THE THIRTEENTH ANNUAL REPORT
OF THE FIBRODYSPLASIA OSSIFICANS PROGRESSIVA (FOP)
COLLABORATIVE RESEARCH PROJECT

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Maps of the ancient world reveal a quaint and astonishing glimpse of what explorers and cartographers of the time knew to be reality. An early map from the 13th century depicts ships going over the edge, while a later map from the 17th century reveals the vague outlines of continents not yet charted and in some cases only imagined. In many ways, FOP research is like exploring a world still shrouded in mystery. FOP research is, after all, the science of discovery and hope. The mission of FOP research is to discover the molecular and genetic topography of the FOP world, and to use that knowledge to design effective preventions, treatments, and eventually a cure. Each year, our maps of the FOP universe improve and our journey and its eventual destination become more clear.

Important highlights in FOP research during 2003 included:

1. The discovery that overactive BMP4 receptor signaling in FOP cells is associated with profound abnormalities in BMP receptor trafficking across the cell membrane.
2. The discovery of ultra–adherent cells from the peripheral blood of FOP patients that spontaneously mineralize in culture.
3. The use of microarray technology to probe the downstream pathways and targets of BMP4 signaling in FOP cells.
4. The addition of three new multigenerational families to the FOP genome-wide linkage analysis bringing the total now to seven traditional multigenerational families worldwide.
5. The refinement and publication of model systems for BMP4-induced heterotopic ossification.
6. Establishment of bone marrow stem cell lines from FOP patients for the study of previously inaccessible BMP pathway analysis in connective tissue cells.
7. The investigation of cell surface heparan sulfate proteoglycans in FOP and control cells and their role in regulating BMP4 morphogen gradients.

8. The investigation of anti-neoplastic and anti-angiogenic properties of aminobisphosphonates in BMP4–induced heterotopic ossification.
9. The publication of the proof-of-concept of noggin gene therapy for disorders of BMP4–induced heterotopic ossification.
10. Collaboration with industry partners on the development of inducible gene therapy vectors for advancing noggin gene therapy for the treatment of FOP.

In addition to new pilot projects and continuing long-term studies, a major focus of our research efforts in 2004 will include:

a. Further molecular characterization of BMP4 receptor trafficking abnormalities in FOP cells.
b. Higher resolution mapping of the FOP gene locus using genome-wide-linkage data from the three additional multigenerational families.
c. Advanced microarray studies of the BMP4 pathway in FOP cells
d. Phenotypic and molecular characterization of blood-derived adherent stem cells from FOP patients.
e. Advanced pre-clinical work on the use of cox-2 inhibitors, aminobisphosphonates, and noggin gene therapy for the treatment of FOP.
f. Exploration of technologies to block BMP signaling in FOP cells at or downstream of the activated BMP receptor.

In this year's Annual Report, we will discuss the progress in the war against FOP. We will describe discoveries in the laboratory and in the clinic as well as broad outlines of our research plans for this coming year. In addition, we will provide substantial background where necessary to help clarify the scientific rationale for this work and to help elucidate how this work fits into the broader picture of developing effective preventions, treatments, and eventually a cure for FOP.

While the mission of FOP research is clear, the research discoveries are not ends in themselves, but mileposts along a journey that will ultimately end with a cure for FOP. Much of the work that we reported to you last April in the Twelfth Annual Collaborative Research Report continues to evolve. Many of these projects are technically complex, and will take several years to complete. In some projects, valuable information emerges regularly and can be transmitted to you incrementally as it is verified and reviewed by our peers. In other projects, useful information emerges unexpectedly and serendipitously, and we are always excited about those clarifying insights as anyone involved in medical research can attest. There have been several such discoveries in 2003 and we are excited to tell you about them.

The process of scientific inquiry is among the most structured of all human endeavors. Yet, progress in scientific and medical research rarely follows a straight path. At times, scientific discoveries creep forward, and at times they leap forward with a verve that leaves everyone speechless. There is rarely, if ever, any advanced sign for such discovery. It happens, as any child trying to solve a puzzle will recognize, with an immediate sense that something new is happening - that pieces that previously seemed discordant begin to fit together in a completely new way. Such was the case this past year when we discovered that a cell from the peripheral circulation of FOP patients can give rise to clusters of mineralized adherent cells in culture, thus suggesting the presence of a circulating bone-forming stem cell; but more on that later.

Recently, we featured the proof-of-concept that a genetically engineered form of the noggin protein can, through a gene therapy approach, successfully prevent bone morphogenetic protein 4 (BMP4)-induced heterotopic ossification in a mouse model that is relevant to the treatment of FOP. Such approaches to inhibiting the overactive BMP4 pathway in FOP cells provide great hope for the future. A major scientific paper

describing this groundbreaking work was published in December, 2003. Eventual treatments for FOP will be only as good as the basic knowledge supports, and that knowledge comes from a fundamental understanding of the abnormal genetic networks and signaling pathways of the FOP cells.

This year, one of our two featured breakthroughs builds upon last year's discovery in the field of molecular signaling. We have known for several years that FOP cells produce too much BMP4, and not enough of the BMP4 antagonists, noggin and gremlin. It is as if the gas pedal is stuck at full throttle and the emergency brakes fail to engage. We learned recently that FOP cells are also unable to properly sense, monitor, and regulate the concentration of BMP4 in their environment. That essentially adds a blacked-out windshield to a runaway car. These discoveries point from two different areas of inquiry to the same region of cell signaling - to the BMP receptors and to their associated molecules that sense, monitor, and regulate the levels of BMP4 in the environment of the cell.

In 2002, we confirmed that FOP cells have an over-abundance of one of the BMP4 receptor proteins on the cell surface, and that this protein which should properly regulate the concentration of BMP4 is stuck in the on-position once it is activated. In 2003, we began investigating the mechanism by which this occurs. While we don't have all of the answers yet, we have found something very important; that BMP receptors are not properly internalized in FOP cells.

The transfer of the BMP receptor to and from the cell surface is called receptor trafficking. We discovered in 2003 that BMP receptor trafficking is profoundly disturbed in FOP cells. Specifically, the activated BMP receptor fails to move inside the cell, and as a result remains active on the membrane. In other words, there appears to be a

molecular traffic jam at the cell membrane that causes the BMP pathway to remain active, once engaged. This important molecular link between signaling and trafficking is one of the hottest areas in molecular cell biology and seems intimately involved in the pathophysiology of FOP. While much is known about the relationship between trafficking and signaling in the transforming growth factor-beta receptors (the parent class of molecules to the BMP receptors), almost nothing at all is known about how the BMP receptors traffic across the cell membrane. Much more knowledge and insight is likely to follow in the upcoming year in this extremely important and exciting field of FOP research.

The second featured breakthrough in this year's annual report is in the field of adult stem cell biology. It involves a serendipitous discovery (from the FOP laboratory in late May, 2003) of the existence of a highly adherent and mineralizing population of stem cells derived from the peripheral blood of FOP patients. The implications of both of these developments are potentially enormous, but a lot more on both of them later. As before, this year's Annual Report will be divided into nine major sections that most clearly organize and highlight the vast amount of work and activity from the FOP core and collaborative laboratories. These sections are:

I. Genes

II. Pathways

III. Cells

IV. Models

V. Triggers

VI. Treatments

VII. Presentations, Meetings, Reports, and Publications

Once again, we hope that you will find this year's annual report interesting, engaging, and hopeful. That is the attitude with which we approach our work every day.

I. GENES

Multigenerational Families & FOP Gene Localization Studies

Identification of the gene that (when mutated) causes FOP will be a key to understanding FOP as well as many more common conditions of skeletal development. The mutations responsible for most genetic conditions are identified by tracking the inheritance patterns of signature pieces of DNA (called markers) through large multigenerational families. However, for FOP no such large families with many affected individuals have been identified anywhere in the world. Recently, however, our work began to pay-off. In the area of multigenerational family identification, several fortuitous developments occurred. These developments were the unexpected discovery of two additional multigenerational families – one from the United Kingdom (thanks to the work of our valued colleagues and collaborators, Dr. Roger Smith & Dr. James Triffitt of Oxford University) and the other from the United States. We reported last year that each of the families had been contacted. In 2003, FOP was confirmed in both families, all relevant DNA samples obtained and a genome-wide linkage analysis begun both at Penn and at Oxford. In addition, a third multigenerational FOP family from Brazil was identified in October, 2003. This new family attended The First Latin-American FOP Family Meeting in late October, 2003. All members of this new mutigenerational family were seen and examined, FOP confirmed, and blood samples obtained for DNA analysis.

A large scale genome-wide linkage analysis is currently being performed on all members of the three new multigenerational FOP families (from the US, the UK, and Brazil), and

the results of those data will be added to the information already available from the four previously identified FOP multigenerational families. At this point, it is impossible to determine whether the addition of three new multigenerational families will allow us to substantially narrow the genetic interval where the FOP gene is located (and thereby allow us to pursue positional cloning of the gene), but the new data will clearly bring us a big step closer.

Additional multigenerational families likely exist but may have had little contact with the medical community. The increasing amount of medical research on FOP and the growing international collaborative network of physicians and scientists who recognize FOP is changing that predicament. Also, we cannot overestimate the power of the Internet to disseminate new information and to attract the attention of physicians, scientists and the families themselves. The discovery in 2003 of the new multigenerational FOP family from Brazil was due to the hard work of Dr. David Glaser from Philadelphia and Dr. Patricia Delai from Sao Paulo, Brazil, and the serendipity of being in the right place at the right time. Hard work is a necessary ingredient in solving the puzzle of FOP but a little good luck from time to time is always welcome.

What is a Genome-wide Linkage Analysis?

For the past several years, we have emphasized the importance of genome-wide linkage analysis in locating and identifying the FOP gene, but to date we have not explained what it is or how it works. It is would be helpful to do that now. So, what is genome-wide linkage analysis? The simple answer is that it is a scientific tool that allows one to correlate the clinical features of a condition (such as FOP) with distinguishing molecular and genetic markers that travel with the damaged gene of interest through various

generations of a family, and then to compare the information gathered from many such families to narrow the location on the human chromosomes where the gene is most likely located. But to better understand the power of the concept and the utility of the approach, let us digress for a moment and review some important background information and genetic principles.

It is important at this stage in the discussion to note a very important point. We often speak of "the FOP gene." But, of course, the gene is not in the human genome to cause FOP, but to encode a protein that does something very important (and as yet unknown) in the regulation of our normal skeleton. When the gene is damaged (or mutated), FOP results. Whatever the gene turns out to be, people with FOP will have one damaged copy and one normal copy. People who do not have FOP will have two normal copies. So, we call it "the FOP gene," but it is really a normal gene that when one of the two copies is damaged can cause FOP.

Although FOP is a genetic disease, most people with FOP do not have affected parents or affected relatives. In most affected individuals, FOP arises as a spontaneous new mutation in an as yet unidentified gene. We say "spontaneous mutation" because it has originally occurred in that person and was not inherited from a parent. We have six billion pieces of genetic information encoded in the DNA in the nucleus of each of our cells. When a cell divides, the DNA gets copied with nearly perfect fidelity. Nevertheless, when six billion pieces of information are copied, sometimes a few mistakes may be made. When these mutations occur within genes, they often disturb the

amount or quality of protein made by that gene. Once a person has a mutation in the gene that causes FOP (whatever that gene is), there is a 50% chance that the affected individual could pass-on the damaged copy of the gene to each child that he or she may conceive.

Each nucleated cell in our body contains a full compliment of 6 billion basepairs of DNA including two copies of each gene, one from the father and one from the mother spread out over 23 pairs of chromosomes (22 autosomes) and two sex chromosomes (XY in males and XX females). However, the gonads (testes and ovaries) through the process of meiosis or genetic reduction, cleverly decrease the number of chromosomes in half so that each sperm cell and each egg cell has only one copy of each chromosome rather than two. If this did not happen, then in each generation we would have more and more chromosomes. This might sound like a good thing, but it is not. Too much of a good thing can be very bad. Three copies of a chromosome (as in three copies of chromosome 21, for example) can cause Down syndrome. The body must make sure that there is not too much or too little genetic information, but just the right amount in each cell. So, in the production of each sperm cell and each egg cell, the genetic information is reduced by half so that upon recombination of the father's and mother's DNA at fertilization, there is exactly the right number of genes and chromosomes in each cell.

For the sake of simplicity let us imagine that there is just one pair of chromosomes in each cell in our body – one copy from mom and one copy from dad. In the process of forming the sperm cell or egg cell, each sperm cell or egg cell ends up with half the

number of genes in the parent's somatic (body) cell, as we said above. But, it is not as if one chromosome goes to one sperm cell or egg cell, and the other chromosome to the other sperm cell or egg cell. Before a chromosome gets distributed to a sperm cell or an egg cell, the DNA gets reshuffled (in the process of meiotic recombination) so that each chromosome is a hybrid of the parental chromosomes.

Imagine, for example, that in a parent's cell that contains two copies of the same chromosome, one copy is blue (originally from the grandfather) and one copy is red (originally from the grandmother). What happens in the formation of each sperm cell or egg cell is that the genetic information gets reshuffled at each locus (location on the chromosome) so that the two resulting chromosomes are like candy canes with blue stripes and red stripes from the two original chromosomes. So, instead of two chromosomes, one red and one blue, each cell ends up with two chromosomes each mixed like a candy cane. Then, one candy-cane (hybrid) chromosome goes to one sperm cell and the other reciprocal "candy-cane" chromosome goes to another sperm cell (in the case of the father; or to two different egg cells in the case of the mother). In essence, this is how grandmom's and grandpop's DNA get shuffled in the gonads of each parent before getting redistributed to each sperm cell or egg cell. The pieces (each red or blue band) that get reshuffled in the formation of the germ cells (to comprise each new hybrid chromosome) may be half-a-million to a million basepairs in length. Each region of DNA has its own individual identity from person-to-person that functions in essence as a molecular bar code identifying the origin of each segment of DNA along the length of the new hybrid chromosome. It is these unique molecular signatures that we use to trace

each candy-cane like block of DNA that gets re-shuffled in each generation, and it is that re-shuffling of genetic information in the formation of the sperm cell or egg cell that is the foundation of linkage analysis.

