

Ninth Annual Research Report

Frederick S. Kaplan, M.D. and Eileen M. Shore, P.h.D.

Nineteen ninety-nine was a year of important developments for FOP research. Advances were made in the fields of genetic mapping, molecular genetics, gene regulation, immunology, cell biology, developmental biology and advanced therapeutics. Here we describe the progress and developments in FOP research during the past year.

Role of Noggin

Noggin does not appear to be the gene that causes FOP, but may become an important therapy for treating FOP.

Promising Candidate Genes

Data from laboratory and clinical studies have identified several promising candidate genes as the cause of FOP. The candidates include genes that are involved in bone morphogenetic protein (BMP) pathways as well as those that control skeletal and bone marrow development, immune function and/or inflammation. One such gene, critical in the regulation of BMP function, is noggin.

History of Noggin Research

Data from laboratory and clinical studies have identified several promising candidate genes as the cause of FOP. The candidates include genes that are involved in bone morphogenetic protein (BMP) pathways as well as those that control skeletal and bone marrow development, immune function and/or inflammation. One such gene, critical in the regulation of BMP function, is noggin.

In order to better understand the significance of noggin in the FOP story, we will turn the clock back several years. In 1996, studies in the FOP laboratory showed that bone morphogenetic protein 4 (BMP4), a powerful bone-inducing hormone, was overproduced in lymphocytes (cells involved in both the blood and immune systems) and lesional cells of patients who have FOP. At exactly the same time, Richard Harland, a colleague and collaborator from the University of California-Berkeley and the scientist who discovered the noggin gene, had just discovered the function of the noggin protein. Noggin avidly binds to BMP4 like a magnet and inactivates it.

Dr. Harland's Discovery

Dr. Harland showed that animals genetically engineered to develop without any noggin protein had an overabundance of BMP4 and formed excessive amounts of bone during development. These and other findings suggested immediately that the noggin gene may play an important role in the FOP story and that the noggin protein may become important in eventually treating FOP.

Noggin Studies and FOP

As these dramatic new insights emerged, we conducted studies to determine if mutations in the noggin gene were the cause of the overproduction of BMP4. However, we did not find any mutations in noggin after examining a number of FOP patients. Genetic linkage data (using markers available from the human genome

project) indicated that the noggin gene was not the most likely cause of FOP, although there was still a strong indication that it could be playing a role in the process.

Detailed Investigation of Noggin Gene

In 1999, prompted by the discovery of noggin mutations in two other rare human disorders of bone formation, we revisited the noggin gene and thoroughly investigated a wider array of DNA samples from FOP patients in search of possible mutations. We sequenced the noggin gene using more advanced techniques not available to us even several years ago, but again found no mutations in noggin in FOP.

We learned in early December, through one of our patient-members in France, that a French geneticist, self-proclaimed French nationalist and independent researcher from Reims, France (whose work is sponsored by the Pierre Yves Association) claimed to have found mutations in the noggin gene in FOP patients from France. This claim was described in a "Letter to the Editor" of the journal *Clinical Genetics* in December 1999.

An Unverified Claim

The French geneticist had informed us during the summer of 1999 that he had identified mutations that could cause FOP but refused to disclose the identity of the gene or any other salient details. He refused to share any relevant information with the scientific community, but later announced his findings to patients, families and the press in France.

We reported to the FOP community in December 1999, immediately upon obtaining documentation of this claim, that the finding was, as yet, unverified by any independent source. We felt strongly that the claim warranted detailed evaluation, which we and our esteemed colleagues in Paris undertook immediately. Any scientific discovery of potentially great importance must be verified by independent investigations.

Testing the Claim

In order to determine the veracity of the claim, we investigated the possibility of noggin mutations once again with even more detailed experimentation on the numerous blood samples that patients have so graciously provided to us. We told the FOP community in December 1999 that we were in a position to check this rapidly and thoroughly with intense scrutiny on a very wide array of blood samples obtained at various times during the FOP process, and we promised to keep everyone well-informed of our progress in this matter. We now have substantial additional information to share.

Linkage Studies

First, elevated levels of BMP4 in cells of patients with FOP, along with a lack of identifiable mutations in the BMP4 gene, suggested that the mutation in FOP may reside in a BMP4 inhibitor. The secreted protein noggin is a powerful antagonist of BMP4, a prime regulator of BMP4 concentrations and a critical factor in human joint formation. A recent article reported a heterozygous mutation (a mutation in one of the two copies of a gene) in noggin in a patient with FOP. In order to determine if heterozygous mutations in noggin were the cause of FOP, we performed a

comprehensive mutational analysis in a large worldwide sample of FOP patients. In addition, we conducted a detailed linkage analysis (gene localization analysis) in four classically affected multigenerational FOP families using an array of highly informative genetic markers that are closely linked to the noggin gene.

These studies were undertaken with our esteemed colleagues and collaborators at The Association Française Contre Les Myopathies (Paris, France), Oxford University (Oxford, England), The University of Glasgow (Glasgow, Scotland) and The National Institutes of Health (Bethesda, Maryland). The data from these very powerful studies showed conclusively that FOP was not linked to the noggin gene in three of the four multigenerational families. Although the analysis could not exclude the possibility that one family might be linked to the noggin locus, sequencing of the noggin gene in all affected members of that family failed to reveal any mutations in the gene.

