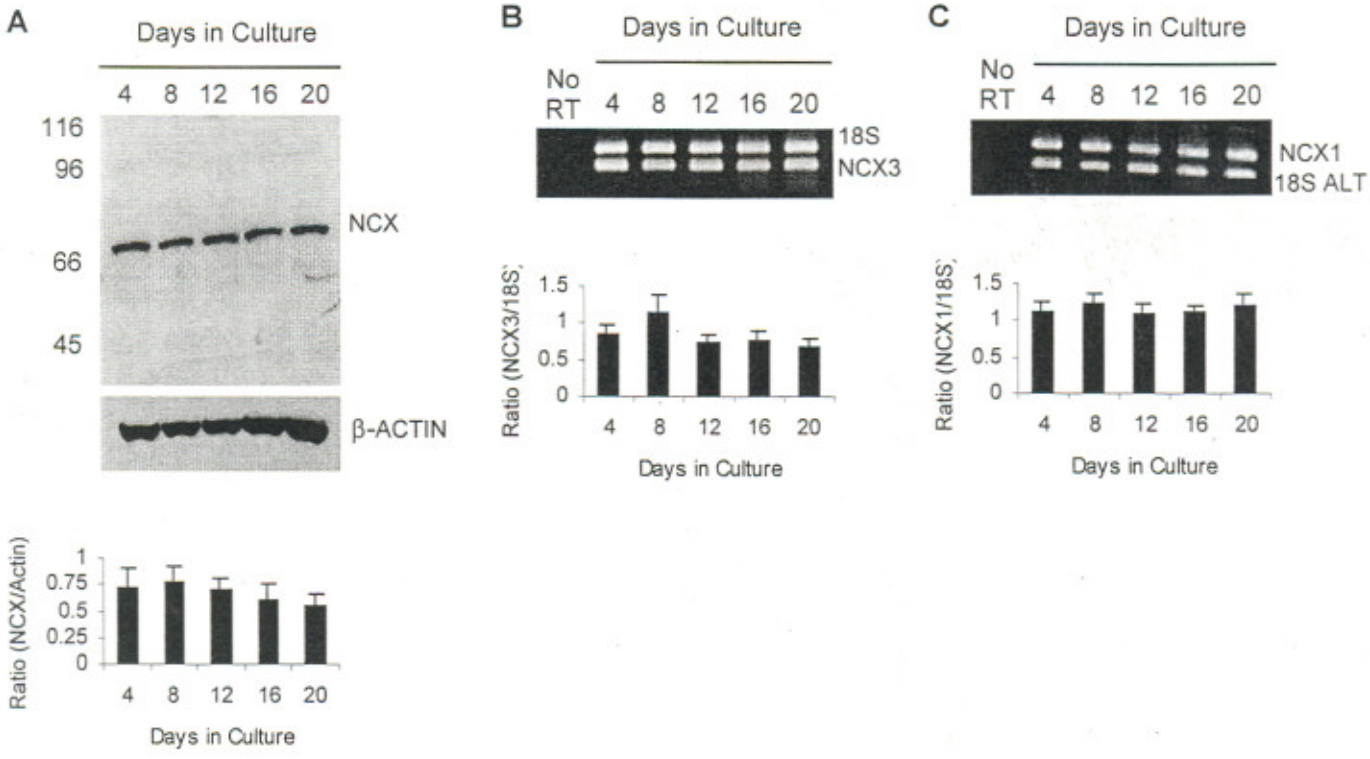

Fig. 6. Time course of NCX protein expression by cultured osteoblasts. ***A)*** *Osteoblast plasma membrane proteins were prepared from cells cultured for 4, 8, 12, 16 or 20 days. The protein extracts (20 μg) were electrophoresed on a 7.5% SDS-PAGE gel, blotted to PVDF membrane and probed with antibodies against canine cardiac NCX. A single 70 kD product is detected. [The mammalian NCX1 is ~120 kD. We tried a number of approaches to determine if the chicken NCX has been fragmented, but found no evidence for this, as discussed in Stains and Gay, 1998 {*PR-5}.] As a loading control, the blots were reprobed with antibodies against β-actin. Molecular weights (in kD) are indicated to the left. The ratios of NCX to β-actin as determined by volume quantitation of immunoblots are indicated. The level of NCX protein appears unchanged throughout the time course. The data are presented as the mean ± standard deviation (n=3).* ***B)*** *Time course of NCX3 mRNA expression by cultured osteoblasts. Agarose gel of the products generated by relative quantitative RT-PCR from osteoblast total RNA using NCX3 gene specific primers and a 3:7 ratio of classic 18S rRNA competimer primers. The ratios of NCX3 to 18S rRNA PCR product as determined by volume quantitation are indicated. (Mean ± standard deviation; n=4).* ***C)*** *Time course of NCX1 mRNA expression by cultured osteoblasts. Agarose gel of the products generated by relative quantitative RT-PCR from osteoblast total RNA using NCX1 gene specific primers and a 2:8 ratio of alternate 18S rRNA competimer primers. The ratios of NCX1 to 18S rRNA PCR product as determined by volume quantitation are indicated. The levels of both NCX1 and NCX3 message remain nearly static throughout the time course of culture. (Mean ± sd; n=4).*

5. Effects of donor age on expression of key proteins by osteoblasts. Osteoblasts were isolated from tibia of 3-4 month old and 14-15 month old rats using the same isolation procedure as used for isolating chicken osteoblasts. Proliferation was assessed using the CyQuant Assay (Molecular Probes). The effects of TGF_{β} and 1,25 vitamin D_3 on proliferation were assessed. Both agents enhanced proliferation cells from young donors and were ineffective on cells from old donors (PR-12). The rat osteoblasts were also compared for their ability to express proteins relevant to osteoblast function as a function of donor age (PR-13). PMCA and osteopontin mRNA were invariant. However, NCX and PTHrP receptor messages were reduced in osteoblasts from old donors. The latter observations suggest a reason why osteoblasts function poorly during aging. This study shows specific effects of aging on osteoblast proliferation and protein expression; the study also encouraged us to continue working with primary chicken osteoblasts since the latter are easier and less expensive to obtain and maintain in culture.

Publications from this project:

PR-1 Gay, C. V. and Lloyd, Q. P., Characterization of calcium efflux by osteoblasts derived from long bone periosteum. Comp. Biochem. Physiol., 111A:257-261, 1995.

*PR-2 Lloyd, Q. P., Kuhn, M. A. and Gay, C. V., Characterization of calcium translocation across the plasma membrane of primary osteoblasts using a lipophilic calcium-sensitive fluorescent dye, calcium-green C_{18}. J. Biol. Chem, 270:22445-22451, 1995.

PR-3 Gay, C. V., Avian bone turnover and the role of bone cells. In: Dacke, C et al., eds., The Comparative Endocrinology of Calcium Regulation. pp: 113-121, J. Endocrinology Ltd, 1996. (Invited Review)

*PR-4 Troyan, M. B., Gilman, V. R. and Gay, C. V., Mitochondrial membrane potential changes in osteoblasts treated with parathyroid hormone and estradiol. Exp. Cell Res. 233:274-280, 1997.