Now, let us imagine that a man with FOP marries a woman who does not have FOP and they decide to have a family. Let us further imagine that they decide to have a very large family of 100 children. By chance, 50 of the children will have FOP and 50 will not. Each of the 50 children who have FOP has received the damaged copy of the FOP gene (whatever it is) from their affected father while each of the 50 children who do not have FOP has received a normal copy of the FOP gene (whatever it is) from their affected father. All of the children, of course, would have received a normal copy of the FOP gene from their mother, as both of mom's copies of the FOP gene (whatever gene it is) are normal. Since all the genes (and the vast amount of DNA surrounding them) have their own unique molecular bar code, it is easy to track which copies of each gene have gotten passed to each child in the hybrid candy-cane like chromosome that comes from each parent in the sperm cell or egg cell.

The first step in the genome-wide linkage analysis is to identify clinically which children have FOP and which do not. That part is easy! The next step is to take a blood sample from mom, dad, and all of the 100 children, and isolate DNA from the blood cells. In FOP, of course, it is the simplest way to get DNA since blood tests themselves do not cause FOP flare-ups as long as they are done carefully. As we mentioned before, in each small segment of DNA along the chromosome, there are unique markers like molecular

bar codes that are different from one individual to another that can be used to identify the parental original of each small segment of DNA.

So, how do we determine where on the chromosomes the FOP gene is located? What we do next is to perform an analysis using the unique markers for each segment of DNA and ask the following questions: Take any two children in this family who have FOP, and determine which pieces of Dad's DNA they share (since in our hypothetical example, Dad has FOP). There will be thousands of pieces of DNA from the affected father that the two affected children share just by chance. Then add another affected child to the analysis and ask the same question. Which pieces of Dad's DNA do all three affected children share? There will also be hundreds or thousands of pieces of DNA that they share by chance, but there will be fewer pieces of DNA than shared by just two affected children. By adding additional affected children to the analysis, one will be able to exclude the randomly shared pieces of DNA. If enough children are added to the analysis, one should be able to find the single segment of Dad's DNA (the affected parent in our example) that is commonly shared between all 50 affected children (in other words the piece of DNA that contains the damaged copy of the FOP gene). Similarly, if one does the same analysis on the 50 unaffected children, one should be able to find the single non-randomly shared piece of DNA from dad that all of the unaffected children have in common (which should be the undamaged copy of the same gene). Eventually, if there are enough children in the family (as in our example of a single hypothetical family with 100 children), one should be able to narrow down the region of DNA that contains the gene of interest that all of the affected children share and that which none of the

unaffected children (who don't have FOP) share. In other words, one should be able to distinguish the paternally-inherited segment (locus) of damaged DNA that is shared in common by all affected children and the undamaged copy of the same locus shared in common by all of the unaffected children. And, it is at that locus where the FOP gene must be located.

At this point in the analysis, however, one still does not know what the gene is but one knows where on the chromosome it is located. This is, in fact, the process of linkage analysis. Thus, one links the affected trait of FOP to the molecular bar code where the FOP gene must be located by the process of elimination. For some genetic conditions where large multigenerational families do exist, it is entirely possible to pinpoint the location of the disease-causing gene by performing genetic analysis on one such family. For FOP, of course, such single large multigenerational families do not exist and it is necessary to piece together the clues from many smaller families.

In the next step, one analyzes the DNA determined to contain the gene of interest to discover what genes might be there. Depending on the size of the multigenerational family or the number of multigenerational families analyzed, the segment of DNA may contain between five and 50 genes. Perhaps, there is a gene that has already been identified and that might "fit the bill" for the FOP gene. This is what we call a candidate gene. Then one sequences the candidate gene to look for a mutation. Until now, we have been searching for candidate genes within suspected linkage regions as well as within functional candidate pathways.

A candidate gene search in an area of the genome where linkage analysis predicts the presence of the FOP gene is more strategically focused than sequencing all candidate genes regardless of where they may reside in the genome. Until now, because we have not had enough power in our linkage analysis (lack of enough multigenerational families and lack of enough people within each family) we have had to rely more heavily on the more global candidate gene approach than on the candidate gene approach narrowed to a defined chromosomal region. We think this is about to change.

It is also possible that the segment of DNA where the FOP gene is located may not contain any obvious FOP candidate genes. How could this happen? Well, it could happen because all of the genes have not yet been identified in that region of DNA. We know the entire sequence of DNA along each chromosome, but we do not yet know exactly where all of the genes are nor what proteins they encode. But, if linkage analysis predicts the location of the FOP gene (to within a few hundred thousand base pairs), one can dispatch the molecular SWAT team to the location without knowing exactly what one is going to find, but knowing that that is the most likely spot where the culprit FOP gene is located. The more multigenerational families there are and the larger each family is, the easier it will be to identify the precise location and the identity of the FOP gene.

An important question that often arises is, "Once the FOP gene is discovered, how will that help us treat FOP?" Once the FOP gene has been identified, one of the first steps will be to try to reproduce the same genetic damage in a mouse to verify that this is truly the FOP gene. It is possible that the same genetic damage in a mouse might not cause

FOP, but it is likely that it will. By generating mice that have FOP, it will be much easier to study the true biology of FOP and to first evaluate treatments in the mouse rather than in adults and children who have FOP. The identification of the FOP gene is clearly the most important piece of knowledge we need to solve the riddle of FOP.

Sequencing Candidate Genes

We previously reported the identification of several FOP families whose DNA analysis could complement our genetic mapping studies. While none of the families was a traditional multigenerational family, they each possessed unique genetic characteristics that could help us decipher clues about the chromosomal location of the FOP gene. The utility of these studies in further narrowing the position of the FOP gene is important, and new genomic mapping data from these genetically non-traditional FOP families are being thoroughly analyzed together with the data from the seven traditional multigenerational FOP families. The analysis to date has suggested small regions on several chromosomes that may also hold clues to FOP, and we are vigorously pursuing these leads. In addition, we continue to examine candidate genes both inside and outside of the linkage regions; genes that if mutated, could plausibly cause FOP. The candidates include genes that are involved in the bone morphogenetic protein pathway, genes that control skeletal and/or bone marrow development, as well genes involved in immune function and/or inflammation. We have, through our targeted DNA sequencing efforts, excluded many promising candidate genes, but there are still many more to evaluate. As work continues to annotate the human genome in laboratories around the world, the potential relevance of newly identified genes provides valuable information and perspective on genetic signaling networks relevant to FOP. While this targeted DNA sequencing effort for promising candidate genes is laborious, it is essential that we leave no potential FOP gene uninvestigated.

Genes Without Proteins; Genes With Many Proteins

Determining the association between a gene and a disease is just the first step in genomic medicine. During the past year, several very important insights have emerged as a result of advanced work on the human genome project. Of the estimated 30,000 genes in the

human genome, approximately half encode presently unidentified proteins. With the use of available genetic techniques, the function of these proteins will be elucidated. Although our FOP candidate gene search includes both well-known and obscure genes known to be involved in the regulation of skeletal formation, it is entirely possible that the FOP gene encodes a protein that is not yet known. Furthermore, many genes do not even encode proteins, but rather small ribonucleic acid (RNA) molecules that have structural or regulatory functions in major cell signaling and trafficking networks.

Recently (through the process of linkage analysis described in the previous section of this report) a gene was discovered for a skeletal condition, but the gene did not encode a protein. Rather, it encoded a small RNA that controlled the regulation of important genetic switches. Also, many genes encode more than one protein. The coding portion of a gene (in distinction to the regulatory or "switch" portion of a gene) is called an exon. Most human genes are composed of many exons, each of which is widely spaced by interspersed non-coding segments called introns. When a gene is "transcribed" or "read," the entire DNA sequence (excluding the regulatory region) is copied. Then, the "intronic" portions are spliced-out so that only the "exonic" portions remain. This spliced "exonic" RNA is called messenger RNA (mRNA) because it contains the "message" for the native protein. Portions of the RNA at the beginning of the message may also be regulatory in a nature and are commonly not "translated" into proteins.

It used to be thought and, in fact, had been accepted as dogma by the biological community, that one gene encoded one messenger RNA, and that one messenger RNA

encoded one protein. In the human genome, it is now abundantly clear that for many genes, the exons can be spliced together in various combinations to encode many messenger RNAs (mRNAs) and thus many proteins. For example, if a gene has 6 exons, perhaps one mRNA may consist of exons 1,2,3,4,5,6. Another mRNA encoded by the same gene may consist of exons 2,3,4,6. Yet another mRNA encoded by the same gene may consist of exons 1,3,4,6. Thus, from a single gene, there may be a vast number of possible mRNAs, and a vast number of corresponding proteins. Some mutations in a gene might affect all of the mRNAs and proteins encoded by that gene whereas other mutations might only affect some of the mRNAs and proteins encoded by that gene. And, it is not even necessary for all of the proteins encoded by the same gene to affect the same pathway. One protein encoded by the gene could play an important role in the brain, another in the kidney, another in fat, and yet another in connective tissue and bone. So, from a single gene, there may be a bewildering array of proteins that have similar or dissimilar functions.

So, what does any of this have to do with FOP? Perhaps not much; perhaps a great deal. It is too early to know for certain. But what is certain is that the gene responsible for progressive osseous heteroplasia (the "sister condition to FOP; both the disease and the causative gene were discovered in our laboratory) is a very complex gene precisely like the hypothetical example described above. The POH gene, named GNAS and located on human chromosome 20, is composed of 13 exons that are alternately spliced to encode several different mRNAs and proteins in many different tissues, (some of which affect the metabolism of the brain and the heart, some of which affect the metabolism of the

kidney, some of which affect the differentiation and metabolism of fat, and some of which affect the differentiation and metabolism of bone). Mutations in various exons of the GNAS gene can lead to the disabling heterotopic ossification that we know as POH. While this is not the same gene that causes FOP, it has taught us (and continues to teach us) a great deal about the extraordinary complexities of a single gene – and it is those lessons that are very much in mind as we travel along a similar path to identify the gene responsible for FOP.

Molecular Inheritance Patterns in FOP: Unusual Clues

In a promising and very exciting new approach, which we first described last year, we are exploring targeted regions of the genome where the FOP gene may be located (identified through our genome-wide linkage analysis) to look for loss of inheritance of one of the two copies of parental DNA. This type of investigation is called "a loss-of-heterozygosity (LOH) study." In this context, "hetero" means different while "zygous" means parental origin. The concept of loss-of-heterozygosity is an important one as well for the mitotic recombination project and is a great example how work in one area may foster progress in another area in unanticipated ways.

For most genes, we have two copies – one copy from our mother and one copy from our father. Due to genetic variations between individuals, it is possible to trace the father's copy of the gene and the mother's copy of the gene using specific markers associated with each copy of the gene, as we described previously in the section on genetic linkage analysis. In some individuals who have FOP, it is possible that a small piece of DNA could be missing, and that the missing piece could contain one copy of the FOP gene. But that piece of DNA might be too small to notice microscopically and might only be identified by detailed molecular studies. Therefore, if one of the parental copies is

missing, the genetic signature at a specific location along the chromosome would seem homogeneous (rather than heterogeneous) because one is able to identify only one of the two parental copies. Such homogeneous-appearing regions at the molecular level might provide a more precise clue to where a small piece of DNA may be missing, and where the FOP gene might be located.

In 2003 using loss-of-heterozygosity analysis, we discovered a substantial deletion of genomic DNA within a linkage region for FOP in a child severely affected with the condition. The genomic deletion was not found in either of the child's parents, providing strong evidence that the deletion was not a normal or neutral variation. Although the deletion of genomic DNA did not appear to interrupt the coding region of any known gene, the possibility of a deletion in a regulatory region of a gene cannot be excluded. The clinical significance of this finding is presently unknown and is under intense investigation in the FOP laboratory at the present time.

The Mitotic Recombination Project

In the past two years, we reported a novel approach to finding the FOP gene called *the mitotic recombination project.* The mitotic recombination project, an alternative genetic approach that circumvents the need to obtain DNA samples from large multigenerational families, could expand our ability to identify the genetic cause of diseases like FOP for which large families are difficult or impossible to obtain. The idea behind the study is a quite simple and novel one that might allow us to capitalize on the rare shuffling of genetic information that occurs from time to time in our body's somatic cells (in contrast to our body's germ cells like the sperm cell or egg cell). This novel idea proposes a method to use FOP lymphocytes, available to us in the laboratory from the blood samples

you have given us, to generate "cellular families" that could be used to narrow the genetic interval where the FOP gene is located.

While the strategy for this novel mitotic recombination project is simple and elegant, the logistics and tactics have been fraught with numerous technical challenges. During the past year, we have devoted much attention to this project in an attempt to circumvent the technical obstacles, but they are formidable. However, we have learned a great deal about the behavior of FOP cells from this work that has allowed us to advance other projects in ways we had not anticipated. Many of the techniques that we have developed and used on this pilot project are applicable to several other projects that we are pursuing in the FOP laboratory including the loss of heterozygosity analysis described in the previous section of this report and to the BMP pathway studies that require the stable transfection of lymphocytes. This continues to be one of the most technically difficult projects in the laboratory, but regardless of its outcome has already produced a great deal of knowledge on the behavior of FOP cells and has revealed new technological approaches that can be used to aid in the discovery of the FOP gene.