Noggin is not the FOP Gene

To confirm our findings, we sequenced the noggin gene in affected members of all four families, as well as in more than 25 patients with sporadic FOP worldwide, and failed to detect any mutations in the noggin gene. In order to exclude the remote possibility of a mutation of the noggin gene in a small subset of cells (mosaicism), we sequenced numerous subclones of the noggin gene from a given patient (using blood specimens obtained during severe flare-ups of FOP), but again found no disease-causing mutations. Thus, our findings of the past several months strongly indicate that noggin is not the FOP gene, at least in the vast majority of affected individuals worldwide.

These results are further supported by the recently published genome-wide linkage analysis (published in January 2000 by the FOP Collaborative Research Group) that located the FOP gene to the long arm of human chromosome 4, a site that excludes the noggin gene or any currently mapped BMP antagonist.

Importance of Independent Verification

The results of our extensive studies are in sharp contrast to the claim of disease-causing heterozygous mutations in the noggin gene in patients with FOP by the French doctor. Just last week, a member of the collaborative research group saw and examined the one patient with the reported mutation, and the patient's DNA is currently being processed for independent mutational screening. We will, of course, report to you on the progress of these and related efforts as soon as more information is available.

Although the French doctor firmly believes that noggin is the FOP gene, our data currently suggest otherwise. We have found no evidence for mutations in the noggin gene and no evidence for linkage of FOP to the noggin locus. Furthermore, a careful analysis of the published data from the French doctor raises the possibility of alternate interpretations of his data.

As a Nobel Laureate in Medicine said recently, "Science is a method whereby a notion proffered by anyone must be supported by experimental data. This means if someone is interested in checking on the notion presented, that person must be allowed access to instructions on how the original experiments were done. Then he can check things out for himself. It is not allowable in science to make a statement of fact based solely on your opinion."

Candidate Genes

The FOP gene has been localized. Several important candidate genes on human chromosome 4 have been excluded from consideration as the FOP gene.

Looking for the FOP Gene

We reported last year that the FOP gene appeared to be localized to a small region on the long arm of human chromosome 4 based upon a genome-wide linkage analysis in four multigenerational families. This work was published in January 2000 in the prestigious American Journal of Human Genetics.

Several promising candidate genes for FOP have been identified within the linkage region on chromosome 4. During the past year we have performed extensive screening of several of these FOP candidate genes. Detailed mutational analysis of these candidates has failed to uncover any mutations, but several additional candidate genes within the linkage interval are currently being examined with intense scrutiny.

Human Genome Project

The human genome project will provide a bounty of new data for gene identification.

How This Helps FOP Research

The FOP linkage region on chromosome 4 may contain more than 100 anonymous (unidentified) genes, and the FOP gene may be an as yet undescribed gene in that region. The first complete sequence of the human genome is anticipated later this year, a remarkable scientific accomplishment that will provide a bounty of data and insight into the conquest of human disease. The FOP Collaborative Research Project will likely be an immediate beneficiary of that milestone achievement of mankind. Data from the complete human genome sequence will enable us to determine the location of additional genes within the linkage region and whether these newly discovered genes should be considered in our FOP search.

BMP4-Key to the Puzzle?

Differences in the molecular and physiologic relationship between BMP4 and BMP4 receptors in FOP patients and normal controls may provide a key to the puzzle of FOP.

Role of BMP4 in FOP

Hormones and hormone-like substances are molecular signals that are produced in a particular cell, and then secreted from the cell. After they are secreted, they may act on the same cell from which they were secreted (autocrine factor), on a cell in the local environment (paracrine factor), or on a cell in a distant tissue or organ (hormone). In each case the factor must bind to a receptor on (or in) the target cell in order for the signal to be transmitted to the nucleus of the cell where genes are turned-on or turned-off. BMP4 can act in each of these three capacities depending upon its developmental and physiologic context.

Our preliminary studies indicate that all key molecules of the BMP4 signaling pathway are present in lymphoblastoid cells. In FOP, the level of BMP4 messenger RNA is dramatically elevated in the lymphocyte cell lines of FOP patients, however the level of BMP4 receptor RNA is the same in FOP patients and in normal controls. One possible interpretation of these findings is that the numbers of BMP4 receptors is normal, but at an inappropriate ratio compared to the level of BMP4 in FOP. Messenger RNA is a transient and short-lived intermediary between the gene and the protein. We are conducting studies to further examine the genetic and molecular circuits that regulate the protein levels of BMP4 and its receptors in FOP and normal cells. Such detailed knowledge is enormously important not only in identifying the FOP gene but also in identifying the relevant pathways that lead to disease activation in an FOP flare-up.

BMP4 and the FOP Gene

The gene encoding one of the BMP4 receptors is also a candidate gene for FOP, as it is located near the linkage region ("the one mile stretch") on human chromosome 4 where the FOP gene has been localized. A mutation that inappropriately activated a BMP4 receptor could cause many of the clinical features of FOP and would be consistent with the elevated levels of BMP4 that we have found. Several collaborators have recently published important papers on the function of the BMP receptors in animal models. In their studies, they selectively eliminated BMP receptors from the cells of growing mouse embryos and demonstrated that BMP receptors have important functions in the formation of the skeleton, especially the fingers and the toes.