*PR-5 Stains, J. P. and Gay, C. V., Asymmetric distribution of functional sodium-calcium exchanger in primary osteoblasts. J. Bone Miner. Res. 13:1862-1869,1998.

PR-6 Gay, C. V., Gilman, V. R. and Sugiyama, T., Perspectives on osteoblast and osteoclast function. Poultry Science 79:1005-1008, 2000. (Invited Review)

*PR-7 Luan, Y-J., Praul, C. A. and Gay, C. V., Confocal imaging and timing of secretion of matrix proteins by osteoblasts derived from avian long bone. Comp. Biochem. Physiol. 126:213-221, 2000.

*PR-8 Armen, T. A. and Gay, C. V., Simultaneous detection and functional response of testosterone and estradiol receptors in osteoblast plasma membranes. J. Cell. Biochem. 79:620-627, 2000.

*PR-9 Weber, J. A. and Gay, C. V., Expression of translation initiation factor IF2 is regulated during osteoblast differentiation. J. Cell. Biochem. 81:700-714, 2001.

*PR-10 Stains, J. P. and Gay, C. V., Inhibition of Na^+/Ca^{2+} exchange with KB-R7943 or bepridil diminishes mineral deposition by osteoblasts. J. Bone Miner. Res. 16:1434-1443, 2001.

*PR-11 Stains, J. P., Weber, J. A. and Gay, C. V., Expression of Na^+/Ca^{2+} exchanger isoforms (NCX1 and NCX3) and plasma membrane Ca^{2+} ATPase during osteoblast differentiation. J. Cell. Biochem., in press, 2001.

PR-12 Shiels, M. J., Mastro, A. M. and Gay, C. V., The effect of donor age on the sensitivity of osteoblasts to the proliferative effects of TGF_β and $1,25(OH_2)\ D_3$. Life Sciences, in press.

PR-13 Zeng, Y., Mastro, A. M. and Gay, C. V., Aging effects on regulatory, calcium transporting and matrix protein expression by osteoblasts. Exp. Gerontology (under review).

Data Base Entries:

PR-14 Weber, J. A., Stains, J. P. and Gay, C. V., Gallus gallus osteoblast regulatory factor 3A mRNA. May 18, 1999. GenBank Submission AF144636.

PR-15 Weber, J. A., Stains, J. P. and Gay, C. V., Identification of novel cDNAs from cultured chick osteoblasts by similarity to the sodium calcium exchanger. May 18, 1999. GenBank Submission AF144637.

PR-16 Weber, J. A., Stains, J. P. and Gay, C. V., Gallus gallus osteoblast 6D12C protein mRNA. May 18, 1999. GenBank Submission AF145780.

PR-17 Weber, J. A., Stains, J. P. and Gay, C. V., Gallus gallus membrane protein mRNA. July 27, 1999. GenBank Submission AF172844.

D. RESEARCH DESIGN AND METHODS

D.1 To examine NCX isoform function in osteoblasts by selective reduction of expression of NCX1, NCX2, and NCX3 using antisense technology.

Rationale and Hypothesis. We have shown that inhibition of Na-Ca exchange results in marked reduction in mineralization (Stains and Gay, 2001 {*PR-10}). Furthermore, using specifically designed primers for RT-PCR, we have found that NCX3 exists in osteoblasts and predominates over NCX1 (Stains *et al.*, in press {*PR-11}). Since the highly effective inhibitors of Na-Ca exchange and mineralization, bepridil and KB-R7943, do not allow precise distinction among NCX isoforms, we will now focus on specific isoform reduction of NCX1 and NCX3 using sequence specific oligonucleotides designed to inhibit expression of NCX at the message level. We will also attempt to develop antisense oligonucleotides to NCX2 from highly conserved sequences of the rat NCX2 gene; currently, NCX2 has been sequenced only for rat. *The hypothesis to be tested is that specific loss of functional NCX3 results in impaired mineral deposition, whereas ablation of NCX1 or NCX2 has little effect on mineralization.*

D.1.a Cell Sources and Culture. ***Primary chicken osteoblasts*** will be used since these cells are fully functional, easy to obtain and, as demonstrated in the Progress Report, we have extensive experience with their isolation, propagation and characterization. Use of the ***mouse cell line, MC3T3-E1*** (Choi *et al.*, 1996), is also planned in order to obtain parallel data in a mammalian osteoblast system that is also capable of forming a mineralized matrix. Further, the mammalian system provides a model in which data on NCX2 can most likely be obtained since primer design is based on rat NCX2.

Chicken osteoblasts are acquired from the periosteal surface of 2-3 week old chicken tibias, after removing the periosteum, using mild sequential enzymatic digestion with 0.05% hyaluronidase, 0.03% trypsin and 0.1% collagenase followed by firm scraping of the bone surfaces with a curette. The cell suspension is plated on fibronectin-coated 35 mm culture dishes at a density of 10^4 cells/cm^2. The cells are maintained in Dulbecco's Modified Eagle's medium (DME) plus 10% fetal bovine serum (FBS), 50 μg/ml ascorbic acid, 5 mM β-glycerophosphate to promote mineralization at 37°C under a humid atmosphere of 5% CO_2 and 95% air. The medium is changed by half every 2-3 days.

The MC3T3-E1 cells are a well-established osteoblast cell line that produces a calcifiable matrix and, therefore, retains the osteoblast phenotype exceptionally well. We have been provided with these cells by Dr. Norman Karin, University of Delaware. Growth conditions for these cells have been defined (Quarles *et al.*, 1992). Stock cultures are grown in the same medium and under the same conditions as the primary chicken cells.

Both cell types will be seeded at densities to achieve confluence in 6 days and will be cultured in the presence and absence of oligos designed to ablate NCX1, NCX2 or NCX3.

D.1.b Oligonucleotide design for knockdown studies. Antisense, nonsense and sense oligonucleotide sequences are shown in Table I. The ***antisense oligos*** are designed to selectively block expression of NCX1, NCX2 or NCX3; ***non-sense oligos***, which have the same base composition as the antisense oligos but with a rearranged sequence, will control for non-specific or toxic effects; ***sense oligos*** will ensure that knockdown effects on expression are due to sequence blocking and not secondary structure. Design of the oligos, shown in Table II, follows the methods outlined by Marcus-Sakura (1988). The antisense oligos will consist of a tandem pair, as used by Slodzinski and Blaustein (1998) in a study of NCX knockdown in arterial myocytes. These are designed to encompass the ***atg*** start codon for each NCX isoform (Van Eylen *et al.*, 1998; Takuma *et al.*, 1996). By performing NCBI BLAST searches, we have found each sequence to be unique to its corresponding isoform (Table II) and thus will only block expression of a selected NCX isoform. The sense and antisense oligo sequence designs were derived from the GenBank database sequence for mouse NCX1 and NCX3 (accession # AF004666 [nt –26 to +6] and AF321404 [nt +1 to +34]) and for chicken NCX1 and NCX3 (accession # AJ012580 [nt –1 to +33] and AJ012579 [nt –1 to +33], respectively). The sequence for the NCX2 gene in mouse and chicken is currently not available. However, in the start codon region of NCX1 and NCX3 there exists a 100% sequence identity between rat and mouse. Therefore, the oligos for NCX2 will be derived from the available Genbank rat NCX2 sequence (accession # U08141 [nt +1 to +34]) and will be used with the mouse cell line since high levels of sequence homology are likely to occur between rat and mouse.