II. PATHWAYS

FOP: A Disorder of Skeletal Regulation

In bone diseases such as fibrous dysplasia or osteogenesis imperfecta, the bone formed is abnormal. In contrast, in FOP, there is nothing at all wrong with the extra bone except that it should not be there. FOP is not a disorder of skeletal structure or of skeletal composition, but of skeletal regulation.

The lessons of progressive osseous heteroplasia (POH), the sister disease to FOP, provide an important counterpoint to the next section of the report. For POH, we have discovered the causative gene, but have no knowledge yet of the pathways that the gene uses to make heterotopic bone. For FOP, we do not yet know the precise causative gene, but we are gaining tremendous knowledge and insight into the signaling pathways used by the cells to make heterotopic bone. For both conditions, it is necessary to understand not only the causative gene, but also the regulatory pathways through which the genetic damage leads to heterotopic ossification.

Establishing an effective treatment for a genetically-based developmental disorder like FOP will result from identification of both the gene mutation and the dysregulated developmental pathway that causes FOP. For FOP, family pedigrees are scarce and genetic linkage and positional cloning are difficult. An alternative approach to identify the primary pathology in FOP involves strategies to isolate the dysregulated molecular pathway and from there trace back to the damaged gene. During the past year, we have continued to devote much effort to this approach, and the results are beginning to pay-off.

In this section of the Annual Report, we will describe one of the major discoveries in FOP research during the past year: that the BMP receptor on the surface of FOP cells is chronically activated and associated with profound abnormalities in its movement and trafficking across the cell membrane. Before describing this exciting new avenue of research, it would be helpful to review some background material, first described in last year's annual report, as this year's advances build directly upon them. We will, therefore, highlight the following six sections: "What is a morphogen?" "Autoregulatory negative feedback loops," "Dysregulation of autoregulatory negative feedback loops in FOP cells," "BMP4 pathway dysregulation in FOP," "Elevated BMP receptor levels at the cell surface," "BMP receptor traffic jams at the cell membrane," "Who are the BMP

traffic cops at the cell membrane and what is their role in BMP signaling?" and "Which road to the target?" In these sections, we will describe some of our newest findings and the hypotheses that guide this seminal branch of our FOP research effort.

What is a Morphogen?

Bone morphogenetic protein 4 (BMP4) is a special type of protein referred to as a morphogen and is encoded by the gene that bears its name. BMP4 is highly conserved throughout evolution in the animal kingdom and has many different functions during development and following birth.

BMP4 is a morphogen, and a morphogen is a secreted signaling molecule that organizes a field of surrounding cells into patterns. In the case of the fruit fly (that has no bones), BMP4 organizes adult stem cell niches, patterns the body plan, and specifies structures such as the wing. In the case of a human being, BMP4 organizes the cellular pattern of various organs including the hair follicles, middle ear, and the skeleton itself. BMP4 forms a concentration gradient emanating from a localized group of cells, and the BMP4 protein determines the arrangement and fate of responding cells according to the concentration of BMP4 perceived by the cells in the environment.

The idea of a morphogen gradient is intimately associated with the concept of positional information. A cell in the environment of a BMP4 molecule reads its position in a concentration gradient, and determines its developmental fate accordingly. Morphogens turn on different genes at different concentrations. For example, at low concentrations BMP4 turns on one set of genes, at intermediate concentrations, it turns on a different set of genes, and at a high concentrations, it turns on yet a third set of genes. This, of course, is an oversimplification, but the concept that morphogens have entirely different effects at different concentrations is an extremely important and relevant one for FOP.

How then are the gradients of a morphogen such as BMP4 established and maintained to allow the body to create exactly what it needs, exactly when it needs it, exactly where it needs it and absolutely nowhere else?

Autoregulatory Negative Feedback Loops

The concept of autoregulatory negative feedback loops is central to attempts to understand the concept of morphogen gradients and the extraordinary fidelity of their control. During the past five years, numerous steps have been identified and elucidated in the molecular relay switches from secreted morphogens (such as BMP4) to downstream gene control at a molecular level. At each step in the molecular relay from the secreted morphogen to the downstream effects on target cells, nature has built-in negative feedback control switches. These negative feedback switches are a set of highly regulated brakes that sense the concentration of intermediate molecules in the pathway and work together as a molecular guidance system to ensure the proper functioning of the pathway.

Negative feedback switches allow cells to operate with high fidelity within an extremely narrow range that never allows the morphogen gradient to waver far from its set-point in any particular tissue of interest. The presence of autoregulatory negative feedback switches in gene circuits provide enormous stability, thereby limiting the range over which the concentration of network components fluctuate. The stability of a morphogenetic pathway controlled by autoregulation is far superior to that of an unregulated pathway. Autoregulatory negative feedback switches have been identified at every single step in the BMP4 pathway.

Dysregulation of Autoregulatory Negative Feedback Loops in FOP Cells

With this background in mind, it will be easier to understand one of the most important basic science discoveries from the laboratory in the past several years – the finding that FOP cells are unable to properly regulate the BMP4 pathway in response to a BMP4 signal. The failure of FOP cells to appropriately upregulate expression of some secreted BMP4 antagonists in response to a BMP4 signal suggests a possible loss of negative feedback by which BMP4 expression levels and thus BMP4 activity may be markedly elevated and sustainable in FOP.

Heterotopic ossification in the setting of FOP begins in childhood, and can be induced by surgical trauma, soft tissue injury, viral illnesses, intramuscular immunizations, or injections for dental procedures. BMP4 is produced by skeletal muscle and its expression can be upregulated at sites of soft tissue injury. Under normal circumstances, BMP4 dramatically stimulates the expression of at least several BMP antagonists. A blunted BMP4 antagonist response following soft tissue trauma would permit the rapid expansion of a BMP4 signal conducive to progressive bone formation. The growth of highly vascular preosseous fibroproliferative tissue seen locally in response to BMP4 overexpression would be magnified in the setting of a blunted BMP4 antagonist response, and could explain the explosive bone induction seen during an FOP flare-up. These findings from FOP illustrate the importance of a critical balance between an inductive morphogen (BMP4) and its secreted antagonists in the formation of an ectopic organ system and suggest the potential for developing BMP antagonist based strategies for the treatment of FOP.

In addition, FOP cells have an intrinsic defect in the ability to regulate BMP4 levels across a wide range of metabolic and cell cycle events *in vitro*. In normal cells, the BMP4 levels are held tightly in-check throughout all phases of the cell cycle, while in FOP cells, the concentrations seem to vary dramatically. The inability of FOP cells to

properly regulate the concentration of BMP4 throughout the cell cycle may reflect a basic defect in the regulation of the BMP4 pathway. The primary action of a damaged gene in the BMP4 pathway may affect one or more components of the BMP4 pathway, like a guard that regulates several checkpoints. Alternatively, a gene defect that affects only one aspect of the BMP4 pathway may have secondary repercussions that are widespread. This suggests that genes encoding proteins that regulate BMP4, BMP4 receptors, and perhaps proteins that degrade BMP4 or its cognate receptors may be dysfunctional in FOP cells.

BMP4 Pathway Dysregulation in FOP

In order to test the hypothesis that the BMP4 pathway is dysregulated in FOP, we conducted a detailed examination of various autoregulatory negative feedback switches within the BMP4 pathway and found defects at multiple points. Such defects included not only the overexpression of BMP4 messenger RNA and protein, and the inability to upregulate multiple secreted BMP4 antagonists, but also the overabundance and the inappropriate activation of BMP4 receptor proteins on the cell surface. These findings have been documented in numerous FOP cell lines and provide striking evidence that the BMP4 pathway is intrinsically dysregulated in FOP. This work is presently the focus of intense research in the FOP laboratory. The first part of this work was the subject of a major scientific paper published in 2003 in **The Journal of Bone & Joint Surgery (JBJS)** entitled, "Paresis of a bone morphogenetic protein-antagonist response in a genetic disorder of heterotopic skeletogenesis."

The peer-review of the **JBJS** article stated, "This is an extremely interesting, well-written, and important article providing further evidence for the role of bone morphogenetic proteins in the pathogenesis of FOP. While prior reports have indicated that this protein's overexpression may be essential to the cause of FOP, the possibility

that an impaired regulatory control loop involving secreted protein antagonists may be involved in the cause of this disease is potentially revealing and important. The ability of the FOP laboratory to obtain sufficient samples of lymphoblastoid cells from patients afflicted with this rare disorder makes this report a unique and valuable opportunity to learn much important new information. The concept that BMP function in humans is closely regulated by an interplay between BMP-BMP receptor binding of morphogens and their antagonists certainly raises questions regarding its potential therapeutic role in orthopaedic surgery. That perspective, in addition to the findings of this report, make for a very important contribution on the role of osteoinductive proteins in human disease and therapeutics."

Elevated BMP Receptor Levels At the Cell Surface

As we have noted, the BMP4 signaling pathway is profoundly dysregulated in the cells of patients who have FOP. Recent studies show that FOP cells fail to properly regulate ambient concentrations of BMP4 and fail to appropriately regulate the transcription of BMP4 antagonists. Other recent discoveries indicate that one of the BMP receptors is present at very high levels on the surface of FOP cells while another BMP receptor is present at very low levels. These data are consistent with developmental studies showing that postnatal overexpression of one of the BMP receptors (the one that is overabundant) can cause heterotopic ossification and that embryonic underexpression of the other BMP receptor (the one that is underexpressed in FOP cells) can cause toe malformations that are nearly identical to those seen in patients who have FOP. There are no mutations in the coding sequences of BMP4, multiple BMP4 antagonists, or the BMP receptors in FOP patients. Taken together, these data suggest that a primary defect exists in the BMP4 signaling pathway in FOP cells and that one of the BMP receptors may be constitutively active and unresponsive to normal feedback signals in FOP cells.

Cells derived from FOP patients provide a unique opportunity to gain insight into the role that altered BMP receptor signaling plays in the induction of endochondral bone formation such as occurs ectopically in patients with FOP. This led to our current hypothesis that abnormal BMP signaling results from increased amounts of one of the BMP receptors on the cell surface and mediates the extra bone formation in FOP while the great toe malformations result from reciprocally decreased amounts of and/or activity of another BMP receptor on the cell surface.

BMP Receptor Traffic Jams at the Cell Membrane

The recent discovery of the overabundance of BMP receptors on the cell membrane stimulated an intense amount of new work in the FOP laboratory this past year. First, we wanted to know: "Why do FOP cells have increased amounts of BMP receptors on their surface? The two most obvious possibilities are: they make more or they degrade less. The easiest way to think about this is like cakes in a bakery. If all of a sudden you walked into a bakery one day and found 10 times as many cakes on the shelf, then there are two likely reasons. Either the baker is baking more cakes, or the baker is making the same amount of cakes as before but no one is buying them and they are accumulating on the shelf. In order to answer the question of why there are too many BMP receptors on the surface of FOP cells, we designed and conducted experiments to distinguish between these two possibilities. We discovered that FOP cells manufacture BMP receptors at the same rate as control cells, but they cannot get rid of them properly, much like the bakery whose shelves are filled with cakes not because the baker is making too many cakes but because no one is buying them and removing them from the store.

In the cell, things are slightly more complex, but the same idea prevails. When a ligand or signaling hormone such as BMP4 latches on to its receptor, the event triggers the addition of a phosphate molecule to the receptor. This phosphate molecule is like a

molecular baton that activates the receptor. This molecular baton is passed on to the next molecule downstream in the signaling cascade. As in a relay race, the molecules that pass and receive the molecular baton, move from place to place to do their job. When a BMP receptor becomes activated by the addition of a phosphate molecule, it relays the signal to the next molecule downstream, inside the cell. Later, the phosphate group is removed from the receptor, thus deactivating the receptor. The inactive receptor is either degraded or recycled to be used again. However, the deactivation process cannot happen if the activated receptor does not enter the cell.

Normally, after a BMP receptor is activated by BMP4, the activated receptor should be removed from the cell membrane and taken back into the cell by a process known as internalization or endocytosis and then subsequently degraded. In some cases, instead of being degraded, some of the internalized receptors are recycled back to the cell membrane for re-use. We discovered that in FOP cells, one of the BMP receptors was not being properly degraded inside the cell after it was activated.

In order to determine whether the problem was truly in the degradation process, we designed experiments to see whether the activated receptor was first being internalized (that is, removed from the cell surface) as that step must occur before the receptor can be degraded or broken down. We found, to our great surprise, that the BMP receptor was not being properly internalized. The discovery of impaired internalization of BMP receptors in FOP cells is of profound importance in understanding the pathophysiology of FOP. This year, we will conduct experiments to determine why the BMP receptor is not properly internalized and inactivated in FOP cells.

One of the most seminal questions to emerge from our work during the past year is: How does the disordered intracellular trafficking of BMP receptors modulate the signaling

pathways within the FOP cell? Despite the tremendous expansion of knowledge about signal transduction pathways over the past several years in many areas of biology, we know almost nothing about the molecular trafficking that dictates how signal transduction molecules are moved about in cells and how such molecular trafficking maybe hijacked in diseases like FOP. In fact, it is the necessity of understanding these interactions in FOP cells that will in all likelihood provide valuable insight for scientists in other laboratories who seek to understand how BMP signaling works normally and how it may be disrupted in other diseases. It is becoming clear that controlling the trafficking of molecules from one cellular compartment to another can profoundly affect the efficiency of information transfer along the signaling pathway. This has become a major focus of cancer research and cancer therapy, and has led to the development of signal transduction inhibitors (STIs), drugs like Gleevec for the modulation of promiscuous tyrosine kinase receptor activity in several forms of leukemia. It may even be possible to develop STIs to block the promiscuously activated BMP receptors in FOP cells. It is quite clear that laboratory investigations in BMP receptor trafficking and signaling will continue to be a major focus for the foreseeable future in FOP research.