Work is on-going in our laboratory to explore the relationships between BMP4 and its receptors as well as the relationship between the hormone-receptor complexes and the downstream molecules (called SMADs) that transmit the BMP signal. BMP4 activates BMP receptors which activate downstream SMADs and allow them to move into the nucleus to "turn on" and "turn off" genes in the BMP4 pathway. SMAD molecules have gained a great deal of attention recently not only because of their importance to bone formation, but also because of their central role in the molecular biology of cancer. This is a yet another example of how research in FOP can also enhance our understanding of more common diseases such as cancer.

New Technology Helps Research

Using a revolutionary new technology, we are able to examine and compare the expression profile of thousands of genes in FOP cells to the expression profile of the same genes in the cells of unaffected individuals.

Microarrays Help in Profiling Gene Expression

By comparing the activity of thousands of genes in the cells of an FOP patient with the same genes in the cells of a person who does not have FOP (and also in patients with active flare-ups vs. those with disease quiescence), we will better understand the complex molecular circuits involved in FOP. DNA "microarrays" enable us to profile the gene expression pattern of many thousands of genes in a single experiment. While the human genome project will produce an inventory of all genes used to assemble a living human being, the interrelationship and pattern of expression of these genes in health and disease is essential for understanding complex diseases like FOP. As one investigator said, "The traditional gene-by-gene approach will not suffice to meet the sheer magnitude of the problem. It will be

necessary to take "global views" of biological processes; simultaneous read-outs of all components. Microarrays offer the first great hope for such global views of genetic variation. It seems that they will become a standard tool of both molecular biology research and clinical diagnostic research."

The use of microarray technology is in its infancy but will allow us to better define the complex "wiring diagram" of BMP4 signaling that triggers the explosive bone formation of FOP. As we compare the genes that are turned on immediately before and immediately after a flare-up begins, we may better understand the triggering mechanism of the explosive bone formation. Since many genes are involved in this process, the microarray technology will be essential in understanding the timing sequence by which these genes are activated. Knowledge of the genes and proteins that control the BMP4 switch in FOP will be critical in effectively preventing "the bomb" from exploding.

Molecular Switch the Controls FOP

The on-off switch of the human BMP4 gene is elaborate and complex. FOP patients may lack a protein that turns the switch off.

FOP Cells and BMP Activity

The promoter (switch) of the BMP4 gene is directly attached to the portion of the gene that encodes the BMP4 protein. The BMP4 promoter is a complex molecular switch that regulates BMP4 gene activation in different ways at different times in different cells. Recent data suggest that the FOP cells may be lacking a protein or proteins that turn off the BMP4 switch. The nature of this protein or protein-complex is the subject of intense investigation in the FOP laboratory. The preliminary findings of this novel work were presented in an award-winning poster at the Annual Meeting of the American Society for Bone and Mineral Research in St. Louis, Missouri in October 1999 and at a special meeting of The Howard Hughes Medical Institute in Washington, D.C.

Role of Mast Cells

Inflammatory mast cells (cellular time bombs residing in connective tissue) are intimately involved in flare-ups of FOP.

Early Flare-up Process

Two of the most puzzling features of FOP are the intense muscle edema (swelling), fibroproliferation, and angiogenesis (new blood vessel formation) characteristic of early pre-osseous (pre-bony) FOP lesions and the rapid spread of the lesion into adjacent tissue. As most patients and families know, these lesions may appear within hours and can reach an alarming size literally overnight. The sudden appearance and rapid spread of these lesions suggest involvement of an armada of inflammatory mediators along with an abnormal connective tissue wound response and points to a potential role for inflammatory mast cells in the the disease process.

What are Mast Cells?

What are mast cells? Mast cells are indigenous cells in the body's connective tissues and arise from the bone marrow. They circulate through the blood as committed but undifferentiated cells and migrate into numerous tissues including skeletal muscle

where they mature and reside as harmless bystanders until provoked by a traumatic or inflammatory stimulus. Mast cells are found in close proximity to blood vessels and nerves. In normal skeletal muscle, mast cells are found very sparsely distributed in the connective tissues between the muscle bundles. Mast cells are conventional bombs (not atom bombs) that contain granules of very potent stored chemicals that induce edema, fibroproliferation and angiogenesis (new vessel formation) when the granules are released into the surrounding tissue.

Role of Mast Cells in Wound Response

For many years, the role of mast cells was unknown, but it now appears that they play an important role in tissue repair and wound response. When mast cell recruitment and activation goes awry, the process can lead to severe inflammatory reactions such as asthma and hives. This is true with mast cell activation in the skin and lungs. However, very little is known about mast cells in the deeper tissues of the body such as the skeletal muscles. Mast cells are not easily visible under the microscope unless special stains are used to detect them. Mast cells are simulated by a myriad of different external and internal influences such as immune responses and tissue injury.

What Mast Cells Do

Mast cells contain granules whose sequestered contents include histamine, heparin, angiogenic proteins (proteins that attract and stimulate the formation of new blood vessels) and matrix-degrading enzymes that allow injured tissue to repair itself. Potent angiogenic proteins released by mast cells include basic fibroblast growth factor, vascular endothelial growth factor, and transforming growth factor beta. Mast cells also release a litany of inflammation-causing molecules including tumor necrosis factor alpha, prostaglandins, and leukotrienes. Upon release from the mast cells, these substances influence a vast array of biological processes including inflammation, immune function, angiogenesis, fibrous tissue formation, extracellular tissue remodeling, and tissue repair. Mast cells are also hijacked by invading tumors. Mast cells accumulate at the leading edge of invading tumors where they are conscripted for angio-genesis and local tumor invasion, but mast cells are not found in the core of the invading tumors.