Table II. Specific tandem pair sequences for performing antisense knockdown studies of NCX1, NCX2 and NCX3. The **atg** start codons and their antisense complementary **cat** sequences are shown in bold.

Mouse NCX1	Sense:	5' agcagttggaagtc 3'	5' ttattgtacaac**atg**ctt 3'
	Antisense:	5' taagacttccaactgct 3'	5' aag**cat**gttgtacaataa 3'
	Nonsense:	5' tagactatcaccatctg 3'	5' gactatgtaacacagat 3'
Mouse NCX2	Sense:	5' **atg**gctcccttggcttt3'	5' ggtaggggttgccctcc 3'
	Antisense:	5' aaagccaatttag**cat** 3'	5' ggagggcaacccctacc 3'
	Nonsense:	5' gaccaatatatagactc 3'	5' agggaagcctcgcccat 3'
Mouse NCX3	Sense:	5' **atg**gcgtggttacggct 3'	5' gcagcctctcacctctg 3'
	Antisense:	5' agccgtaaccacgc**cat** 3'	5' cagaggtgagaggctgc 3'
	Nonsense:	5' cagcagtaaccgcatcc 3'	5' agacgtgaaggcgtggc 3'
Chicken NCX1	Sense:	5' **cat**gtctctcattgaag 3'	5' tcatcacatcccaagaa 3'
	Antisense:	5' cttcaatggagga**cat**g 3'	5' ttcttgggatgtgatga 3'
	Nonsense:	5' ctatcgaggatacggat 3'	5' cttgttaggtggatatg 3'
Chicken NCX3	Sense:	5' **cat**ggcatctatagagg 3'	5' tcattacatcgcaagag 3'
	Antisense:	5' cctctatagatgc**cat**g 3'	5' ctcttgcgatgtaatga 3'
	Nonsense:	5' tctccagattgacatcg 3'	5' tcctcgagcttagatag 3'

All oligo sets defined in Table II were examined by the sequence analysis program, Oligo 6, to assure that excessive formation of interfering secondary structures is avoided as recommended by Schwaller *et al.*, 1999. The first and last four bases of each oligo will be phosphorothioated to increase resistance to cellular nuclease activity and improve transport into the cells (Fisher *et al.*, 1993; Zhoa *et al.*, 1993). Synthesis and purification of the oligos will be done by MWG Biotech (High Point, NC), a company we have utilized in the past.

D.1.c Introduction of oligonucleotides into osteoblasts. The cells will be cultured in the presence or absence of the panel of oligos for 1, 2 and 4 weeks, following the protocol of Slodzinski *et al.*, 1995. Optimal concentrations of oligos added to culture media will be determined; however, they are expected to be ~1 μM, as found by Marzia *et al.* (2000). Media will be changed 3 times weekly as used in other antisense knockdown studies (Slodzinski *et al.*, 1995). To determine that sufficient uptake of the oligos by the osteoblasts has occurred, fluorescein-tagged oligos (MWG Biotech, High Point, NC) will be added to the media of confluent cultures (Schwaller *et al.*, 1999). Confocal microscopy will be used to confirm that the oligos have entered the nuclei of the osteoblasts. To determine possible cytotoxic effects of the oligos, cultures will be assessed for viability, using propidium iodide exclusion, and cell proliferation using the CyQuant assay (Molecular Probes, Inc). Also, immunoblots of key matrix proteins (osteopontin, bone sialoprotein, osteonectin and osteocalcin) will be evaluated to assess normal cell function [see sections D.4.c and D.4.d for the immunoblotting method and for sources

of antibodies]. Reversibility of antisense knockdown of NCX expression will be assessed as further indication that the antisense oligos are specific. For this, cells will be cultured 7 days with antisense oligos, then cultured 4 more days after removal of the oligos. The knockdown effect should disappear after wash-out of the oligos. Slodzinski and Blaustein (1998) found that oligo washout out of NCX1 oligos required several days in the arterial myocyte system. The long recovery time may be due to relatively long oligo half-life. Reversibility of the knockdown effect will be quantified by measuring Na/Ca exchange rates as defined in Section D.1.e.

D.1.d Effects of oligonucleotides on NCX isoform expression. Expression of NCX1, NCX2 and NCX3 will be determined at the RNA level using relative quantitative RT-PCR. Cells will be cultured with and without antisense, sense and nonsense oligos and processed for extraction of RNA and protein using Trizol (Life Technologies, Rockville, MD) as detailed in Section D.4.a. First strand cDNA synthesis will be performed on the extracted RNA using the RETROscript kit (Ambion, Austin, TX); RT-PCR will then be run on the cDNA using the primers listed in Section D.4.b. Relative amounts of mRNA will be measured using the QuantumRNA system (Ambion) as described in section D.4.b. Feasibility for detecting NCX1 and NCX3 expression by RT-PCR in chicken osteoblast lysates has been demonstrated previously in this laboratory (Stains *et al.*, in press {*PR-11}).

Immunoblotting of SDS-PAGE gels will also be used to assess NCX expression at the protein level as detailed in Section D.4.c. Only two antibodies are currently available, anti-NCX, and NCX1, (listed in Section D.4.d). However, even with this limited set of antibodies, insight into isoform expression at the protein level can be gained. The general anti-NCX antibody can be used to detect all three isoforms simultaneously. Distinction of the isoforms can be partially clarified by removing NCX1 from samples by immunoprecipitation with anti-NCX1. More detailed analysis can be done by reducing NCX2 or NCX3 with the appropriate antisense oligo. Band intensities will be assessed by ImageQuant analysis (Molecular Dynamics, Sunnyvale, CA).