Who Are the BMP Traffic Cops at the Cell Membrane and What is Their Role in BMP Signaling?

Endocytosis is a dynamic process that functions by internalizing, sorting, and degrading molecules involved in signal transduction. Endocytosis regulates the activity of signaling receptors at the cell surface. The endocytic machinery begins with the primary endocytic vessicles which bud off of the cell membrane. Internalized membrane proteins such as cell surface BMP receptors enter early endosomes that can either be recycled to the cell surface or sorted to late endosomes and lysosomes for degradation.

Until recently, it was thought that all cell surface receptors follow the same

internalization pathway and are internalized by clathrin-coated pits, by a process in which the cell surface receptor proteins bind to clathrin–associated proteins.

An important paper in **Nature Cell Biology** in 2003 reported that TGF-beta receptors (related to BMP receptors) can be internalized either by clathrin or caveolin dependant trafficking. What was particularly striking about the article, however, was that the fate of the internalized receptors was different depending upon the entry route into the cell. The authors suggested that internalization via the clathrin-pathway triggered signaling from inside the endosomes whereas internalization via the caveolin-pathway resulted in accelerated receptor degradation.

Although similar in many respects, the TGF-beta signaling pathway is different from the BMP signaling pathway in many ways. In one of the most important differences, the TGF-beta receptors are constantly undergoing internalization regardless of whether or not the TGF-beta ligand is bound to the receptor. BMP receptors seem to act differently in that internalization does not occur at a robust level unless BMP4 has bound to the BMP receptors. Furthermore, data from our laboratory suggest that BMP receptor signaling is downregulated when the activated receptors are internalized, whereas in the TGF-beta pathway, receptor signaling is upregulated when the activated receptors are internalized. In other words in the TGF-beta pathway, the molecular baton is passed once the receptors have been internalized whereas in the BMP pathway, the molecular baton is passed when the receptor is at the cell membrane. While tremendous advances have been made in the past year in understanding the relationship between trafficking and signaling in the TGF-beta receptor pathway, very little is presently known about the normal relationships between trafficking and signaling in the BMP pathway.

An analysis of the molecular pathology of BMP receptor trafficking and signaling in FOP

cells is beginning to provide critical insight into the molecular mechanisms underlying the earliest events in the pathogenesis of FOP. A fundamental understanding of the molecular and genetic regulation of BMP trafficking and signaling in FOP cells will lead to a more rational therapeutic approach to FOP as well as to a wide variety of disorders involving the induction of bone formation in humans.

Which Road To the Target?

The standard description of a cell signaling pathway goes something like this: A morphogen (such as BMP4) is made and secreted by a cell and binds to a cell membrane receptor causing activation (phosphorylation) of the receptor. This receptor activation leads to a cascade of events inside the cell that activates or represses downstream target genes that trigger events such as bone formation.

One of the major mysteries in biology concerns how cells regulate their downstream targets with such specificity and sensitivity. For the BMP pathway, there are multiple morphogens, multiple receptors, and multiple intermediate molecules. However, until recently, it was thought that there was only one major pathway through which BMPs could regulate downstream targets. In the past several years, reports have emerged from several laboratories, including ours, suggesting that there are at least two (and perhaps even three) coordinate (or alternate) pathways through which a BMP signal may affect its downstream targets.

For the past decade, it has been thought that BMPs affect their downstream targets only through the SMAD pathway. Recently, we have discovered that a coordinate or alternate pathway known as the p38 mitogen–activated protein kinase (MAPK) pathway also plays an important role in FOP cells and may be the predominant pathway by which the cell surface BMP receptors regulate downstream signaling components in the lymphoblastoid

cell system we are presently using. New data emerging from several laboratories suggest that the relative amount and configuration of BMP receptors on the cell membrane may primarily determine whether the cell uses the SMAD pathway or the p38 MAPK pathway for downstream signaling, and ultimately therefore which downstream target genes will be stimulated or repressed. While the exact meaning of these findings remains unknown, it is clear that the more that can be learned about the pathways through which BMP4 signals its downstream target genes, the better able we will be to design treatment strategies that interrupt and correct any molecular imbalance in these pathways.

Large Scale Microarray Gene Expression Studies

Comparison of the expression pattern of multiple genes in the BMP4 pathway and related pathways in FOP cells vs. non-FOP cells is an extremely important approach to deciphering the wiring diagram of FOP cells. We have said in previous reports that FOP research is much like trying to decipher the wiring diagram of an atom bomb built by a "molecular terrorist" (the mutated FOP gene). A more comprehensive analysis of gene expression patterns in FOP cells will enable us to determine the relevant wiring diagram of the BMP4 pathway and to more quickly determine how to effectively de-activate the bomb.

Two revolutionary developments in technology are providing the tools needed to probe deeper into the mysteries of the human genome, and have opened-up new and more productive avenues of inquiry for understanding the molecular basis of genetic diseases. These developments are highly relevant to our ongoing research in FOP and include:

- large-scale microarray gene expression studies

- high-speed computer analysis of comparative genome databases

Rapidly emerging DNA microarray technology now enables us to monitor thousands of genes simultaneously and constitutes a major technological advance that is beginning to give us unprecedented insights into gene expression in FOP.

Synthesis of information can often be as important as the data itself. Large-scale microarray gene expression studies, and the computer power and software necessary to analyze the overwhelming flood of data generated by such technology permits us now to mine the bounty of the human genome for the purpose of better understanding the damaged genetic pathways in FOP. Large-scale microarray analysis of gene expression patterns in FOP cells require the use of specialized core facilities and computing power outside of our FOP laboratory. Those facilities are now in place at The University of Pennsylvania and are available to us.

With the support of the Weldon Family FOP Research Endowment, we are conducting this exciting new work. A state-of-the art core facility for microarray analysis was established at The University of Pennsylvania and has been a tremendous asset for the design and accomplishment of this approach to study FOP. Studies using FOP cell lines and control cell lines are well underway, and several experimental data sets have been compared for similarities and differences. Results to date strongly suggest that there is a differential expression of downstream genes in the BMP4 pathway in FOP and control cells, and that while some of these gene targets and patterns were predictable, others were not. The predicted target genes provide an important control for future experiments,

while the unpredicted gene targets provide a novel set of clues and tools to better understand the relationship between receptor trafficking and downstream signaling that is so profoundly disturbed in FOP cells. The availability of new inhibitors for the downstream p38 MAPK pathway discussed above are providing important molecular tools to dissect the downstream pathways to determine how different BMP receptor levels and configurations trigger different downstream gene responses such as heterotopic bone formation. While no one method of experimentation is likely to answer all of the outstanding questions about BMP signaling in FOP cells, the microarray experiments and data sets will continue to provide great insight into the BMP4 signaling pathway in FOP cells.

Microarray experiments generate large and multivariate data sets that require computer-assisted evaluations to identify significant similarities and differences among compared samples. We are currently conducting the statistical analysis of the generated data sets in our core FOP laboratory and are excited to note the addition to our laboratory of Chaitanya Kommidi, a graduate student in bioinformatics whose presence has already added greatly to the value of this project.

BMP-Interacting Pathways

For reasons outlined above, most of our attention has focused on the BMP4 signaling pathway in FOP. However, it is likely that other signal transduction pathways may be involved in the FOP process. Nearly all investigations in developmental biology relevant to the study of FOP suggest the possible involvement of five major inter-related signal

transduction pathways. These include the BMP signaling pathway, the hedgehog (HH) signaling pathway, the WNT signaling pathway, the NOTCH signaling pathway, and the fibroblast growth factor (FGF) signaling pathway. Each of the four latter signaling pathways has critically important interactions with the BMP signaling pathway, and we have been investigating these interactions as they may relate to FOP.

While we have, to date, found no primary pathology with the HH signaling pathway, the WNT signaling pathway, or the NOTCH signaling pathway in FOP cells, our discovery that basic fibroblast growth factor 2 (FGF2) is overexpressed in FOP lesions has provided a focus for several of our ongoing collaborative research efforts.

Last year we reported preliminary findings of a collaborative research project supported by the Developmental Grants Program of The Center for Research in FOP & Related Disorders. Dr. Hyun Duck Nah, Research Associate Professor in the Department of Biochemistry at the School of Dental Medicine at Penn, has been investigating the interactions of FGF2 and BMP4 signaling in endochondral ossification as a potential pathogenic mechanism for FOP. Interestingly, her data show that FGF2 significantly upregulates BMP4 gene expression in both cartilage precursor cells and differentiated cartilage cells. This has led to the hypothesis that FGF2 is an upstream transcriptional regulator of the BMP4 gene, and that integrated FGF2 and BMP4 signaling have essential and coordinate roles in endochondral ossification.

During 2003, Dr. Nah studied how FGF signaling regulated the transcriptional activation

of the BMP4 gene in cartilage cells in an attempt to identify the region of the BMP4 promoter (regulatory DNA sequences of the BMP4 gene) that confers FGF2 responsiveness. The results so far show that FGF2 induces a 2½-fold increase in the transcriptional activity of the BMP4 promoter. Dr Nah is currently analyzing the BMP4 promoter to identify the responsive region in cartilage cells as well as in pre-cartilage connective tissue cells. Preliminary data suggest that the responsive region is a stretch of regulatory DNA between 700 base pairs and 1600 base pairs upstream of the transcriptional start site of the BMP4 gene.

A confluence of studies at the molecular, cellular, and clinical level from numerous laboratories over the past several years has shown an important relationship between the FGF signaling pathway and the BMP signaling pathway in numerous developmental and regenerative events. Very often there is a molecular interaction between these two signaling pathways as between FGF10 and BMP4 during lung morphogenesis, between FGF8 and BMP4 during tooth development, between FGF2 and BMP4 during cranial suture closure, between FGF2 and BMP4 during limb bud formation, and between FGF2 and BMP4 during fracture healing.

The case of FGF2 and BMP4 interaction during cranial suture closure is particularly relevant to the study of FOP. In a series of elegant studies, Warren and colleagues showed recently that the BMP antagonist noggin regulates cranial suture fusion in both the mouse and in human beings. They showed that the FGF2 signaling pathway dramatically inhibits the ability of BMP4 to upregulate the expression of BMP

antagonists, thus tipping the balance in favor of bone formation; not by increasing BMP4 levels but by inhibiting the noggin response that would normally result from a BMP4 signal. Although there are no cranial suture abnormalities noted in FOP, the overactivity of the FGF signaling pathway in FOP and the intimate interaction between these two highly regulated and conserved signaling pathways demands extremely close attention in future FOP studies.

In summary, studies that carefully define the interactions between the FGF and the BMP signaling pathways have provided new insights into potential mechanisms underlying the formation of early FOP lesions and are beginning to influence our thinking on the potential utility of drugs that modulate the interactions between these pathways in controlling the growth of FOP lesions.

III. CELLS

Insights on the Identity of FOP Target Cells

An important piece of the FOP puzzle that must be solved in order to effectively prevent the complications of FOP is the identity of the target cells involved in heterotopic bone formation. It is essential to determine not only which molecules activate the BMP4 pathway in FOP, but also which cells receive and process the message that leads to the formation of heterotopic bone. Defining the origin of FOP lesional cells would represent a major breakthrough in FOP research, as it would suggest precise targets and "stealth strategies" for therapeutic intervention. Knowledge of the identity of the cells that incorrectly become bone cells during the process of heterotopic ossification will be an

important component in developing treatments and therapies not only for FOP, but also for a plethora of common disorders of heterotopic ossification as well as for FOP's "sister condition" progressive osseous heteroplasia (POH). During the past two years, our studies to examine the cellular origins of heterotopic cartilage and bone formation have advanced dramatically producing definitive and surprising results.

The Developmental Grants Program of The Center for Research in FOP & Related Disorders, established by the Cali Family Research Endowment, has funded collaborative work for the past several years with the laboratory of Dr. David Goldhamer to identify the responding cells in BMP4-induced heterotopic ossification, a model relevant to the study of FOP. This work, first described last year, and which will be explained below, represents a major breakthrough in FOP research, as it begins to identify targets for therapeutic intervention. But first, we must review some background information.

The original focus of these studies was to test whether satellite cells, the adult stem cells of skeletal muscle tissue, are precursors of BMP4-induced heterotopic bone. The impetus to address this question was based upon two observations: 1. FOP lesions most often occur in skeletal muscle tissue. 2. Under certain cell culture conditions, muscle cells exhibit characteristics of bone cells.

Two other cell types that reside in skeletal muscle tissue - perivascular cells (smooth muscle cells that surround the small blood vessels) and endothelial cells (cells that form the inside of the blood vessel walls) suggest that these cell types may be possible

contributors to heterotopic bone formation. Both cells types are found in early FOP lesions, and both cell types exhibit bone forming characteristics in other settings. Therefore, the experimental scope of the original work has expanded to include the investigation of these additional cell types. The experiments to investigate the cell lineages of heterotopic bone use a sophisticated molecular cell lineage tracing approach in mice to follow the fate of satellite cells, smooth muscle cells, and endothelial cells after stimulating heterotopic bone formation with recombinant human BMP4 protein.

Until recently, cell lineage tracing experiments were not technically possible due to the inability to stably mark specific cell populations. This technical hurdle was overcome by methods that allow permanent genetically-based marking of specific cell types using a sophisticated genetic labeling approach.

We have previously shown that the injection of BMP4 into the muscle of mice causes heterotopic bone formation that is histologically identical to that seen in FOP lesions. By injecting BMP4 into the muscles of mice whose satellite cells, perivascular cells or endothelial cells are genetically-labeled, it is now possible for us to determine both the presence and the magnitude of the contribution to heterotopic bone formation of each cell type.