New FOP Studies

The intense muscle edema, fibroproliferation, and angiogenesis characteristic of early pre-osseous FOP lesions and the rapid spread of these lesions along muscle planes into adjacent tissue suggested a potential role for mast cells in the FOP process. As little is known about the resident mast cells in skeletal muscle, we undertook a comprehensive analysis of mast cell distribution in normal skeletal muscle, in uninvolved FOP muscle, in FOP lesions, in inflammatory and genetic muscle diseases, and in experimentally-induced animal models of heterotopic ossification.

The findings of our studies were startling and unexpected. We found mobilization and activation of inflammatory mast cells at all stages of FOP lesional development. Mast cells have long been known to be involved in bone maintenance, but this is the first study to implicate their involvement in newly forming bone. The data from our study document an important role for mast cells in the pathology of FOP lesions.

New Hypothesis

We have developed the following hypothesis based on our observations and

experimental data. Tissue injury in patients with FOP leads to lymphocyte migration into normally appearing skeletal muscle. Some of these lymphocytes overproduce BMP4 and appear to lead to mast cell mobilization, a finding which is supported strongly by the FOP pathology and by our experimental models of heterotopic ossification using recombinant BMP. Mediators released by mast cells stimulate a cycle of inflammatory edema, fibrosis, and angiogenesis, which is potentiated at the leading edge of an advancing FOP lesion. Reactive fibroblasts within the muscle tissue produce proteins which lead to further proliferation of mast cells and a self-sustaining escalation of the disease process known as a flare-up. Eventually, transforming growth factor beta, released by mast cells and other lesional cells, limits the lymphocytic recruitment and migration and thus the size and extent of the expanding lesion, while endogenous overexpression of BMP4 in the fibroproliferative core drives the fibroproliferative lesion towards ossification through an endochondral pathway.

The observation of mast cell mobilization in FOP lesions provides a novel and previously unrecognized opportunity to evaluate anti-mast cell therapies in limiting the spread of FOP lesions. Studies are underway in animal models of genetically inhibited mast cell activity to assess that possibility.

Chain Reaction

Fibroproliferative cells in the very early FOP lesion arise from within skeletal muscle tissue but produce smooth muscle proteins.

Molecular Signals Lead to Explosive Bone Growth

It is essential to identify not only the molecular signals that trigger the explosive bone formation in FOP, but also the cells that receive and propagate the signal in the "chain reaction" that leads to heterotopic ossification.

We reported last year that a cell within muscle tissue, but a non-skeletal muscle cell itself (possibly one residing near or around a small blood vessel), is involved in the formation of the early lesion. Our recent studies, in collaboration with colleagues at Cambridge University, have focused on identifying the molecular signature of the responding cell. The preliminary results from these ongoing studies suggest that the fibroproliferative cells in the early FOP lesion express smooth muscle proteins, similar to the proteins expressed in the cells that surround blood vessels.

The results so far suggest that excessive BMP4 may stimulate the cells surrounding the blood vessels (the smooth muscle pericytes) to begin an uncontrolled fibroproliferative response. While the exact details of the triggering mechanism will require a great deal of additional work, the identification of important players such as BMP4, lymphocytes, mast cells, and perivascular cells helps to narrow the molecular and cellular stage on which the FOP process explodes. The continued integration of vital information from genetic, molecular, and cellular studies will enable us to decipher the events involved in the cascade of explosive bone formation in FOP.

POH-A Sister Disease to FOP

We have discovered many new mutations in the gene responsible for progressive osseous heteroplasia (POH), the sister-disease to FOP.

How POH Relates to FOP

We discovered and named POH in 1994 and described it as a distinct disorder of heterotopic ossification in human beings. Children with POH were originally thought to have FOP. After seeing a number of these children, we clearly recognized a very different condition than FOP. Although bone forms in the deep skeletal muscles in POH, children with POH also have bone formation in the skin, which is a feature not seen in FOP. In late 1998, we discovered the gene responsible for the condition. We described the discovery of the POH gene in our Annual Report last year. During the past year, we have identified more than 20 affected patients with POH who have various mutations in the disease-causing gene. The gene was discovered after seeing several patients whose symptoms provided a clue to a plausible candidate gene. Examination of that candidate gene in the POH patients revealed disease-causing mutations. Currently, we are investigating this gene in all possible patients with POH worldwide.

Finding the POH Gene

It was an enormous thrill to identify the gene for POH—this discovery will enable us to study how this gene leads to ectopic bone formation, a necessary step in effectively treating and eventually curing POH. The POH gene is called *GNAS1* and is located on the long arm of human chromosome 20. The structure of the gene and the regulation of its expression are extraordinarily complex. *GNAS1* encodes a protein called Gs-alpha which is located on the inside of the cell membrane in nearly every cell in the body. The protein is versatile and appears to have different functions in different cells. Generally, Gs-alpha functions as a component in a three-part relay switch that is linked to membrane-spanning receptor molecules that monitor the environment of the cell and sends signals to the nucleus (the site of the chromosomes), instructing the cell not only what to do, but exactly what type of cell to become.