D.1.e Effects of reduced NCX isoform expression on Na-Ca exchange. Osteoblasts, cultured in the presence/absence of antisense oligos for NCX1, NCX2 and NCX3 for 8 days, will be analyzed for Na-dependent Ca^{++} flux using the time course software integrated into the confocal imaging system (TCSM software program, Bio-Rad), as we have previously reported (Figures 2, 3 of Progress Report; *PR-5; *PR-10). Osteoblasts, on fibronectin-coated coverslips, are rinsed in 10 mM HEPES buffer (pH 7.4) which contains 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM glucose and 0.1% bovine serum albumin. Cells are loaded with 2 μM Calcium Green-1 (CG-1) AM ester. Since the Ca^{++} detecting fluorescent reporter dye is placed inside cells the Na-Ca exchange process is operated in reverse, *i.e.* Ca^{++} enters the cell in exchange for intracellular Na^+. The CG-1 loaded cells are placed in medium which contains low sodium (5 mM), low sodium + 0.3 mM ouabain or high sodium (140 mM). When both Na^+ and Ca^{++} are high externally, exchange is blocked. Ouabain is added to inhibit Na/K-ATPase, the enzyme which maintains low intracellular sodium; this enhances the reaction by allowing slightly higher intracellular [Na^+] to develop, so when external Ca^{++} is added a greater level of exchange can occur. In low sodium buffers, isotonicity is maintained by adding choline chloride. Cells are placed in a live cell observation chamber in their appropriate buffers and a bolus of $CaCl_2$ (1.5 mM final concentration) added at 15s. The relative pixel intensity of ~30 cells from 3 cell isolates will be collected at 5 sec intervals using the time-course software program (TCSM, Bio-Rad). Pixel intensities will be normalized to a common starting pixel intensity to aid in data

interpretation. Data similar to that shown in Fig. 3 and Table I in the Progress Report will be collected. The data are expected to reveal the relative contributions of each isoform. NCX3 is expected to predominate. Analysis of variance will be used to determine degrees of significance.

D.1.f. Effects of reduced expression of NCX isoforms on mineralization. Osteoblasts cultured for 4 weeks in the presense of antisense, sense and nonsense oligos for NCX1, NCX2 and NCX3 will be evaluated for the extent to which extracellular mineralization has/has not occurred. Up to 10-days exposure to NCX antisense oligonucleotides has been used in other studies (Slodzinski and Blaustein, 1998). The cultures will be rinsed 3x in distilled water to lyse cells and remove aqueous Ca^{++}. Mineral deposited into extracellular matrix will be solubilized in 5% trichloroacetic acid according to Gronowitz *et al.* (1989). Calcium content of triplicate cultures will be assayed using a colorimetric O-cresolphthalein-based calcium quantitation kit (Sigma). The data will be evaluated as percent of untreated values. Means will be compared by Student's t-test. Von Kossa staining will also be done. These methods are in place in the lab (*e.g.* Stains and Gay, 2001 {*PR-10}). The data should reveal which NCX isoform(s) are responsible for mineralization. NCX3 is expected to support mineralization.

Pitfalls and Expected Results. We expect to find that reduced expression of NCX3 will impair mineralization of extracellular matrix from both chicken and mouse osteoblasts, since NCX3 dominates over NCX1 and since mineralization is a major process supported by sodium-calcium exchange. The extent of the impairment will depend on the extent of antisense knock down that can be achieved. There is a possibility that upregulated expression of the other NCX isoforms or PMCA may occur as a compensatory mechanism. If necessary, experiments will be designed to determine the contributions of compensating expression. It is expected that NCX1 is important in intracellular Ca^{++} homeostasis and that NCX3 serves in the mineralization process. We do not expect that specific NCX isoform reduction will have adverse effects on the cells, since cytotoxic effects have not been observed in myocytes (Slodzinski and Blaustein, 1998), pancreatic β-cells (Van Eylen *et al.*, 1998), astrocytes (Takuma *et al.*, 1996) or distal renal tubule cells (White *et al.*, 1998). Use of antisense oligonucleotides in other cell types has resulted in complete or nearly complete ablation of NCX function. For example, in arterial myocytes (Slodzinski and Blaustein, 1998) where rates of $[Ca^{++}]_{cyt}$ decline were measured, a highly significant ($P<0.001$) exchange of Ca^{++} for external Na^{+} was found in control cells, whereas the antisense treated cells did not exchange Ca^{++} for Na^{+} ($p>0.05$). Also, antisense knockdown via the highly conserved region of NCX1 in β cells of the pancreas obliterated sodium-calcium exchange (van Eylen *et al.*, 1998). Further, Takuma *et al.*, (1996) using NCX antisense oligos similar to our design, found greatly diminished protein expression in immunoblots. These antisense knockdown studies indicate that our experimental approach has a good chance for success.

D.2 To determine the distribution of NCX isoforms and other critical proteins in osteoblasts in relation to the development of osteoblast polarity.

Rationale and Hypothesis. A question that intrigues us is: at what point in development do osteoblasts become fully polarized cells capable of forming a mineralized matrix? More specifically, when do NCX and plasma membrane Ca-ATPase (PMCA) become sorted into apical and basal membrane domains? As mentioned earlier, we have previously shown that mature, functional osteoblasts possess NCX on the secretory side of the cell and PMCA on the opposing

cell surface (Stains and Gay, 1998 {*PR-5}; Stains *et al.*, in press {*PR-11}). Interestingly, separation of PMCA and NCX into distinct plasma membrane domains has also been found in other cell types, *e.g.* arterial myocytes (Slodzinski and Blaustein, 1998).

First, we will explore an *in vivo* model. At six days of development in embryonic chick, bone formation begins as a collar of osteoblasts circumscribing the cartilage anlage at the center of the diaphysis of the femur (Anderson, 1973). The collar of osteoblasts arises from mesenchyme on the surface of the central cartilagenous shafts of the femurs. With time, the collar of osteoblasts widens, and by 8 days, bone matrix is secreted and begins to mineralize. Eventually, the central cartilage shaft is removed to form the marrow cavity. When Soares *et al.* (1992) fixed 17 to 19 day rat embryo bones with a glutaraldehyde fixative that contained lanthanum chloride, electron microscopic analysis revealed that electron dense $LaCl_3$ had moved freely between immature osteoblasts at the periphery of the collar. At central sites, where osteoblasts were mature and adherent to each other, $LaCl_3$ was excluded from the bone fluid space and was present only in marrow spaces. This indicates that tight connections form between mature osteoblasts and it is likely that the time just preceding lateral cell-cell contact is when PMCA and NCX isoforms are sorted into their respective membrane domains.

Several recent studies have shown that cell-cell interaction is necessary for osteoblasts to function normally. In one study, over-expression of connexin 43, i.e. the dominant isoform of gap junction protein in osteoblasts, lead to increased matrix mineralization (Gramsch *et al.*, 2001). In a study using cadherin-11 (also called OB-cadherin) null mice, there was a substantial reduction in the volume and density of the bones (Kawaguchi *et al.*, 2001). Both connexin 43 and cadherin-11 are a key linking proteins between osteoblasts and are critical to normal development. In another report, expression of a truncated N-cadherin in the MC3T3-E1 osteoblast cell line resulted in a 50% reduction in cell-cell adhesion, in reduced expression of several key matrix proteins and in reduced matrix mineralization (Cheng *et al.*, 2000).

The hypothesis to be tested in this aim is that segregation of plasma membrane domains with respect to NCX and PMCA occurs just prior to the development of lateral contacts between osteoblasts. In Aim 2a we will employ an immunocytochemical approach using developing embryonic chick bone as a model. This will be followed in Aim 2b by a cell culture approach so that expression levels of NCX isoforms, PMCA, cadherin-11 and connexin 43 can be quantified over time in relation to each other. Expression levels of both mRNA and protein will be determined.