The first part of the study identified two precursor cell populations that contribute to BMP4 induced heterotopic cartilage and bone formation. Results with the MyoD-Cre transgenic mice (which label satellite cells - the cells that regenerate muscle after an

injury) show participation of satellite cells in the induced heterotopic ossification consistent with their previously identified osteogenic potential, although they did not seem to be the major cell type involved.

A numerically more significant contribution to heterotopic cartilage and bone formation was observed for the cells tagged by Tie-2 gene expression (from the Tie-2-Cre mice). Although blood-forming cells express Tie-2, bone marrow transplantation experiments suggested that a major bone marrow contribution to BMP4-induced heterotopic cartilage or bone in the early lesion was less likely. The most likely source of these Tie-2 positive cells are therefore endothelial (blood vessel) cells of the local vasculature within skeletal muscle or Tie-2 stem cells resident in muscle tissue.

Experiments with smooth muscle myosin heavy chain-Cre mice in which the Cre recombinase enzyme is expressed exclusively in smooth muscle, showed no labeling of cartilage cells in the BMP4 implant studies. These data exclude mature smooth muscle cells as progenitors to BMP4-induced heterotopic ossification in this model system. This result was unexpected as smooth muscle cells express osteogenic markers in cell culture and occur in abundance amongst fibroproliferative cells in early FOP lesions.

The results of these experiments demonstrate the incredible and unexpected plasticity of stem cells derived from the muscle and local vasculature in the evolution of the BMP-induced lesion and their ability to express various markers during their transformation towards a cartilage and bone phenotype.

These data, along with observations of FOP lesions themselves, strongly suggest that the FOP lesional cells are not likely derived from a smooth muscle cell lineage, but rather express smooth muscle cell markers along their path of differentiation into cartilage and bone. However, despite its similarities to FOP, it is important to keep in mind that the BMP4-induced model system for FOP may differ in critical ways from those of the FOP lesions, although current interpretation seems more likely.

Thus, an important and novel project in cell biology has produced extraordinary insight into the origin of cells in BMP-induced FOP-like lesions. Our research has identified at least two sources of progenitor cells that contribute to heterotopic bone formation in mice. A minor contribution was observed by presumed muscle satellite cells that expressed MyoD at some point in their developmental history. Future work will investigate whether these precursor cells represent quiescent or activated satellite cells or whether a resident stem cell population that expresses the MyoD-Cre transgene is responsible. A more significant contribution is provided by Tie-2 expressing cells. Endothelial cells represent a major Tie-2 positive cell type resident in skeletal muscle. Our working hypothesis is that mature Tie-2 positive endothelial cells from skeletal muscle, in the presence of abundant BMPs, can undergo a phenotypic conversion to cartilage and bone cells. Ongoing experiments will investigate the precise identity of this Tie-2 positive precursor and the mechanism by which vascular markers are expressed during the evolution of BMP-induced FOP-like lesions. This work relates directly to the

cell surface markers found on blood derived adherent cells, a new and important discovery from the lab in 2003 that we will discuss later in this report.

In summary, we have found that BMP4-induced lesions contain cellular contributions from muscle satellite cells as well as endothelial cells resident within the skeletal muscle. Taken together with the findings of the collaborative work from Catharine Shanahan of Cambridge University, FOP lesions express smooth muscle markers during the course of evolution although it is unlikely that the cartilage and bone cells in FOP lesions arise from smooth muscle cells. Furthermore, our cell lineage tracing experiments indicate that additional cell populations contribute to the fibroproliferative cells, cartilage cells and bone cells in the evolving FOP-like lesions. The origin of these cells is presently undetermined, but other precursors and stem cell populations are being actively pursued using the Cre/lox lineage tracing technology that has been so successful in our hands to date. Suffice it to say, that an extremely powerful molecular cell lineage tracing technology has been developed and refined by the FOP laboratory and its collaborators to determine the definitive origin of cells in the BMP4-induced FOP-like lesions.

The results of the cell lineage tracing experiments (in collaboration with the Goldhamer laboratory at The University of Pennsylvania) and the results of the FOP lesional cell study (in collaboration with the Shanahan laboratory at The University of Cambridge) strongly suggest that a substantial portion of the early lesional cells arise from blood vessels within skeletal muscle. A major peer-reviewed scientific paper describing the findings of the Shanahan collaboration was published in 2003 in **The Journal of**

Pathology. The results of the Goldhamer collaboration are currently being prepared for publication.

The Role of the Vasculature in Endochondral Ossification: Insights from Developmental Biology

In an important and related Developmental Grants project undertaken by the Pacifici laboratory at the University of Pennsylvania and reported previously, a developmental biology approach was used to study the role of blood vessels in initiation, progression and completion of bone formation in the normal skeleton. It is well-established that blood vessels are required for replacement of cartilage with bone during both fetal and early postnatal life. Blood vessels are also required for fracture repair as well as for ectopic bone formation. However, several lines of experimental evidence suggest that blood vessels may have additional and previously unsuspected roles in much earlier stages of skeletal formation and heterotopic ossification. To test these hypotheses, the Pacifici lab carried out two sets of studies which provided extremely important insights into the relationship between the developing skeleton and the developing vasculature and identified factors that may mediate these interactions. Taken together with the findings from the Goldhamer and Shanahan laboratories, these data add to the understanding and importance of the vasculature in the formation of FOP-like lesions. If we keep in mind the occurrence of lymphocytic infiltration around the blood vessels of skeletal muscle in the early FOP lesions, a powerful picture begins to emerge of the interaction of the immune system and the vascular system in the formation of heterotopic bone.

Powerful insights from the FOP Core Laboratory, the Goldhamer Laboratory, the Shanahan Laboratory and the Pacifici Laboratory, strongly implicate the importance of the immune system and the vasculature in general and the lymphocytes and endothelial cells specifically in the evolution of BMP4-induced heterotopic bone formation. Intense investigation has continued in this area in 2003. In an upcoming section of the annual report entitled "An historic email and a breakthrough in FOP stem cell research," we will describe one of the most exciting and serendipitous discoveries in FOP research in the past several years; but, first some background on tissue regeneration and stem cells.

FOP: The Far Side of Tissue and Organ Regeneration

Embryonic development is a mesmerizing metamorphosis that ends in the formation of differentiated cells, tissues, organs, and organisms. Phenotypes, once specified retain their identity and are controlled by the regulatory networks they encode. The maintenance of stable phenotypes is one of the great unexplored realms of modern biology and lies at the heart of understanding FOP. Few organs have the potential to repair themselves following tissue injury. Bone, however, is the exception and can completely regenerate itself following injury. But in FOP, regeneration runs wild and occurs in a temporally and spatially erroneous manner. As a result, soft connective tissues undergo replacement by bone. From where does this unexpected metamorphosis arise? Adult stem cells, multipotent progenitor cells that betray their true identity only at times of metabolic and regenerative crisis, hold some of the answers to these questions.

The studies described in the previous section of this report begin to explore these issues

by attempting to identify these co-conspirator stem cells using stable tissue markers to see what they might be masquerading as under resting conditions – much like an assassin who holds a normal job when not provoked to his alternate occupation.

It is now widely recognized that adult stem cells or multipotent progenitor cells exist and lie in waiting in many adult tissues as we have seen convincingly with the Tie-2 positive cells described in the previous section of this report. Adult stem cells have been described for many tissues, and a common emerging theme is that the fate of these adult stem cells can be determined by the environment within which they reside rather than by their specific lineage. There is no predictable location or appearance of stem cells in most adult tissues, and we still possess only limited tools for identifying them. Furthermore, increasing evidence suggests that adult stem cells, like metastatic tumor cells, use common molecular mechanisms to home to damaged tissues. Presently, very little is known about the molecular codes that allows this stealth-like homing.

While many investigators look to stem cell biology as a promise for regenerating tissues, the FOP research team must understand the origin and fate of stem cells in order to halt the ill-timed and ill-placed regenerative response.

What exactly are these stem cells? Where do they reside? What normally keeps them quiescent and more importantly, how do overactive BMP pathway signals launch them on a wild journey with catastrophic consequences?

In the next section of this report, we will briefly examine the biology and property of stem cells as a prelude to describing one of the biggest advances in FOP cell biology in the past several years.

What are stem cells?

There are two major classes of stem cells: Embryonic stem cells and adult stem cells. Regardless of whether we are speaking of embryonic stem cells or adult stem cells, a true stem cell must satisfy several functional criteria. First, a stem cell must be clonogenic, or capable of unlimited self-renewal. Second, a stem cell must be able to give rise to another stem cell upon division and also must be capable of forming multiple differentiated cell types. Third, a stem cell must have the ability to functionally reconstitute tissues *in vivo*, not just in cell culture. Thus, stem cells have the unique capacity not only to give rise to more stem cells (self renewal) but also to generate differentiated progeny *in vivo*. Stem cells are present at all stages of development and probably exist in all multicellular organisms.

Embryonic stem cells arise from the inner cell mass of the early embryo (called the blastocyst) and have the capacity to become any cell in the body. Embryonic stem cells are truly pluripotent and do not undergo cellular aging. They are easily derived from early embryos and grow indefinitely in culture. Embryonic stem cells can be manipulated genetically to correct a genetic defect and can be coaxed into becoming any cell type through the use of specific culture conditions or genetic manipulation.

In contrast to embryonic stem cells, adult stem cells are multipotent, not pluripotent and

undergo cellular aging in culture. Adult stem cells are difficult to obtain due to their generally low numbers in most differentiated tissues and are found widely scattered through tissues following birth. In general, the therapeutic potential of adult stem cells is less than that of embryonic stem cells. In other words, embryonic stem cells can give rise to any cell type in the body whereas adult or tissue specific stem cells have a more limited repertoire of differentiation. While embryonic stem cells can be manipulated genetically to correct a genetic defect with current technology, adult stem cells can be genetically manipulated only through the introduction of retroviral transgenes.

While there is extraordinary interest, potential, and controversy surrounding the use of embryonic stem cells, the study and use of adult stem cells has gained much attention over the past several years in a much less controversial atmosphere. An excellent review article on the subject of adult stem cells for tissue repair was published by Martin Corbling and Zeev Estrov in **The New England Journal of Medicine** in August 2003.

Adult stem cells that reside in different tissues have different repertoires of renewal and differentiation. Among the most studied types of adult stem cells are those in the bone marrow. Studies over the past decade have elucidated two types of bone marrow stem cells. These are the hematopoietic stem cells that give rise to all cell types in the peripheral blood and the mesenchymal stem cells that give rise to numerous differentiated cell types including cartilage bone, tendon, fat, and muscle. The mechanisms by which bone marrow stem cells may be recruited into various differentiated tissues are not well understood. It is possible and, in fact, likely that the body has multiple sources of stem

cells that can be recruited under various conditions to subserve the process of tissue repair and regeneration and that ultimately go awry in conditions like FOP.

As Corveling and Estrov explained, we are only beginning to understand the circulating blood as a distribution conduit of adult stem cells that have the potential to participate in the repair and regeneration of various solid organs. A comprehensive understanding of both the BMP-mediated environmental signals and the locally and systemically available population of adult stem cells that respond to those signals will eventually enable us to better solve the riddle of FOP. It will be clear from the next section of this report that a major breakthrough was made in 2003 in understanding the adult stem cell repertoire involved in renegade tissue regeneration in FOP.

An historic email and a breakthrough in FOP stem cell research

In an editorial in ***Scientific American*** in November 2003, the physicist Brian Greene said, "The difference between making a breakthrough and not can often be just a small element of perception" or, as the world-renowned biologist Louis Pasteur said, "Chance favors the prepared mind." These qualities are well-exemplified in an email we received on Tuesday, June 3, 2003 from Robert Pignolo, M.D., Ph.D., a postdoctoral fellow who was working in our laboratory on a POH-related project. Dr. Pignolo is a physician-scientist who specializes in geriatric medicine with a research interest in the cellular biology of aging and regeneration. He joined our laboratory last year as a Hartford

Foundation Fellow in the Study of Aging, and in July 2003, joined the faculty of The University of Pennsylvania School of Medicine.

From: Robert J. Pignolo

Sent: Tuesday, June 3, 2003: 8:23 PM

To: Fred Kaplan and Eileen Shore

Subject: New FOP Data

Fred and Eileen:

Although I have been working mostly on POH-related projects, it has not stopped me from thinking about FOP. Specifically, I have been thinking about ways to approach the problem of studying a condition where lesional tissue is not readily available. I have come up empty until about a month ago when two interesting observations came together. The first was a report less than two years old that I recently read describing blood derived adherent stem cells (BdACs) which have osteogenic (bone-forming) potential. When studied across species, it turns out that guinea pigs have BdACs that by far have the greatest osteogenic potential. The second observation was something mentioned in lab meeting soon after I came to the lab; that is, guinea pigs have high rates of heterotopic ossification. This stimulated the question: Are BdACs from FOP patients different in any way from those of unaffected individuals? The opportunity to answer this question came very soon after I formulated this question. There was enough blood from the samples from the FOP family meeting at Disney (in early May) to derive BdAC cultures

from FOP patients and unaffected individuals. I derived BdAC cultures from 14 individuals (seven FOP patients and seven unaffected individuals). The three striking results are as follows: 1. BdACs are rare in unaffected individuals, but common among patients with FOP. 2. Compared to unaffected individuals, BdACs from patients with FOP are much more likely to proliferate *in vitro*. 3. BdACs derived from patients with FOP acquire a morphology distinct from cells initially present in culture and distinct from cells obtained from unaffected individuals. These results are very exciting to me and I would like to show you these new data. I have a meeting tomorrow between 9 am and 10:30 am but otherwise can meet any time after that or on Thursday.