An enormous amount of additional research is necessary to understand exactly how mutations in the *GNAS1* gene (and the corresponding abnormalities in the Gs-alpha protein) trigger ectopic bone formation. Early indications are that Gs-alpha protein normally acts as an inhibitor of bone formation in soft connective tissue (skin, fat, and skeletal muscle). When the G-protein relay switch is broken, the inhibition ceases, and the cell becomes a bone cell by default. In children who have POH, bone formation occurs in the skin and in the fat tissue underneath the skin and then progresses into deeper tissue such as muscle, tendon and ligament. In POH, bone formation occurs directly rather than through a cartilaginous precursor as in FOP.

POH and Other Diseases

POH can be as disabling as FOP depending on the extent of ectopic bone formation. Additional data suggests that part of the bone-inducing pathway that is mis-activated in POH is also involved in FOP, although the genes that cause the two conditions are different. It is interesting and important to note that the *GNAS1* gene that is damaged in POH is also damaged in several other genetic bone diseases including fibrous dysplasia (or McCune Albright's Syndrome and its variants), Albright Hereditary Osteodystrophy (AHO), and pseudohypoparathyroidism (PHP).

The discovery of the POH gene in late 1998 and its further elucidation during this past year is an important development in bone biology and of paramount importance for understanding the earliest cellular and molecular pathways in bone formation. Identification of the POH gene has profound implications for developing medications

to treat POH, for the study of FOP, and also for the study and conquest of many more common diseases of bone formation. It is also a spectacular example of how the candidate gene approach ("best guess approach") can sometimes pinpoint a disease-causing gene without requiring detailed family studies. Several multigenerational families with POH have been identified worldwide, and will be extremely helpful in deciphering the genetic pathways for POH. The attention that we received from the FOP documentaries (ABC and BBC) enabled us to discover more patients with FOP and POH, a development that has been instrumental to the research progress in both conditions.

Current Studies

At the present time, we are completing mutational studies on all known POH patients, and are in the process of writing this important discovery for the peer-reviewed scientific and medical literature. Within the next year or so, we anticipate that as many as four additional major papers on POH will be published in the medical and scientific literature. We would also like to announce that our lab will be the recipient of a prestigious National Institutes of Health grant on POH, just as it has been for FOP. The initial description of the genetic mutations in POH was presented at the 21st Annual Meeting of the American Society for Bone and Mineral Research in St. Louis, Missouri in October 1999. You will be hearing much more about POH at the symposium in November 2000.

Squalamine

The cellular and molecular mechanisms of squalamine action have been elucidated.

Squalamine as a Potential Treatment for FOP

Squalamine was discovered in 1992 in the FOP laboratory by Dr. Michael Zasloff. Dr. Zasloff isolated squalamine from the body tissues of the dogfish shark. Squalamine is a naturally occurring cholesterol-like molecule that inhibits the proliferation of endothelial cells (blood vessel cells) and exhibits potent-antiangiogenic (blood vessel inhibiting) activity in laboratory animals and in humans. During the past year the cellular mechanism of action of squalamine has been elucidated. Squalamine modifies the response of endothelial cells (small blood vessel cells) to proteins that organize their structure and shape. This work, conducted by Dr. Zasloff, has been submitted for publication in a major peer-reviewed scientific journal.

Role of Blood Vessels in FOP and Cancer

New blood vessels are formed during tissue growth and repair, during bone formation, during the normal female reproductive cycle, and during the development of the fetus. Endothelial cells, the cells that form the blood vessels, have a remarkable ability to divide and migrate. Angiogenesis, the process of forming new blood vessels from endothelial cells, is critical for tissue growth and repair, and is hijacked for the progression of disorders like cancer and FOP. In cancerous tissue, tumors cannot grow or spread (metastasize) without the development of new blood vessels. In FOP, new blood vessel formation is a prominent part of the early FOP lesion. Blood vessels are necessary for bone formation. The goal of anti-angiogenic therapy in FOP is to inhibit new blood vessel formation in order to slow down or inhibit the subsequent production of new bone formation.

Squalamine is currently produced synthetically under sterile conditions and does not have to be obtained from sharks. In pre-clinical studies, squalamine has been shown to inhibit angiogenesis and the subsequent growth of solid tumors.

Antio-angiogenetic Drugs and Cancer Treatments

Numerous anti-angiogenic drugs are now in clinical trials for the treatment of cancer. Many of these drugs were designed to target specific molecules involved in blood vessel formation while other drugs were designed to inhibit endothelial cell function. In general, four strategies are currently being used to inhibit angiogenesis:

- Inhibit endothelial cells directly
- Block factors that stimulate endothelial cells
- Block the ability of endothelial cells to invade surrounding tissues
- Block tissue-recognition molecules on the surface of endothelial cells

Squalamine inhibits these cells directly.

Squalamine and Cancer

Phase I human safety trials of squalamine have been successfully completed in patients with cancer.

Understanding How Squalamine Works

During the past year, tremendous progress has been made in understanding the safety profile for squalamine, in understanding its molecular mechanisms of action, and in deciphering the role and timing of blood vessel formation in the early stages of BMP-induced bone formation. Pre-clinical and clinical research conducted at Johns Hopkins Medical School, the Institute for Drug Development, the Dana Farber Cancer Institute, the University of Wisconsin, the University of Virginia, Georgetown University and UCLA have demonstrated that anti-angiogenic approaches using squalamine have application across many cancer types and in other angiogenic diseases. Comprehensive data on the safety of squalamine in humans, as well as comprehensive biochemical data on the identification, location and distribution of squalamine in endothelial cells, enables us to proceed to the next step in designing clinical protocols for testing the safety of squalamine in patients with FOP.