D.2.a. Immunocytochemical staining of longitudinal sections of developing whole bone. Femurs from chick embryos at 8 days of age will be excised, fixed in 4% buffered paraformaldehyde and embedded in either paraffin or water soluble polymer. Longitudinal sections will be made to include the entire span of the collar of bone on the surface of the cartilagenous femur shafts. The osteoblasts at the edges of the collar will be young, those at the center mature. Initially we will examine sections that have been immunostained with anti-cadherin-11 (OB-cadherin) and anti-connexin-43 (the dominant gap-junction protein of osteoblasts). The sources of antibodies to be used are described in section D.4.d. Osteoblasts at the periphery of the collar of bone will not have adhered to each other (Soares *et al.*, 1992). Therefore, expression of cadherin-11 and connexin 43 is expected to be nil between immature

osteoblasts. In some embryos, a low MW dextran (3000 daltons) tagged with fluorescein, rhodamine or Texas red will be injected (via the easily accessible omphalomesenteric artery in the chorioallantoic membrane using a #30 needle), a technique the PI has experience with. Fixation with a lysine-based fixative will be used to lock the dextran in place (Molecular Probes, Eugene, OR). Immunostaining of adherent cells, as indicated by lateral membrane staining with anti-cadherin 11 and anti-connexin 43, should correlate with exclusion of fluorescent dextran from bone matrix spaces. Next, longitudinal sections will be immunostained with anti-NCX and anti-PMCA. Tightly adherent cells are expected to display distinct domains for NCX and PMCA; immature osteoblasts at the periphery of the collar are expected to display a random distribution of NCX with respect to PMCA. We have previously shown segregation of NCX and PMCA in plasma membrane of cultures of confluent osteoblasts using dual immunostaining (Stains *et al.*, in press {*PR-11}). In that study, we did not focus on the time of appearance of NCX and PMCA in relation to cell-cell contact. The value of using longitudinal sections of chick embryo femur is that it provides a spectrum of osteoblast ages, *i.e.* from the oldest at the center of the collar to the youngest at the edges, in a single section. Also unique to this approach is the ability to study the osteoblasts in their natural setting. As before, antibodies will be used at optimal conditions. Each new batch of antibodies acquired will be tested to determine optimal dilutions. For controls, primary and secondary antibodies will be replaced by an irrelevant antibody. We will select the fluoroprobe for each primary antibody so that dual or triple staining can be used (e.g. anti-cadherin-11 (green), anti-NCX (red) and anti-PMCA (orange) by selecting fluorescent tagged secondary antibodies from the numerous choices available. Mineralization of matrix will be assessed by von Kossa staining

D.2.b. Changes in message and protein levels of NCX isoforms as development proceeds *in vitro*. In order to quantify the expression of key proteins as osteoblasts become fully polarized cells we will utilize a cell culture system. Both primary chicken osteoblasts isolated as before (Section D.1.a) and mouse MC3T3-E1 cells will be seeded at densities to achieve confluence in ~6 days. Both pre- and post-confluent cells will be lysed, e.g. at 4, 6 and 8 or 10 days. However, the exact timing may need to be refined. Expression of cell contact proteins (cadherin-11, Cnx43), NCX isoforms and PMCA will be assessed over time at both message and protein levels. Total RNA and protein will be isolated at each time point using the Trizol reagent (Section D.4.a). The timing of expression of NCX1, NCX2, NCX3 and PMCA in relation to cadherin-11 and connexin- 43 protein will be assessed at mRNA and protein levels using methods detailed in Section D.4.b and c. Antibody sources are provided in Section D.4.d. Plasma membrane fractions will be collected in the presence of a protease inhibitor cocktail containing EDTA, leupeptin, pepstatin and phenylmethyl sulfonyl fluoride, as we have done previously (Stains and Gay, 2001 {*PR-10}). SDS-PAGE and immunoblotting will be performed as detailed in section D.4.c.

To further test the association of NCX3 expression with the occurrence of cell-cell contact, cultures will be seeded at low, intermediate and high densities. In our previous work a seeding density of ~10^4 cells/cm^2 resulted in confluence at 6 days and had peak NCX3 expression at 8 days (Stains and Gay, 2001 {*PR 11}). By seeding at lower and higher densities the timing of confluence and cell-cell contact will be delayed or enhanced. Expression of NCX3, cadherin-11 and connexin-43 will be delayed or enhanced if the timing of their expressions are linked.

An expected outcome is that NCX3 will be expressed after confluence is achieved but prior to cadherin-11 and connexin-43 expression (*i.e.* prior to the formation of stable lateral contacts). We already have evidence that NCX3 message is more abundant at 8 days of culture whereas NCX1 and PMCA message is constant between days 4 and 20 of culture (Progress Report, Fig. 6).

Pitfalls and expected results. The proposed experiments in this Aim will allow correlations to be made with regard to expression and deployment of NCX and PMCA into distinct domains just preceeding the time when osteoblasts become adherent and linked into a functional "syncytium." It is likely that a random membrane distribution of NCX and PMCA will be found in osteoblasts which have not made lateral contacts. Conceivably, NCX3 will be expressed in abundance near the time lateral contacts are made, while the other isoforms are expressed early since their role is likely to be in intracellular Ca^{++} homeostasis.

We are well experienced with immunostaining and other microscopic methods. The P.I. has examined the ultrastructure of chick embryo femurs, which are 3 mm long at the 6 day stage, in relation to initial mineralization (Schraer and Gay, 1977) and has performed i.v. injections on 6 day embryos. While we have not obtained preliminary evidence to show that osteoblast polarity development requires cell-cell adherence, studies sited above (Gramsch, 2001; Kawaguchi, 2001; Cheng *et al.*, 2000) clearly indicate that the development of mature functional osteoblasts requires cell-cell contact. Cell-cell contact is necessary for many developing tissues. To cite just one example, in developing heart the positioning of Na/K-ATPase occurs as cell-cell contacts are made (Lisnak *et al.*, 2001). Further, it has been shown that fibronectin enhances osteoblast survival (Globus *et al.*, 1998). Fibronectin is a component of bone matrix and is used in our culture system to coat the tissue culture surfaces.

We will use anti-NCX to follow the developmental changes in NCX distribution in experiment D.2.a, since that antibody recognizes all three isoforms. Immunolocalization of the NCX1 antibodies for specific NCX isoform can also be performed since anti-NCX1 is now commercially available (Section D.4.d). However anti NCX2 and NCX3 with cross-reactivity for immunocytochemistry of chicken and mouse tissue are not currently available.

D.3. Conclusions and future directions. Collectively, these studies will determine 1) the timing of expression of the Na-Ca exchanger isoforms in osteoblasts, in relation to other key proteins (*i.e.* plasma membrane calcium ATPase, cadherin-11 and connexin-43); 2) the timing of the distribution of these proteins in relation to the development of cell polarity; and 3) whether or not NCX3 is the isoform responsible for Ca^{++} translocation to sites of mineralization. Thus, new understanding of osteoblast development and function will emerge.