Sincerely,

Bob"

The importance and implications of this finding will be immediately obvious to everyone. Dr. Pignolo's findings suggest that in FOP there may be a source of blood-derived stem cells that have the capacity of self-renewal and the property of forming mineralized tissue *in vitro* and possibly (yet to be determined) *in vivo*.

It is well known that hematopoietic stem cells from the bone marrow give rise to the blood and that mesenchymal stem cells from the bone marrow give rise to cells of the musculoskeletal system. A fundamental question is whether circulating stem cells can

give rise to cells of the musculoskeletal system and, if so, what is their tissue origin, physiologic role, and regenerative potential in disorders like FOP?

Work on this new discovery has continued with extraordinary enthusiasm during the second half of 2003 (since the initial discovery) and work on this project will continue for the next several years sponsored by a grant from the Center for Research in FOP and Related Disorders. Specific aims for the upcoming two years will include determination of the molecular phenotype of BdACs in patients with FOP, determination of the osteogenic potential of BdACs from FOP patients and unaffected individuals and determination of the origin of BdACs by cell lineage tracing methods similar to those outlined in previous sections of this report. This work in adult stem cell biology has the potential to revolutionize our understanding of FOP.

In addition to the original blood samples obtained from the Disney Trip, numerous additional blood samples were obtained from generous patients and family members at the First Latin-American FOP Family Meeting in Sao Paulo, Brazil in October 2003 (organized by Dr. Patricia Delai) and are being used in ongoing experiments. As always, we are grateful to patients and families for participating in these important studies.

The Gift of Bone Marrow Stem Cells

While the tissue of origin of the blood derived adherent cells (BdACs) described in the previous section of this report has not yet been determined, those lineage studies will be a major focus of our work during this coming year. It is well-known, however, that

mesenchymal stem cells derived from the bone marrow have the distinct ability to generate all connective tissue lineages. Much of the work on FOP to date, especially work that involves the delineation of BMP pathways in FOP cells, has been restricted to cells of hematopoietic origin such as the lymphocytes, as those have been among the only cell types widely and safely available for study from FOP patients.

It has become clear to us over the past several years that a stable source of adult stem cells derived from FOP patients would greatly enhance the range of experiments we could perform and the subsequent knowledge we could obtain. Although it is possible to isolate such cells from umbilical cord blood, very few such specimens have been obtained prospectively from FOP patients. Furthermore, such cord blood might be precious for future use if gene correction therapy is some day possible.

The process of obtaining adult stem cells from the bone marrow of FOP patients is highly invasive and traumatic and would likely cause flare-ups of FOP. The best and safest approach therefore, to obtaining adult mesenchymal stem cells from FOP patients is through a post mortem gift. This past year, such gifts were generously given to the FOP laboratory from the families of three very special individuals: Andy Sando, Heidi Hostettler, and Robert Waterman. As Nancy Sando, Andy Sando's wife noted in her holiday greeting this past year, "In death, there is even life. There is a very viable and valuable part of Andy's make-up still very much alive in the Philadelphia FOP lab. Our local pathologist was able to extract bone marrow that was requested from the FOP team that contained a remarkable amount of live stem cells. The team of researchers had never

before received live stem cells from an FOP patient and had been waiting for over 10 years for a donation of this nature. What a miracle! There have since been other donations of this nature in the past year. So, even in his death, Andy gave the gift of life to future generations of those who are diagnosed with FOP." We could not say it better ourselves. Although we cannot help Andy, Heidi, or Robert any longer, they will always be with us to help us help others.

Continued Need For FOP Lesional Tissue

Finally, it is essential to reiterate that many of the important experiments, findings, and discoveries from the FOP laboratory in the past decade would have been impossible without the all-important FOP biopsy samples that you have so graciously provided to us. While many of the experiments described above used either blood samples or sophisticated animal models of heterotopic ossification, they were all based upon primary findings from FOP lesional biopsy specimens. While we realize that these biopsy samples were obtained prior to the definitive diagnosis of FOP, and should never be obtained prospectively, these biopsy specimens provide us with extraordinary insight into FOP that could not otherwise be reproduced by any other means currently available. To those of you in the FOP community who read this report and are reminded of biopsies that you may have had performed and whose samples we do not yet have in the FOP laboratory, we would ask you to please contact us so that we could help you obtain those samples for further investigation. Just as the multi-generational families are so important for the process of gene identification, and the blood samples are so important for the BMP pathway and stem cell studies, the FOP lesional biopsies are invaluable for determining the features of FOP at a cellular and tissue level.

If you contact us, we will provide you with the appropriate forms that will authorize the hospital or clinic (where a biopsy was performed), to release the specimens for review

and study.

IV. MODELS

The Importance of Animal Models for FOP

The development of relevant animal models for FOP is a major stepping-stone in the development of effective treatments. While FOP-like conditions have been described sporadically in domestic house cats, in several pigs, and recently in a dog, no known living animals are currently available for study. It is even doubtful whether the FOP-like condition in the cat, pig, or dog is truly FOP. Guinea pigs form heterotopic bone sporadically, but it is not FOP. The achievement of a truly reliable animal model for FOP in humans will likely have to await the discovery of the gene responsible for FOP. After that discovery occurs and is verified, immediate attempts will be made to develop a truly relevant animal model based upon manipulation of the identical gene in the mouse.

The BMP4-Matrigel System: A Useful Animal Model

The most reliable model system to date for the induction of isolated FOP-like lesions is recombinant (genetically-engineered) human BMP4 protein mixed with a heterogeneous carrier substance called matrigel that is injected into a muscle of a mouse. This continues to be the most useful system for reproducing all of the known stages of FOP-like heterotopic ossification. These stages include lymphocytic and mast cell infiltration, the death of skeletal muscle cells, the formation of a highly angiogenic fibroproliferative lesion, the transformation of the fibroproliferative lesion into cartilage, the calcification of cartilage, and the eventual replacement of calcified cartilage with mature heterotopic bone containing bone marrow elements. We have used this model to study the early inflammatory events associated with BMP-induced heterotopic ossifications including lymphocytic and mast cell infiltration as well as to test the effects of anti-angiogenic

compounds and BMP antagonists. The recombinant BMP4-matrigel mouse muscle implant model continues to be a useful model system to assess various treatments for FOP and will likely continue to be so until a better animal model can be developed based on the precise gene mutations(s) causing FOP. We will refer to this animal model later in the treatment section when we review the recent "proof-of-concept" discovery using noggin gene therapy to inhibit BMP4-induced heterotopic ossification in the mouse (first described last year). A major scientific paper from the FOP laboratory containing the description of the BMP4-matrigel system was published in the December 2003, in the **Journal of Bone and Joint Surgery.**

Lymphocytes from FOP Patients Induce Early FOP-like Lesions in Nude Mice

During the past year, we have continued the development and validation of a lymphocyte cell culture model system relevant to the early molecular pathology and histopathology of FOP. The validity of this lymphocyte-cell system is based on a series of observations and experimental findings in FOP lymphocytes as well as in BMP4 signal transduction pathways in relevant cells:

1. Perivascular accumulation of B-lymphocytes and T-lymphocytes (with subsequent infiltration and death of skeletal muscle) are the earliest histopathological findings in FOP.
2. BMP4 signaling regulates hematopoietic stem cell commitment and early lymphocyte differentiation.
3. BMP4 is overexpressed in lesional lymphocytes in FOP patients.

4. Routine immunizations (iatrogenic activation of the immune system) lead to heterotopic ossification of skeletal muscle at the injection site in FOP patients but not in normal controls.
5. Cells derived from circulating lymphocytes in FOP patients exhibit dysregulation of BMP signaling.

These data suggest that the lymphocytes are an informative model cell relevant to the early molecular pathology and histopathology of FOP. The readily available and safely obtainable lymphocytes from peripheral blood (through routine venipuncture) can be immortalized in the laboratory and used for studies in an animal-based system.

In order to determine the ability of FOP lymphoblastoid cells to induce FOP lesions, we subcutaneously implanted lymphoblastoid cells obtained from FOP patients and from unaffected family members into athymic nude mice (immune compromised mice that will not reject cells from a different species such as human). Cells from unaffected individuals either did not grow or formed small masses with little evidence of a fibrotic or angiogenic response. In dramatic contrast, cells from FOP patients gave rise to solid tumor-like masses in the animals.

Histopathologic evaluation of these lesions indicated that FOP cells induced angiogenesis and a fibrotic response in the host mouse, similar in appearance to early FOP lesions. FOP-like cell-induced lesions were probed for human-specific genetic sequences, confirming that the cellular masses contained human cells as well as host cells. These results suggest that cells of FOP patient origin induce changes in cell growth and/or differentiation and mimic events in early FOP lesions. In 2003, we began using the noggin gene therapy system (described later in this report) to test the responsiveness of the lymphocyte-induced lesion formation to the BMP antagonist noggin. The

implantation of FOP-derived cells in nude mice is beginning to provide a useful cell model system for examining the early stages of FOP lesion formation, and is being studied as an intermediary model system for testing potential therapies.

Multiple Hereditary Exostoses and FOP: Exploring the Connections

Imagine for a moment the surface membrane of a cell - a dense bubble studded with receptors that grab hormones, growth factors, morphogens, and signaling molecules from its immediate environment and after binding them transmit messages to the inside of the cell in a molecular relay that turns on and turns off downstream targets. Interspersed between the receptors on the cell surface are channel proteins - molecular gateways that act as a series of dams and locks to let water molecules and ions in and out of the cell after small expenditures of energy. Now imagine for a moment that around this cell-specific molecular machinery of receptors and channels is a dense forest comprised of proteins anchored to the cell membrane at right angles like trees that dwarf the protein channels and receptors on the forest floor below. Spread from these protein trunks, is a dense network of perpendicular branches composed of long chains of repeating sugar molecules. These large and numerous tall tree-like molecules with their dense network of sugar-like branches are called cell-surface heparan sulfate proteoglycans (HSPGs). The sugar molecules which extend like large branches off of the protein cores are rich in sulfate groups that allow for the nesting of growth factors, hormones, and morphogens. Among the growth factors, hormones, and morphogens that have a particular affinity for the sulfated branches of these giant tree-like molecules are the fibroblast growth factors,

the hedgehog proteins, and the bone morphogenetic proteins and their antagonists noggin and gremlin.

The tall tree-like protein trunks of the HSPGs that support this vast canopy of sulfated sugar branches are called syndecans and glypicans and are encoded by genes of the same names. The sugar branches are not encoded by genes, but their assembly on the protein trunk is regulated by enzymes encoded by genes called EXT1 and EXT2. The addition of sulfate groups to the sugar-like branches is also regulated by enzymes and turns them into molecular velcro for the various morphogens and morphogen antagonists that adhere to them.

These HSPGs (the tall tree-like protein cores with their densely sulfated sugar-like branches) are regulators of molecular encounters on the cell membrane. Many HSPGs act as co-receptors in morphogen signaling. These HSPGs have recently received a great deal of attention in BMP signaling and cancer biology and have come to the forefront of attention in bone development. Recent evidence also supports that some HSPGs act as co-receptors in BMP signaling and play an extremely important role in establishing and regulating BMP morphogen gradients by regulating the binding of noggin, a potent BMP antagonist. Thus, it appears that HSPGs may play an extremely important role in regulating the access of morphogens such as BMP4 to its cell surface receptors.

Heterozygous mutations in EXT1 and EXT2, genes that encode the enzymes that catalyze the assembly of the heparan sulfate branches on the HSPGs are the proximate cause of

multiple hereditary exostoses (MHE). Exostoses (also known as osteochondromas) are benign endochondral bone tumors with a cartilaginous cap that grow off the shaft of bones near the growth plates. Patients with multiple hereditary exostoses (MHE) have many such lesions throughout their skeletons, and may suffer chronic pain, immobility, and profound growth disturbances from them.

While much has been written about FOP, it is still a poorly known fact that patients with FOP have a predilection for developing multiple osteochondromas similar to those seen in patients with MHE (although they are usually more limited in number and distribution in FOP). Most patients with FOP develop symmetrical osteochondromas around the knees and also in other locations in a more variable pattern.

At present, it remains unclear how the resultant cellular and molecular anomalies in heparan sulfate proteoglycan (HSPG) metabolism lead to the specific pathology experienced by patients who have MHE and by implication, FOP. It is believed, however, that an abnormality in the ability of HSPGs to appropriately bind and sequester morphogens such as bone morphogenetic proteins and their antagonists may play a critical role in the development of these benign endochondral tumors. The genes encoding EXT1 and EXT2, the enzymes that catalyze the assembly of the sulfate-brimming sugar-like branches on the HSPG protein cores, function as tumor suppressor genes. The loss of one copy of the gene in the germline leads to an increased susceptibility to developing osteochondromas. The loss of the second copy of the gene in perichondral cells near the growth plate enables the formation of these benign

osteochondral tumors.

Although the primary genetic damage responsible for FOP is unknown, cells from FOP patients exhibit numerous defects in the BMP signaling pathway including the inability to respond appropriately to BMP4 and its secreted antagonists, noggin and gremlin. BMP4 and its secreted antagonists are HSPG-binding proteins. The HSPGs with their projecting network of sulfated sugar-like branches that bind BMP4 and noggin and other morphogen-like molecules thus act normally as potent regulators of BMP signaling. Thus, it is reasonable to wonder whether the inability of FOP cells to respond appropriately to both BMP4 and its secreted antagonists may be the result of changes in either the composition or concentration of cell surface HSPGs on FOP cells.

The possibility of a relationship between FOP and MHE at the molecular level is intriguing. There are many similarities between FOP and MHE. It is not difficult to separate the two conditions or to make a definitive diagnosis of one or the other in any given individual. However, patients with MHE and FOP share many common clinical features as noted above. It is also possible that they may share a common link at the molecular level that could help illuminate important pathophysiological mysteries that still shroud both conditions.