Squalamine and FOP

Magainin Pharmaceuticals Announces A New Clinical Program in Fibrodysplasia Ossificans Progressiva (FOP) for the Angiogenesis Inhibitor Squalamine.

Clinical Trial for FOP

Magainin Pharmaceuticals announced (February 22, 2000) a new clinical program in fibrodysplasia ossificans progressiva (FOP) for the angiogenesis inhibitor squalamine. Magainin plans to file an investigational new drug (IND) application with the Food and Drug Administration (FDA) and begin Phase I human safety and efficacy testing in patients with FOP in the second half of 2000.

Dr. Kaplan will be the principal investigator for the study. Anti-angiogenic therapy may have important medical benefits not only in cancer, but also in other debilitating

conditions in which angiogenesis is an important part of the disease process. Fibrodysplasia ossificans progressiva (FOP) is one of these conditions. Our research, conducted in collaboration with Dr. Judah Folkman, has shown that an angiogenic factor, basic fibroblast growth factor, is elevated during flare-ups of FOP. By directly blocking the angiogenic process, squalamine has the potential to inhibit the progression of the FOP lesions in muscle seen in FOP and prevent the muscle from replacement by bone. We are excited to have the opportunity to conduct this initial study designed to evaluate the safety and preliminary efficacy of squalamine in FOP.

Protocol for Clinical Trial

We anticipate that the squalamine clinical trials in FOP will be targeted to the treatment of early and severe pre-osseous flare-ups. The initial study will be designed to evaluate the safety and potential efficacy of intravenous squalamine on the inhibition of angiogenesis and will enroll no more than 10 patients with FOP. Data from the phase I Safety and Efficacy Trial will be used to design a larger controlled phase II Dose-Response Study, and finally a controlled double-blinded phase III efficacy study. We do not anticipate that the limited phase I study will begin before the end of 2000. The study will require full approval by the FDA as well as by the Institutional Review Boards of The Children's Hospital of Philadelphia and The University of Pennsylvania. As additional details of the study protocol become available, the information will be widely and rapidly disseminated throughout the FOP community.

How Clinical Trials Work

Phase I clinical trials represent the first studies in humans and are designed to collect data on dosage, timing and safety —but generally not efficacy—of an investigational treatment in a small number of subjects. By definition, therapy administered during phase I clinical trials cannot be considered to be a proven treatment, and therefore is unlikely to be of real benefit to the patient. The Phase I clinical trial typically evaluates how a drug is absorbed, metabolized, and distributed throughout the body. The Phase I clinical trial of squalamine will be designed to determine drug safety in patients with FOP as well as the ability to target blood vessels in the early pre-osseous lesions.

Phase II clinical trials are designed to provide continued evaluation of the safety of the treatment as well as its efficacy according to specific standards in a larger group of patients. Finally, Phase III clinical trials gather large-scale efficacy data from large numbers of patients to determine whether the treatment under study is better than the current standard of care. Phase III clinical trials are usually randomized and controlled.

Important Note

As we embark upon the Phase I clinical trials, it is important to remember that we have no absolute proof that squalamine will be effective in FOP. We are, however, greatly encouraged by the recent discoveries in the laboratory that enable us to proceed with the testing of squalamine, a new pharmaceutical agent that has a rationale basis for therapeutic application in FOP.

Thalidomide

The Phase I safety-efficacy trial of thalidomide for active flare-ups of FOP continues to enroll patients.

Clinical Trial for FOP

Thalidomide is an inhibitor of fibroblast growth factor-induced blood vessel formation. Basic fibroblast growth factor is overproduced during FOP flare-ups. In addition, thalidomide is an immune-modulator (affects cells of the immune system) and may play a role in the very early FOP lesions where circulating lymphocytes attack the body's skeletal muscles and replace them with bone. Safety data from this limited Phase I study indicates that thalidomide is generally well-tolerated in patients with FOP. The preliminary efficacy data is currently being evaluated and will be presented by Dr. Deanna Mitchell, the study's principal investigator, at the Third International Symposium on FOP in November 2000.

Pre-clinical Research on Noggin

Noggin vectors are produced for pre-clinical gene therapy trials in FOP.

Noggin Inhibits BMP-Produced Bone

The importance of noggin to the FOP story became clear in 1996 after the BMP4 discovery, and we brought noggin to the forefront of development for FOP treatment. Noggin is involved in controlling the amount of skeleton and bone that is formed by regulating the concentrations of BMP4 available in the body's tissue. For the same reason, noggin offers promise for controlling the rampant bone growth of FOP. Although genes for noggin and BMP4 do not appear to be damaged in FOP, the results of pre-clinical studies prove dramatically that noggin can effectively inhibit BMP-induced heterotopic bone formation.