NCX is especially abundant in excitable tissue, i.e. in cardiac tissue, brain and neurons. While osteoblasts are not classified as electrically excitable, they do respond to mechanical stretching, a process that creates electrical fields (Duncan and Turner, 1995). It is possible that NCX in osteoblasts, which is an integral part of the mineralization process, is regulated by biochemical, electrical and/or mechanical forces. The present proposal is designed to provide a basic understanding of NCX isoform expression in osteoblasts so that future studies can focus on regulation of NCX driven Ca^{++} efflux into sites of mineralization.

A fruitful, future direction will be to explore the regulation of NCX expression. For example, a recent study has shown that TGF-β1 upregulates NCX1 expression in cardiac myocytes and proposes that the resultant enhanced Ca^{++} efflux is a mechanism to maintain appropriate cytosolic Ca^{++} levels under conditions of stress (Carrillo *et al.*, 1998). This is fascinating, since osteoblasts are known to both copiously produce and respond to TGF-β1 (Bonewald, 1996). Once we know for certain that NCX3 is the critical NCX isoform that subserves mineralization, we will begin to make plans to study regulation of NCX3 regulation.

D.4 Compendium of Methods.

D.4.a. RNA and protein extract preparation.

RNA and protein are extracted from cell lysates according to standard techniques using Trizol (Life Science Technologies, Gibco BRL). Cultured cells are lysed in 3.5 ml Trizol solution and the lysate extracted with 700 µl chloroform. The aqueous phase is collected and RNA is precipitated with 1.5 ml isopropanol. The RNA pellet is washed with 3.5 ml of 75% ethanol and air dried for 15 min. The RNA is resuspended in 30-50 µl nuclease-free water, quantified and assessed for purity by spectrophotometric readings at 260 nm and 280 nm. All RNA preparations are treated with 1 unit RQ1 DNase (Promega, Madison, WI) prior to cDNA syntheses to remove traces of contaminating genomic DNA.

Proteins are isolated from the phenol-ethanol supernate obtained after removing DNA from the non-aqueous phases. Isopropanol is used to precipitate the cellular proteins. The protein pellet is washed three times with 7 ml 0.3 M guanidine hydrochloride in 95% ethanol and once with 2 ml absolute ethanol. The protein pellet is then vacuum dried and resuspended in a 1% SDS solution.

D.4.b. Design and application of reverse transcriptase primers.

Total RNA (2 µg) will be reverse transcribed from random decamers using the Ambion RETRO script kit (Austin, TX). The cDNA obtained will be used for relative quantitative RT-PCR to determine message levels for cadherin-11, connexin-43, NCX isoforms and PMCA. Conditions, cycle number and primer:competimer ratios for TR-PCR will be optimized for each set of primers. Levels of amplified signal will be normalized to 18S RNA, using Quantum RNA classic 18S internal standards (Ambion). The 18S primers and competimers will be selected so that the amount of 18S RNA product is in the linear range under the same amplification conditions as the gene-specific primers. RT-PCR products will be electrophoresed on 2.0% agarose gels and stained with ethidium bromide. The gels will be documented using an Eagle Eye II digital imaging system (Stratagene, La Jolla, CA). Band intensities will be volume quantitated using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The primers to be used (Table III) have been or will be synthesized by the Penn State Nucleic Acid Facility directed by Dr. Deborah Grove.

Table III. RT-PCR Primers for NCX1, NCX2, NCX3 and PMCA1.

		Forward	Reverse
Chicken	NCX1	5' gttgttcgtgatggagaaataagg 3'	5' tggccaagagatactcgacg 3'
	NCX3	5' acaacaacaaccattcggattt 3'	5' caaagtggaaagaagaaaagc 3'
Mouse	NCX1	5' ttgttttcccatgttgacca 3'	5' aagttatggccgcacacttc 3'
	NCX2	5' ggaatgaaacggtgtccaac 3'	5' acacacaccgggaagaagac 3'
	NCX3	5' ctgcaaggagggtgtcattt 3'	5' aagactcgcaggtgcttgat 3'
Chicken	PMCA1	5' gccatcttctgcacaattgt 3'	5' tcagagtgatgtttccaaac 3'
Mouse	PMCA1	5' tggcaaacaactcagttgcatatagtgg 3'	5' tcctgttcaattcgactctgcaagcctcg 3'

NCX1 and NCX3 primers for chicken (Table III) have been used by us to assess expression in primary chicken osteoblasts (Stains *et al.*, in press {*PR-11}). For NCX1, these primers yield a product of 483 bp, corresponding to nucleotides 269-752 of the published chicken NCX1 sequence (GenBank accession # AJ012579). Alternate 18S internal standards and competimer are used at a 2:8 ratio. The PCR conditions we previously established are: one cycle at 94°C for 3 min, then 32 repetitions of 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec. Chicken cardiac cDNA will be used as a positive control. For chicken NCX3 the primers yield a product of 367 bp, corresponding to nucleotides 71-438 of the published chicken NCX3 sequence (GenBank accession # AJ012580). Classic 18S internal standards and competimer are used at a 3:7 ratio. The PCR conditions used previously are: 94°C for 3 min, then 28 rounds at 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec. Chicken brain cDNA (Clontech, Palo Alto, CA) will be used as a positive control tissue.

Mouse NCX1, NCX2 and NCX3 primers, shown in Table III, were chosen from published sequences for mouse NCX1 (accession # AF004666) and NCX3 (accession # AF321404). For designing NCX2 primers, the rat sequence for NCX2 (accession # U08141) was used (Li *et al.*, 1994) in lieu of non-existent sequence data for mouse using the same strategy as we used for designing the chicken NCX primers. Primers are selected in the region approximately 800 bp following the start codon since there is little sequence homology among the NCX isoforms in this area. A target amplicon size between 300 and 500 base pairs is considered appropriate both for ease of amplification and clear separation from the 18S amplicons used as internal controls. The selected sequences (Table III) were analyzed for matched PCR primer pairs by the computer program Primer3 (www-genome.wi.mit.edu). PCR product sizes for the mouse primers will be 407 bp [nt 172-578], 349 bp [nt 361-719] and 450 bp [at 159-608] for NCX1, NCX2 and NCX3 respectively.

PCR primers for chicken PMCA1 have been previously described by Cai *et al.* (1993) and have been utilized by this laboratory to quantify PMCA1 mRNA in chicken osteoblast cultures

(Stains *et al.*, in press {*PR11}) This primer set yields a 644 bp product; classic 18S internal standards and competimer were used at a ratio of 1:9 for the relative quantitative PCR assay. The PCR conditions were: one cycle of 94° C for 3 min, the 37 repetitions of 94° C for 30 sec, 50° C for 30 sec and 72° C for 60 sec. Primers for mouse PMCA1 have been previously used by White *et al.* (1997). They are derived from the NCBI sequence for rat PMCA1 (accession # J03753) and encompass the region from nt 275 to nt 836, yielding a product of 562 bp (Meszaros and Karin, 1993).