Late in 2003, we began a series of experiments to explore the molecular profile of HSPGs on FOP cells and control cells to determine if there were any differences in their ability to bind BMP4 and its antagonists. While it is far too early to make any definitive comments

about these studies, it is possible that these molecules will play an important primary or secondary role in the abnormal BMP4 morphogen gradients of FOP cells. During the next year, we will continue to focus our attention on this new, intriguing and vitally important aspect of FOP research.

Cancer and FOP: Misdiagnosis and Missed Opportunity: Thinking Outside of Everything

In the strict sense of the word, FOP lesions are "tumors" or "neoplasms" – new growths that should not be where they are. In addition, the osteochondromas (exostoses) that form around the knee in FOP patients are also benign tumors of endochondral origin. Despite this semantic digression into lesions, neoplasms, tumors, lumps, and bumps, patients with FOP are often misdiagnosed as having cancer. While the most common misdiagnosis in patients who have FOP is aggressive juvenile fibromatosis (a benign but aggressive tumor), many patients with FOP are dangerously misdiagnosed as having fibrosarcoma, chondrosarcoma or osteosarcoma (all highly malignant tumors) depending upon the maturational state of the FOP lesion at the time the biopsy was obtained.

While FOP lesions are aggressive in that they destroy the surrounding skeletal muscle and replace it with bone, the bone formation that results eventually stops. However, the histological and pathological appearance of the lesion under the microscope may appear (to the untrained eye) as a malignant lesion, especially during the early stages of formation. Thus, two facts about FOP are abundantly clear: 1. FOP is an example of a dysregulated but controlled tissue repair process that in many ways resembles cancer, and

2. FOP is **not** cancer. While we can take solace in the fact that FOP, despite the renegade regenerative response that results, is not a malignant tumor, the old adage that in every problem there is a promise, also rings true. We should not lose sight of the fact that there are similarities at the cellular and molecular level between cancer cells and FOP cells that could help us better understand both processes. Exploring the similarities and differences between FOP cells and cancer cells provides an opportunity to think creatively to help us better understand the pathophysiology of FOP.

In a recent review article on the origins of cancer in the July 2003 edition of **Scientific American**, W. Wayt Gibbs succinctly describes the six diabolical super powers of cancer. Interestingly, four of these superpowers are intimately shared with FOP.

1. Cancer cells and FOP cells grow even in the absence of normal "go" signals. Most normal cells wait for external messages before dividing, but cancer cells and FOP cells share the property of counterfeiting their own pro-growth messages. Most FOP lesions are triggered by an external injury that stimulates the BMP signaling pathway which is severely deranged in FOP cells. Thus, FOP cells, like cancer cells, possess aberrant "go" signals. What are the normal "go signals" that allows a bone to form or regenerate? And, how exactly are those signals deranged in FOP? In the case of a cancer cell, it may be a promiscuous tyrosine kinase receptor that dysregulates cell division. In the case of FOP, it may be a promiscuous serine-threonine kinase receptor (BMP receptor due to renegade BMP receptor trafficking) that dysregulates the inductive signals for ossification.

2. Cancer cells grow despite "stop" commands issued by neighboring cells. In FOP cells, those "stop" commands, the production and secretion of BMP antagonists like noggin and gremlin, are mysteriously switched off or ineffective. Eventually however, FOP lesion formation does cease, and occasionally early lesions spontaneously regress. What are the signals from neighboring cells that allow FOP lesions to stop growing, and what are the molecular signals that allow an early lesion to spontaneously regress? Such clues from the domain of cancer cells might help us more effectively arrest an early FOP lesion prior to the formation of bone so that FOP became more of a nuisance rather than a lifelong disabling problem.

3. Cancer cells evade built-in auto destruct mechanisms. In healthy cells, genetic damage above a critical level usually activates an apoptosis or cell suicide program. Cancer cells bypass this mechanism. FOP cells occasionally bypass this mechanism but at other times do not. This apoptosis or cell suicide program is hard-wired into the molecular machinery of the cell. Under what circumstances do FOP cells call upon their suicide program to allow lesion regression prior to its development into a mature piece of heterotopic bone? What are the molecular events that drive the activation of these cell suicide programs in FOP cells and how can they be more effectively stimulated to allow lesion autodestruct signals to occur?

4. Cancer cells and FOP cells both have the ability to stimulate blood vessel formation that fuels FOP lesions or tumor growth. Are the mechanisms by which angiogenesis is stimulated in cancer cells and FOP cells the same? Are the same types of "tumor" or "lesional" blood vessel cells stimulated in both conditions, or are they different? Can endothelial cells be differentially regulated in cancer and in FOP?

5. Cancer cells (but not FOP cells) are immortal. They can divide endlessly and have no reproductive limit. Although FOP cells appear to grow uncontrollably at times during early lesion formation, they are completely mortal, and have relatively normal reproductive capacities in culture. While mutations in cell cycle regulatory genes are common in cancer, there is no suggestion that the same genes are primarily disturbed in FOP cells.

6. Cancer cells can invade other tissues and spread to other organs. BdAc type stem cells in FOP appear to have the capacity to circulate, to adhere, and to form bone. What are the chemoattractive molecules by which FOP BdACs home to damaged tissues? How are those chemoattractive molecules similar or different in the Tie-2 positive stem cell of skeletal muscle?, or in the invasive lymphocytes seen in early FOP lesions?

Thus, while FOP cells are distinctly different from cancer cells in that FOP cells are not immortal and have the ability to stop growing and undergo terminal differentiation, they

clearly share many properties of cancer cells such as growth in the absence of normal "go" signals, the disabling of normal "stop" signals, a partial evasion of built-in autodestruct mechanisms, the ability to stimulate blood vessel formation (angiogenesis), and the power to invade other tissues with each new flare-up.

The discussion of the similarities and differences between FOP cells and cancer cells is presented not for the purpose of trying to force cancer or FOP into niches into which they do not belong, but in order to begin to explore similarities and differences at the molecular level that will help us better understand FOP. There is an enormous wealth of information in gene regulation, receptor processing, molecular pathway circuitry, stem cell biology, and signal transduction inhibitor development from the field of cancer research that can be brought to bear on outstanding issues of FOP. Furthermore, the juxtaposition of cellular properties between FOP cells and cancer cells stimulate many important questions that can lead to engaging experiments in the field of FOP research.

V. TRIGGERS

The Immune System and FOP

Mounting evidence from all levels of investigation suggests involvement of the immune system in FOP. The presence of lymphocytes and mast cells in early FOP lesions, lymphocyte-associated death of skeletal muscle, flare-ups following viral infections and immunizations, the intermittent timing of flare-ups and the beneficial response of early flare-ups to corticosteroids are all important pieces of evidence to support involvement of the immune system in the pathogenesis of FOP flare-ups. Some have also indicated that

the clinical and pathological features of FOP suggest an autoimmune component to the condition, perhaps an autoimmune trigger.

Autoimmune diseases, with the exception of rheumatoid arthritis and autoimmune thyroid inflammation, are individually rare, but together affect approximately 5 percent of the population in western countries. Autoimmune disorders are a fascinating but poorly understood group of diseases. An autoimmune disease is defined as a clinical condition caused by the activation of T-lymphocytes or B-lymphocytes or both in the absence of an ongoing infection or other discernible cause. Genetic susceptibility, environmental and internal triggers of autoreactivity, changes in pathologic processes as the disease progresses, and multiple mechanisms of tissue injury have been considered as causes of autoimmune disorders. A useful division of autoimmune diseases distinguishes between those in which there is a general alteration in the selection, regulation, or death of T-lymphocytes or B-lymphocytes and those in which an aberrant response to a particular antigen causes autoimmunity.

Lymphocyte-Endothelial Cell Interaction: Early Markers of Inflammation

The migration of lymphocytes from an intravascular location to a location just outside of the endothelial cell membrane is the earliest microscopically-observed event in an FOP flare-up. How does the lymphocyte leave the blood vessel and gain access to the skeletal muscle where subsequent death of skeletal muscle cells occur? Integrins, cellular sensors that act as signaling molecules, are expressed by most lymphocytes. Integrins interact with integrin receptors such as vascular-cell adhesion molecules on the surface of

endothelial cells to regulate the infiltration of lymphocytes into solid organs such as muscle. Alpha-4 integrin, a glycoprotein, is expressed on the surface of activated lymphocytes and monocytes and plays a critical role in their adhesion to the vascular endothelium and in their subsequent migration into various organs.

We are currently investigating the identity of these integrin markers on lymphocytes and endothelial cells in the limited FOP tissue that we have available. Identification of specific integrins and other cell surface molecules on activated lymphocytes in FOP lesions could provide important therapeutic targets for pharmacologically available humanized monoclonal antibodies at the earliest stages of an FOP lesion**.** Humanized monoclonal antibodies directed against B-cell or T-cell surface markers, or against the overabundant and constitutively active BMP receptors (or receptor-associated proteins) may also provide important targets for therapy. This will continue to be an important topic of investigation in 2004, and you will be hearing more about it in future reports.

VI. TREATMENTS

The ultimate goal of FOP research is to develop treatments that will prevent, halt and eventually reverse the progression of the condition.

BMP4 Antagonists: Paving the Way

In 2002, we described an important discovery in FOP research in the field of gene therapy. The discovery, more appropriately labeled “a proof of concept,” provides the foundation for ongoing work in this area. In order to present a comprehensive account of

this important work, as with other long-term projects in the laboratory, we plan each year to review the relevant background so that those new to this story will be able to more coherently follow its development and progress.

Following the discovery of the overexpression of BMP4 in FOP cells, Brigid Hogan, a distinguished developmental biologist from Vanderbilt University in Nashville, Tennessee wrote in the journal **Science**, *"With so much being discovered about how the BMPs act, it might be possible to develop drugs that would block some part of the BMP4 pathway-and therefore prevent the progression of what is a horrible, nightmare disease."*

Noggin: Blocking the BMP4 Pathway

The protein noggin, discovered by our collaborator Dr. Richard Harland from The University of California–Berkeley, was known to have powerful effects on antagonizing BMP4 activity. The day after our discovery of the overexpression of BMP4 was published in **The New England Journal of Medicine,** Dr. Harland and his colleagues published a seminal paper in the journal **Cell** showing that the noggin protein directly binds to the native BMP4 molecule and prevents it from interacting with its own receptor. The importance of noggin to the FOP story became apparent immediately, and noggin was brought to the forefront of development for FOP treatment.

Noggin is involved in controlling the amount of skeleton that is formed by regulating the amount of BMP4 available to the body's tissues. For this reason, noggin offers promise for controlling the rampant bone growth of FOP. Soon after the discovery of BMP4 overexpression in FOP, we were contacted by

scientists from Regeneron Pharmaceuticals suggesting that a collaboration be established between the FOP laboratory, Dr Harland's laboratory, the scientists involved in the development of gene therapy vectors, and the scientists at Regeneron Pharmaceuticals, to explore the possibility of developing noggin as a treatment for FOP. We have been working together since then.

A Proof-of-Concept Experiment In Noggin Gene Therapy

Several years ago, we began a critical "proof-of-concept" experiment. The results of this experiment demonstrated dramatically that a genetically-engineered form of the protein noggin can be produced by the mammalian liver after targeted delivery of the modified noggin gene and circulate systemically to completely block BMP4-induced heterotopic ossification in the mouse.

The purpose of the study was to develop an effective gene therapy approach for the prevention of BMP4-induced heterotopic ossification that would be applicable in principle to patients with FOP. To achieve this goal, we used an adenovirus–mediated transfer of a genetically-modified noggin gene.

Naturally occurring noggin protein does not circulate in the bloodstream, and has a very short half-life in the body. The reason that noggin does not circulate through the blood is that the noggin protein sticks to complex sugars (the heparan sulfate proteoglycans or HSPGs) on blood vessel walls. (These are the same molecules that play a role in the development of osteochondromas due to their stickiness for noggin). Such "stickiness" of noggin would not be amenable to treating a disease like FOP. Therefore, if noggin is to be applicable for systemic (body-wide) delivery, the gene encoding the noggin protein first must

be modified to allow the noggin protein to circulate in the blood and to have a long half-life in the circulation.

The native human noggin gene was modified by removing the DNA sequences that encode the portion of the protein that allows it to stick to blood vessel walls. This modification of the noggin gene was accomplished, and the protein produced by the modified gene was able to circulate through the blood vessels without sticking to the vessel walls while still retaining its ability to bind and inactivate the BMP4 molecule. Furthermore, this modification of the noggin gene and its associated protein increased the half-life of noggin within the circulation, making it even more desirable for systemic delivery.

The mouse model of BMP4-induced heterotopic bone formation was used in the definitive experiment. This model system, described earlier in this annual report and published in 2003, allowed us to identify the various stages of BMP4-induced bone formation that closely mimic the stages of bone formation of an FOP lesion.

The implants containing recombinant human BMP4 induced an aggressive fibroproliferative lesion with early cartilage formation at seven days and heterotopic ossification at 14 days in control animals. However, in animals pre-treated with the modified noggin gene, the BMP4 implants caused no lesion formation at either seven days or 14 days.

This remarkable study demonstrates that the delivery of a modified noggin protein through a systemic gene therapy approach successfully prevents BMP4-induced heterotopic ossification in a mouse model. It provides “proof-of-

concept" that a genetically-modified morphogen antagonist (noggin) can be produced *in vivo* and act systemically to prevent BMP4-mediated heterotopic ossification that is clinically relevant to the treatment of FOP.