Noggin Gene Therapy

Work on noggin gene therapy has continued at a brisk pace with the development of noggin vectors for experimental testing in animal models of heterotopic ossification. Critical to the development of gene therapy for FOP is the development of methods for regulating noggin gene expression. Work has focused on the development of a novel delivery system. Preliminary data from animal experiments during this past year are quite encouraging. Vectors are being created to drive noggin expression from constitutive and inducible promoters and are being provided to collaborators at Regeneron Pharmaceuticals, who are studying their function in animal models. If pre-clinical animal safety data are satisfactory, we will continue to develop this therapy for human clinical trials.

Gene Therapy and New Technology

Gene therapy is a powerful new technology that enables the production and delivery of a therapeutic protein under pharmacologic control by transferring extra copies of the desired gene to the body for production. Recently, gene therapy has had some very difficult technical and regulatory problems. However, the successful delivery of a therapeutic protein following gene transfer is our best hope for the future. National regulatory boards in conjunction with human gene therapy institutes will establish the safest monitoring protocols possible for this pioneering therapy of human disease.

Technical Problems

Many of the current technical problems with gene therapy are due to the failure to achieve long-term gene expression with early viral vector systems, a critical requirement for correcting many inborn genetic defects. However, with the advent of adeno-associated viral (AAV) vectors, which demonstrate persistent gene expression in animal studies, this technological barrier is beginning to be overcome. These new vectors, which are very different from the old adeno-viral vectors (although the names sound similar), will form the basis for gene therapy protocols for many genetic diseases, including FOP. The toxicity of most gene therapy protocols is due generally not to the introduction of the therapeutic gene, but to the adenoviral vector system. Investigations using the new vector systems show great promise of fewer complications.

Regulation of Gene Therapy

Legislation to more stringently regulate federally-funded human gene therapy trials was recently introduced in the United States Congress. Human gene therapy trials have come under increased scrutiny by Federal regulators during the past year at every institution involved in human gene therapy trials. A gene therapy-associated death in 1999 has prompted a temporary halt in many human gene therapy studies. However, this has not impaired the progress of critically important pre-clinical research. It is very clear that many vital scientific and regulatory issues must be resolved before gene therapy can become good medicine. Nevertheless, recent setbacks in gene therapy shall not dampen enthusiasm for this most logical and vital approach to the treatment of genetic disorders.

Gene Therapy In Its Infancy

To reduce redundant effort and consequent cost, the FDA and the National Institutes of Health have proposed the initiation of "platform studies" for pre-clinical testing of vector toxicity with data collectively pooled in a publicly available database. This information would serve as a baseline for assessing a wide-range clinical applications of a given viral vector, such as adeno-associated virus. The FDA is considering modifications of its requirements so that investigators will need only to conduct toxicity testing for aspects of their protocol that are not addressed by existing data.

Our current approach, therefore, is to carefully monitor the ongoing national studies of adeno-associated viral vector toxicity, and to concentrate our efforts on evaluating the toxicity related to the noggin transgene in preclinical studies. It is important to remember that gene therapy is not just a new medication, but a new way of delivering vitally important medications for the treatment and cure of disease. An editorial in the January edition of *Nature Medicine* stated, "Like most medical interventions, gene therapy is potentially dangerous. It is also in its infancy." Thus, as we move forward into this bold new era, we must temper speed with safety and be constantly vigilant of the risks that any new drug or delivery system might impose. Despite all of the caveats recently raised about gene therapy, we remain extremely encouraged about the prospect of noggin gene therapy for the treatment of FOP.

The topic of noggin gene therapy for FOP will be a major focus of the therapy sessions at the Third International FOP Symposium in November. We anticipate an exciting and robust discussion of progress in this area by leading experts on the

developmental biology and pharmacology of noggin as well as by world experts in the field of gene therapy.

Center for Research in FOP

The Center for Research in FOP & Related Disorders expands research efforts beyond the core laboratories into related disciplines.

Developmental Grant Program

In this first full year of operation, the Center has funded three developmental grant protocols in widely divergent areas of scientific inquiry related to FOP.

One of the developmental grants focuses on the contribution of primitive muscle cells to BMP-induced ectopic bone formation in mice. The goal of the proposal is to assess the role of muscle precursor cells in BMP-induced heterotopic bone formation using an animal model. Substantial progress was made last year on the production of transgenic mice that express a novel muscle cell receptor under the control of a very powerful regulatory gene called myo-D. Five transgenic lines were produced and all five were susceptible to activation by the special receptor. Activation appears to be restricted to the muscle precursor cells, with fibroblasts and other muscle-associated cells remaining unaffected. This approach allows us to isolate the contribution of a single and specific cell type. Two transgenic lines have been selected for subsequent experiments which will determine the role of these genetically-tagged muscle cells in the formation of BMP-induced heterotopic ossification. This important experimental approach will allow us to determine whether skeletal muscle cells contribute to the heterotopic ossification process, or whether the dying muscle cells are simply replaced by heterotopic bone that forms from other connective tissue cells, perivascular cells, or smooth muscle cells within the skeletal muscle environment.

BMP-Activation and Inhibition

The second developmental grant project is studying the BMP signalling pathway. In contrast to the cell biology perspective of the previous project, this project is important in furthering our understanding of "the wiring diagram" of BMP-activation and inhibition, an essential feature of FOP since cells from FOP patients have been found to overexpress BMP4. This study has discovered a gene called SANE that inhibits BMP signalling and is examining the role of SANE in bone formation. A mouse "knock-out" of the SANE gene may form heterotopic bone and provide an animal model for FOP. A clear understanding of the steps through which BMP4 acts on cells to regulate the expression of sets of genes that promote bone formation will provide valuable information in the development of pharmacological approaches of controlling bone formation.