Primers for mouse connexin-43 and cadherin-11 have been previously described (Chung *et al.*, 1999; Kawaguchi *et al.*, 2001) and are shown in Table IV. Resulting PCR products are 498 bp for connexin-43 and 438 b for cadherin-11.. The chicken primer sequences for connexin-43 and cadherin-11 (Table IV) were designed from the published NCBI sequences, accession # M29003 and accession # AF055342, respectively. The connexin-43 primers produce a 476 bp amplicon spanning from nt 208 to nt 706; cadherin-11 primers encompass the sequence from nt 2275 to nt 2711, yielding a PCR product at 392 bp. The regions chosen displayed a high level of sequence homology to the primers previously developed for mouse; matched primer pairs were chosen using the Primer3 software.

Table IV. RT-PCR Primers for Connexin 43 and Cadherin 11.

	Forward	Reverse
Mouse connexin-43	5' gtcagcttggggtgatgaacag 3'	5' atggttttctccgtgggacg 3'
cadherin-11	5' cgtggagggttcagtcggcaga 3'	5' tactgatactcaggtttgat 3'
Chicken connexin-43	5' agtctgcttggggagatgaa 3'	5' caggaaacagtccaccctgt 3'
cadherin-11	5' agacgcgaaggatttagtcg 3'	5' ccatcagggttctgcaaagt 3'

D.4.c. SDS-polyacrylamide gel electrophoresis for immunoblotting.

SDS-PAGE is performed according to the method of Laemmli (1970). Protein concentrations for the plasma membrane fraction of cell lysates are determined by the BCA assay (Pierce, Rockford, IL). Protein samples are placed on 7.5% polyacrylamide gels, electrophoresed and blotted onto Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore. Corp., Bedford, MA). Membranes are blocked in 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T). Primary antibodies are applied at a dilution of ~1:1000, depending on the antibody. The blots are stripped and reprobed with anti-β actin at a 1:5000 dilution, as a loading control. After rinsing with TBS-T, the blots are incubated in horseradish peroxidase (HRP) linked mouse or rabbit antibodies at a dilution of 1:5000. Immunoreactions are detected using the ECL western blotting analysis system (Amersham International, Cleveland, OH). Band intensities are volume quantitated using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA) so that protein/actin ratios may be compared.

D.4.d. Sources and properties of antibodies.

Anti-Canine Sodium-Calcium Exchanger (Anti-NCX), Research Diagnostics, Flanders, NJ. Polyclonal; produced in Rabbits. Broad range species and tissue specificity including chicken and mouse osteoblasts. Detects NCX1, NCX2, NCX3. Used for both immunoblots and immunohistochemistry. Used by us (Stains and Gay, 1998; Stains *et al.*, 2001).

Anti-Rat Sodium-Calcium Exchanger 1 (Anti-NCX1), Chemicon, Temecula, CA. Polyclonal; produced in Rabbits. Broad species reactivity including chicken and mouse. Reacts with NCX1, but not other isoforms. Used for immunoblotting; immunohistochemical staining yet not tested, but likely because reactive site is in the cytoplasmic domain.

Anti-Mouse PMCA ATPase, Research Diagnostics, Flanders, NJ. Monoclonal; clone 5F10. Broad range of species and tissue specificity, including chicken. Results with all 4 known PMCA isoforms. Used for immunoblots and immunohistochemistry. Used by us previously (Stains and Gay, 1998; Stains *et al.*, 2001). Also, **anti-rabbit PMCA1 ATPase**, Affinity Bioreagents, Golden, CO. Polyclonal. Detects rat and mouse PMCA. Used for immunoblots.

Anti-Mouse connexin 43 (Anti-Cnx43), Chemicon, Temecula, CA. Polyclonal; produced in rabbits. Broad species reactivity. Chicken not tested, but likely due to high conservation of sequences. Used for immunoblotting and immunohistochemistry.

Anti-human OB-cadherin (cadherin-11). Research Diagnostics, Flanders, NJ. Polyclonal; produced in goats. Human, mouse and rat reactive. Not tested in chickens, but expected due to high conservation of the C-terminus. Used for immunoblotting and immunohistochemistry.

Most of the matrix protein antibodies are already in use in our lab. Dr. Louis Gerstenfeld provided us with **anti-chicken bone sialoprotein, anti-chicken osteopontin** and **anti-chicken osteocalcin**. Dr. Larry Fisher has given us **anti-chicken osteonectin, anti-mouse BSP (LF123), anti-mouse osteopontin (LF-83)** and **anti-mouse-osteonectin (LF-23)**. **Anti-mouse osteocalcin** is commercially available (Biogenesis LTD), Poole, UK and Biomedical Technologies, Stoughton, MA). All are polyclonal and have been used for both immunohistochemistry and immunoblotting, except the anti-mouse osteocalcin, which will need to be tested for immunoblotting.

Secondary antibodies are all commercially available and will be selected as appropriate, for example anti-rabbit IgG tagged with BODIPY (green) or Cy3 (red) for immunohistochemistry or tagged with HRP for immunoblotting.

Time Table

Year		01	02	03	04
Aim 1a	Antisense studies using Ca flux as an end point	---------->			
Aim 1b	Antisense studies using mineralization as an end point	---------->			
Aim 2a	Correlation of NCX and PMCA distribution with lateral cell adhesion		---------->		
Aim 2b	Correlation of NCX and PMCA expression with lateral cell adhesion			---------->	

Format of Time Table is up to the applicant

E. HUMAN SUBJECTS - none

F. VERTEBRATE ANIMALS

1. Heavy Breed Broiler Chickens, 2-3 weeks of age all of the male gender will be obtained from the Poultry Education and Research Center (PERC) at Penn State. The birds are transported from the PERC to the Animal Facility in S. Frear Building in the morning of the osteoblast isolation days. The chickens are decapitated by a razor sharp guillotine, an Am. Veterinary Assoc. approved procedure. Tibias and femurs are collected and their periosteal surfaces used as a source of primary osteoblasts. A sufficient number of osteoblasts can be obtained from 12 chickens every 2 weeks to perform the studies planned (12 x 25 weeks = 300 chicks per year).

2. Primary osteoblasts, used shortly after isolation, closely resemble cells *in vivo*, as we have documented earlier. The primary cells are obtained to serve as a standard for comparing data obtained from an osteoblast cell line. We have chosen to work with chicken osteoblasts because we have found these cells to be robust when cultured and we have had considerable experience with these cells. The number to be used, 12 every 2 weeks, is based on past experience.

3. The Animal Resource Lab at Penn State is staffed with Veterinarians, some of whom have specific training and interest in avian species.

4. The distress level to the birds is momentary and minimal. (It is USDA Category C.)

5. Euthanasia by guillotine is a preferred method for birds and is recommended by the Am. Veterinary Medical Association.

Penn State has AAALC accreditation.

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H. CONSORTIUM/CONTRACTUAL ARRANGEMENTS

None

I. CONSULTANTS

- Attached are letters of agreement from Drs. Deborah S. Grove and Roland M. Leach.

Gay, Carol V.

PENNSTATE

LIFE SCIENCES CONSORTIUM
The Pennsylvania State University
519 Wartik Laboratory
University Park, PA 16802-5807

(814) 865-3155
Fax: (814) 863-1357

Nucleic Acid Facility
October 22, 2001

Dr. Carol V. Gay
Dept. of Biochemistry and Molecular Biology
The Pennsylvania State University
108 Althouse Laboratory
University Park, PA 16802

Dear Carol:

I will be pleased to assist you and your laboratory staff in the design of primers for RT-PCR and of oligonucleotides for antisense studies for your NIH grant. We will be able to synthesize the primers for you here in the Nucleic Acid Facility. Since the oligonucleotides require phosphorothioation, you will need to have those synthesized elsewhere. As always, I look forward to working with your group.

Sincerely yours,

Deborah S. Grove, Ph.D.
Director of Research Projects

Gay, Carol V.

PENNSTATE

Department of Poultry Science

The Pennsylvania State University
213 William L. Henning Building
University Park, PA 16802-3501

(814) 865-3411
Fax: (814) 865-5691

lnr@psu.edu
814-865-5082

October 23, 2001

Dr. Carol V. Gay
Professor of Cell Biology
Department of Biochemistry and Molecular Biology
The Pennsylvania State University
108 Althouse Laboratory
University Park, PA 16802

Dear Carol:

This is to affirm my interest in your research program and my willingness to serve as a consultant, particularly with regard to your studies on changes in gene expression of sodium-calcium exchange protein and plasma membrane calcium ATPase as osteoblasts mature. This dovetails nicely with our ongoing studies with maturing chondrocytes and osteoblasts in medullary bone. As in the past, I look forward to working with you and your group.

Sincerely yours,

Roland

Roland M. Leach, Jr.
Walther H. Ott Professor in Avian Biology

RML:llh

Principal Investigator/Program Director *(Last, first, middle)*: Gay, Carol V.

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☐ NEW application. *(This application is being submitted to the PHS for the first time.)*

☐ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant No. _____ ☐ SBIR Fast Track

☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant No. _____ ☐ STTR Fast Track

☐ REVISION of application number: _____

(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☒ COMPETING CONTINUATION of grant number: DE09459-08

(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS *(Competing continuation appl. and Phase II only)*

☒ No

☐ Yes. If "Yes," ☐ Previously reported ☐ Not previously reported

☐ SUPPLEMENT to grant number: _____

(This application is for additional funds to supplement a currently funded grant.)

☐ CHANGE of principal investigator/program director.

Name of former principal investigator/program director: _____

☐ FOREIGN application or significant foreign component.

1. PROGRAM INCOME ***(See instructions.)***

All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***

The following assurances/certifications are made and verified by the signature of the Official Signing for Applicant Organization on the Face Page of the application. Descriptions of individual assurances/ certifications are provided in Section III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Pluripotent Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA and Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR) STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☐ DHHS Agreement dated: _____ ☐ No Facilities And Administration Costs Requested.

☐ DHHS Agreement being negotiated with _____ Regional Office.

☒ No DHHS Agreement, but rate established with Office of Naval Research Date 06/14/01

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information. Supplying the following information on F&A costs is optional for for-profit organizations.)*

a. Initial budget period:	Amount of base $	175,000	x Rate applied	41.00	% = F&A costs	$	71.750
b. 02 year	Amount of base $	175,000	x Rate applied	41.00	% = F&A costs	$	71.750
c. 03 year	Amount of base $	175.000	x Rate applied	41.00	% = F&A costs	$	71.750
d. 04 year	Amount of base $	175.000	x Rate applied	41.00	% = F&A costs	$	71.750
e. 05 year	Amount of base $		x Rate applied		% = F&A costs	$	
					TOTAL F&A Costs	$	287.000

*Check appropriate box(es):

☐ Salary and wages base ☒ Modified total direct cost base ☐ Other base *(Explain)*

☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.)*:

4. SMOKE-FREE WORKPLACE ☒ Yes ☐ No *(The response to this question has no impact on the review or funding of this application.)*

APPENDIX - LIST OF CONTENTS

APPENDIX 1 Lloyd, Q. P., Kuhn, M. A. and Gay, C. V., Characterization of calcium translocation across the plasma membrane of primary osteoblasts using a lipophilic calcium-sensitive fluorescent dye, calcium-green C_{18}. J. Biol. Chem, 270:22445-22451, 1995.

APPENDIX 2 Troyan, M. B., Gilman, V. R. and Gay, C. V., Mitochondrial membrane potential changes in osteoblasts treated with parathyroid hormone and estradiol. Exp. Cell Res. 233:274-280, 1997.

APPENDIX 3 Stains, J. P. and Gay, C. V., Asymmetric distribution of functional sodium-calcium exchanger in primary osteoblasts. J. Bone Miner. Res. 13:1862-1869,1998.

APPENDIX 4 Luan, Y-J., Praul, C. A. and Gay, C. V., Confocal imaging and timing of secretion of matrix proteins by osteoblasts derived from avian long bone. Comp. Biochem. Physiol. 126:213-221, 2000.

APPENDIX 5 Armen, T. A. and Gay, C. V., Simultaneous detection and functional response of testosterone and estradiol receptors in osteoblast plasma membranes. J. Cell. Biochem. 79:620-627, 2000.

APPENDIX 6 Weber, J. A. and Gay, C. V., Expression of translation initiation factor IF2 is regulated during osteoblast differentiation. J. Cell. Biochem. 81:700-714, 2001.

APPENDIX 7 Stains, J. P. and Gay, C. V., Inhibition of Na^+/Ca^{2+} exchange with KB-R7943 or bepridil diminishes mineral deposition by osteoblasts. J. Bone Miner. Res. 16:1434-1443, 2001.

APPENDIX 8 Stains, J. P., Weber, J. A. and Gay, C. V., Expression of Na^+/Ca^{2+} exchanger isoforms (NCX1 and NCX3) and plasma membrane Ca^{2+} ATPase during osteoblast differentiation. J. Cell. Biochem., in press, 2001.

PENNSTATE

Department of Biochemistry
and Molecular Biology
Eberly College of Science

The Pennsylvania State University
Paul M. Althouse Laboratory
University Park, PA 16802-4500

(814) 865-5497
FAX: (814) 863-7024

February 6, 2002

Dr. Priscilla B. Chen, SRA
Center for Scientific Review
6701 Rockledge Drive
Rm 4104, MSC 7814
Bethesda, MD 20892

RE: Application # 2 R01 DE09459-09

Dear Dr. Chen:

When submitting my application for the November 1 deadline, I didn't provide a statement for the research support of my collaborator, Dr. Roland Leach. His current research support is provided through his endowed professorship. This provides, in addition to salary, $30,000 per year for research supplies. A graduate assistantship is provided by the Department of Poultry Science and technical support is provided by the College of Agricultural Sciences. Pending support is as follows:

USDA/BARD 07/01/02 - 06/30/08 $50,000 per year
"Disordered Angiogenesis in Tibial Dyschondroplasia: The Role of Dithiocarbamates"
The goal is to identify causes of TD.

Best wishes,

Carol V. Gay, Ph.D.
Professor of Cell Biology

CVG:ekm

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