The third developmental grant project involves the production of noggin gene therapy vectors for animal testing, a requisite feature of the pre-clinical studies necessary for eventual noggin gene therapy in FOP. The rationale for this approach was described above.

Expanding Research Efforts

A major function of The Center for Research in FOP and Related Disorders is to foster FOP research by investigators at The University of Pennsylvania who work in disciplines that complement those of the core laboratory. There has been exciting

progress during this first year. The Center is currently soliciting and evaluating intramural applications for 2000 from programs in molecular biology, cell biology, developmental biology and immunology related to our primary mission of finding the cause and establishing a cure for FOP.

In addition the Center is actively recruiting highly motivated postdoctoral fellows (M.D. and/or Ph.D.) with strong backgrounds in molecular biology and cell biology.

We are grateful to Susan Lippo, the Center Administrator, who provides much needed administrative support for all of the activities of the core laboratory as well as the developmental grants program. We are grateful to Kay Rai, Dr. Kaplan's secretary, who plays an instrumental role in bridging the daily and often urgent concerns of the clinical world of FOP with the vital pioneering activities of the FOP laboratory.

Meetings and Publications

During 1999 major presentations of FOP research were made by members of the FOP Laboratory at many locations.

Presentations included:

- The National Institutes of Health; Bethesda, Maryland
- The National Institute of Dental Research; Bethesda, Maryland
- The Johns Hopkins University School of Medicine; Baltimore, Maryland
- The Howard Hughes Medical Institute; Washington, D.C.
- The American Society for Bone and Mineral Research; St. Louis, Missouri
- Oregon Health Sciences University; Portland, Oregon
- St. Lukes Medical Center; Milwaukee, Wisconsin
- The Marshfield Clinic; Marshfield, Wisconsin
- The University of Bristol; Bristol, England
- The University of Aberdeen; Aberdeen, Scotland
- Charles University; Prague, Czech Republic
- FOPE.V.; Valbert, Germany, and Garmisch-Partenkirchen, Germany

These lectures exposed FOP to a wide audience of scientists from all over the world.

Invited Honorary Scientific Lectures on FOP included:

- The Robert A. Robinson Memorial Lecture in Orthopaedic Surgery - The Johns Hopkins University School of Medicine; Baltimore, Maryland.
- The Ann Stansfield Memorial Lecture - Bristol University; Bristol, England
- The Marshall R. Urist Distinguished Lecture - Oregon Health Sciences University; Portland, Oregon
- The Sir John Charnley Award Lecture - The Arthritis Foundation; Philadelphia, Pennsylvania
- The Distinguished Visiting Scientist Lecture - The Czech Society of Rheumatology; Prague, Czech Republic

During the past year, nine research articles, chapters and reviews on FOP were published by members of The FOP Research Laboratory. Four major papers on FOP

and POH were presented at The American Society for Bone and Mineral Research in St. Louis, Missouri in October 1999.

In addition to the numerous invited scientific presentations and peer-reviewed scientific articles, work from The FOP Laboratory was featured in two internationally-televised programs: The BBC Horizon documentary "The Skeleton Key" and an ABC News 20/20 segment. Work in the research lab was also graciously mentioned in Carol Whelan's beautiful article in Newsweek.

FOP Laboratory

University of Pennsylvania

The current staff of the FOP research laboratory includes 17 researchers: 2 principal investigators, 4 research specialists, 4 post doctoral associates, 1 graduate student, 3 medical students, and 3 premedical students. The FOP Laboratory occupies approximately 2,000 square feet of space, nearly double its size from two years ago. The pictures of our FOP and POH children adorn the hallways and are a constant reminder of our goals and our mission. As we tell the children and adults who visit the laboratory, this is really their laboratory. We love when they come and visit!

Cause and Cure

Making Progress

We are thankful that dramatic progress continues to be made in understanding the genetic, molecular, cellular and physiologic basis of FOP. We are proud that discoveries made in the laboratory are being translated into clinical protocols and new drug therapies that can be tested in a stringent scientific fashion. Cause and cure are the two words that continue to propel all of our efforts and provide the guiding principle for all that we do: to discover the exact molecular cause of FOP and to use that knowledge to develop timely therapies that will be truly effective in preventing, treating and curing FOP.

The year 1999 was a year of important developments for FOP. We are hopeful that 2000 will be a year of even greater milestones in FOP research. It is amazing how far the FOP community has come in nine years, but we are constantly reminded that we have a long way yet to go.

We are all extremely excited and enthused about the Third International Symposium on FOP which will be held November 2-5, 2000 in Philadelphia. All of the invited scientists are exuberant about their participation in this extremely important meeting for the FOP and POH communities. As always, finding the cure for FOP is not a job, it is a mission.

Thank You

All of us in the FOP Laboratory at The University of Pennsylvania, in the developmental grants program and in the affiliated collaborative ventures around the world are extremely proud to be part of this mission and are enormously grateful to those who support this vital research effort.

FOP continues to be one of the most obstacle-ridden and perplexing quandries of the human condition. In the relatively brief existence of the international FOP

collaborative effort, great progress has been made. We will continue to need your help until a cure is found. Thank you for your generous and heartfelt support of this effort.