This major breakthrough in advanced therapeutics proves the concept that the noggin gene can be modified to permit systemic delivery of the biologically engineered protein, that the gene can be inserted into a viral delivery vector, that the liver can act as a factory for producing an active form of the modified noggin protein, that the modified noggin protein can circulate systemically as a hormone, and that at therapeutic concentrations, the modified noggin protein can effectively bind and inactivate ambient levels of recombinant human BMP4 and completely block the formation of even the most rudimentary FOP-like lesion.

Major Noggin Therapy Paper Published in 2003

A major scientific paper describing the "proof-of-concept" of noggin gene therapy was published in the December 2003 edition of **The Journal of Bone and Joint Surgery.** The paper is entitled, "In Vivo Somatic Cell Gene Transfer of an Engineered Noggin Mutein Prevents BMP4-Induced Heterotopic Ossification." The authors of the paper are David L. Glaser, M.D., Frederick S. Kaplan, M.D., and Eileen M. Shore, Ph.D. from The Center for Research in FOP and Related Disorders; Lili Wong, Ph.D. and James M. Wilson, M.D., Ph.D., from The Center For Gene Therapy in The Division of Medical Genetics, The University of Pennsylvania School of Medicine; and Aris N. Economides, Ph.D., Xia Liu, M.S., Robert D. Kimball, Ph.D., James P. Fandel, Ph.D., and Neil Stahl., Ph.D., from Regeneron Pharmaceuticals.

A recent editorial also published in **The Journal of Bone and Joint Surgery** highlighted the work from the FOP laboratory over the past several years. Dr. Lawrence C. Rosenberg, M.D., Deputy Editor for Research of the **Journal of Bone and Joint Surgery** wrote, "BMP signal transduction is regulated by BMP antagonists such as noggin, which bind to BMP extracellularly and block the binding of BMP to its receptors. Recently, it was shown that BMP4 is over-expressed in patients with fibrodysplasia ossificans progressiva, and that whereas cells from normal human subjects exhibit increased expression of BMP antagonists on exposure to high concentrations of BMP4, cells from patients with FOP do not. These observations led to the development of a procedure involving gene transfer of an engineered noggin mutein that prevents BMP4-induced heterotopic ossification in a mouse. Thus, studies that shed light on the pathogenesis of a genetic disorder in humans were based on knowledge of the molecular mechanisms that mediate and regulate BMP activity. Results of these studies led in turn to the development of a new modality of therapy to prevent pathological ossification. This information provides the foundation for the development of new modalities of treatment for skeletal disease."

Our paper in the December edition of the **Journal of Bone and Joint Surgery** highlights these accomplishments and developments, and excerpts from the paper are presented below to help clarify the discoveries and to share the excitement of the work:

"BMP4, a potent skeletal morphogen is overexpressed in cells of patients who have FOP. Additionally, noggin, a secreted protein that functions as a high-affinity antagonist of BMP2, BMP4, and several other BMPs, is underexpressed in the cells of patients who have FOP. Therefore, a rational strategy for the treatment of the disease involves inhibition of BMP4 activity or its signal transduction pathways.

Noggin acts by binding BMPs and preventing the interaction of the BMPs with their transmembrane receptors. Like BMP4 and most osteoinductive BMPs, noggin binds heparan sulfate proteoglycans and binds BMP at the site of noggin expression.

During the embryonic development of vertebrates, noggin expression and secretion is triggered by BMP signaling and acts to define the boundaries of BMP-induced structures. As part of the negative feedback system, BMPs regulate the expression of noggin in a variety of in vitro and in vivo systems. Complete deletion of the noggin gene in mice is lethal soon after birth because of multiple defects. Examination of the skeleton of noggin deficient mice revealed that the lack of noggin expression leads to exuberant heterotopic ossification in utero, fusion of the chest wall, and failure of joint formation throughout the body.

The postnatal role of noggin is less well-defined. BMP4 is produced by bone cells and muscle cells, and its expression is increased at sites of soft-tissue injury. Under normal circumstances, BMP4 upregulates the expression of BMP antagonists such as noggin and gremlin. *In vitro* experiments with the use of cells derived from patients with FOP show

a markedly attenuated response of noggin expression to BMP4 stimulation. An inadequate BMP antagonist response following soft tissue trauma would permit the rapid expansion of the BMP morphogenetic gradient in a patient with FOP and could explain the explosive bone induction seen during FOP flare-ups. These findings in a rare and disabling genetic disorder illustrate the importance of a critical balance between an inductive morphogen such as BMP4 and its secreted antagonists in the formation of an ectopic organ system and suggest the potential for the development of BMP antagonist-based strategies for the treatment of FOP.

We hypothesized that noggin may be useful in the treatment of FOP and other diseases involving abnormal bone formation by blocking an osteoinductive signal produced from exuberant BMP signaling. However, the native form of noggin does not circulate systemically. In order to be useful for the treatment of refractory disorders of widespread heterotopic ossification such as FOP, noggin must first be able to circulate systemically and to actively sequester BMP4 at distant targets. We report here the engineering of a circulating form of noggin and furthermore demonstrate that this noggin mutein (the term "mutein" refers to a protein with altered amino acid sequence usually importing novel properties compared with a wild-type protein) can be delivered *in vivo* either by systemic administration or by somatic cell gene transfer to block BMP4-induced heterotopic ossification in a mouse model of fibrodysplasia ossificans progressiva. The study demonstrates that BMP4-induced heterotopic ossification can be completely blocked by a noggin mutein and provides proof-of-concept for its application in the treatment of BMP-mediated heterotopic ossifications such as in FOP.

The results reported in the present study support a potential clinical utility of a noggin mutein in diseases for which heterotopic ossification cannot be prevented or treated with other approaches. An extreme example of this, of course, is FOP and was the motivation for this study. Although FOP is very rare, other clinical situations of generalized and disabling heterotopic ossification often occur following injuries to the central nervous system or following hip replacement surgery, repeated muscle injury, or burns. Pathologic expression of osteogenic BMPs may also play a role in the bone metastasis seen in prostate cancer and in osteosarcomas. More recently, cranial synostosis has been shown to result from a lack of noggin-expression in cranial sutures that normally stay open. Reconstitution of noggin expression using the noggin mutein or application of noggin protein results in the patency of the cranial sutures. Therefore, there are many potential clinical applications of noggin as a therapeutic protein.

Naturally, before noggin or other BMP antagonists may be considered as potential therapeutic agents in patients, more research and development needs to be performed to determine the effects of their long-term systemic administration on bone as well as on other organs. The effects of noggin overexpression in the skeleton of developing mice results in severe osteoporosis as well as spontaneous fractures, indicating that blocking BMPs during the development and maturation of the skeleton is detrimental to proper bone formation. It should be noted, however, that noggin expression in these transgenic mice wanes by six months of age and, although their bones still display reduced mineral density, there is a lack of new fractures, indicating that some form of healing does take

places as noggin levels start to fall. In addition, we previously showed that noggin inhibits intramembraneous ossification in a model of bone regeneration. Considered together with the fact that BMPs have numerous effects, and are expressed in and presumably act on many different tissues, use of their antagonists as potential therapeutic agents should be contemplated only after extremely careful toxicology studies have been performed.

The availability of a potent and systemically deliverable BMP antagonist, such as the noggin mutein, opens new opportunities for the study of the biologic BMPs. The field of BMP antagonists is not limited to noggin. Other classes of BMPs inhibitors have been identified: the follistatin family, the chordin family, and the DAN/Cerberus family. These molecules each demonstrate unique binding properties, interacting with different subsets of BMPs and displaying different affinities for their cognate BMPs. The potential use of these molecules remain to be explored.

In summary, this study demonstrates that noggin can be used to block BMP-induced heterotopic bone formation in vivo, either by local administration or by means of the systemic delivery of a noggin mutein through somatic cell gene transfer. This study provides proof-of-concept that a secreted morphogen antagonist that normally acts locally can be engineered so that it could act systemically. In the present investigation, it was used to prevent heterotopic ossification in an animal model but is clinically relevant to catastrophic disorders of progressive heterotopic ossification in humans, specifically FOP.

The development of improved animal models based upon improved knowledge of the molecular genetics of FOP, and the development of safe and effective viral vectors with inducible promoters for the systemic and durable delivery of noggin muteins may ultimately lead to more effective treatments for heterotopic ossification. For patients who have FOP, a safe, stable, and regulated gene transfer of an inducible BMP4 antagonist such as noggin may offer a solution when all other modalities have failed."

Noggin Gene Therapy: Recent Developments

There have been six major developments in noggin gene therapy since completion of the groundbreaking "proof-of-concept" experiments (previous section):

1. The development of a highly sensitive and specific radioimmunoassay for measuring the concentration of modified noggin protein in the blood.
2. The exploration, development and use of safer viral vectors (adeno-associated viruses) for systemic noggin gene delivery.
3. Studies to determine if the genetically-engineered form of noggin causes organ-specific toxicity in the mouse.
4. Studies to determine the potential of noggin gene therapy to inhibit early FOP-like lesions induced by FOP lymphoblastoid cells.
5. Exploration of specific cell-based delivery systems for genetically engineered noggin gene therapy.
6. The exploration of collaborative ventures with industry partners to use highly-sophisticated inducible promoters to regulate the timing and dose of noggin gene therapy in BMP4-induced, lymphoblastoid-cell induced, and blood-derived adherent cell-induced (BdAC) FOP lesions.

The development of a highly sensitive and specific radioimmunoassay for measuring the concentration of the genetically engineered noggin protein in the blood is in place and necessary for all future experimental work in animals and eventually in humans. This work was performed in the core FOP laboratories. The noggin radioimmunoassay is critical not only for determining the level of modified noggin protein in the circulation of the experimental animals, but also in determining whether or not the mice are developing antibodies to the modified noggin protein. It is possible that the mouse may, over time, develop antibodies to the native or genetically-modified human noggin protein. Although the human and mouse noggin proteins are extremely similar in sequence, they are perhaps different enough to elicit an antibody response that could confound the analysis.

Of greater concern, however, for human studies, will be whether or not the modification of the native human noggin gene elicits an antibody response in patients. The act of truncating the noggin gene to encode a modified but circulating noggin protein (noggin mutein) that is missing its heparin-binding domain leads to a molecule that has a slightly different shape and confirmation in three-dimensional space than the native noggin protein. Such a modified protein might be recognized as a foreign protein by the human immune system. As a result, detailed and intensive immunological studies will eventually be necessary for further development of noggin gene therapy in humans. We are well aware of those considerations, and they are already figuring largely into our thinking about the design of a more definitive FOP therapy. For example, it might be possible to target the native noggin gene (rather than the modified noggin gene) to the exact cells in skeletal muscle such as the Tie-2 positive cells or perhaps to blood derived adherent stem cells where the FOP lesions may

begin. Emerging knowledge of those cell types described earlier in this report might enable such an approach in the future.

The development and use of safer viral vectors for noggin gene delivery will be critical in considering whether the noggin gene or a modified noggin gene can be administered via a viral-based delivery system for human use. Although adenovirus is an excellent delivery vehicle for demonstrating proof-of-concept of noggin gene therapy, the adenovirus vector elicits an intense systemic immune response and is unacceptable for long-term human use.

Our colleagues in the Division of Human Genetics at Penn have isolated new adeno-associated viruses and are developing them as vectors for human gene therapy. In general, adeno-associated viruses are much less toxic systemically (but still have some toxicity) and elicit a much less robust reaction from the immune system. The hope was that these novel adeno-associated viruses could be developed as vectors for human gene therapy, that they would have improved efficiencies of gene transfer, and that they would not be recognized by antibodies generated to adeno-associated virus infections in humans. That, in fact, was the case. We continue to use these new delivery vectors in our modified noggin gene therapy experiments this past year. We are pleased to report that they are extremely effective in delivering adequate doses of the modified noggin protein systemically to inhibit heterotopic ossification in an animal model.

These novel adeno-associated viruses are much less toxic to the mice than the original adenoviruses we used, and they elicit a much weaker inflammatory response, especially in the liver where the modified noggin protein is

manufactured. Also, this safer viral gene delivery vehicle is necessary in animal studies in order to examine and isolate any potential toxic effects of the modified noggin protein at high-sustained dosage levels. In other words, it is first necessary to minimize the toxic effects of the delivery vehicle (virus), which carries the modified noggin gene into the body before we can effectively study any possible toxic effects of the modified noggin protein itself. The studies to determine whether these novel adeno-associated viruses could be used to deliver the modified noggin protein at high enough levels to inhibit heterotopic ossification were extremely successful.

Preliminary toxicity studies in the mice using the novel adeno-associated virus (AAV) vector delivery system indicate that although the adeno-associated virus vector induces less inflammation in the liver than the adenovirus, the noggin protein and modified noggin protein themselves may be toxic at high-sustained dosages. These findings are compatible with studies showing that BMPs are important in the development and maintenance of the liver. Thus, if noggin is to be considered for human use, it must be precisely regulated and be kept at the lowest effective levels possible for the briefest periods of time to inhibit heterotopic ossification. As a result, it is even more compelling now that we explore regulated and inducible systems for the delivery of BMP antagonists (such as noggin) and then target the noggin gene therapy to cells that are either direct targets of BMP action or to cells in the vicinity of the BMP targets.

Earlier in this Annual Report (Section III. Cells), we discussed important breakthroughs in determining cellular targets of BMP action within skeletal muscle as well as in circulating stem cells (BdACs). These new and exciting discoveries in the cell biology of heterotopic ossification will be exceedingly

important in designing the most appropriate cell-based therapies to target gene therapy. Ultimately, an inducible promoter for the native noggin gene might best be targeted to those exact cells that would respond to the BMP4 signal within skeletal muscle (or in the peripheral blood following the recent discovery of the BdACs) and that begins the transformation into bone. While the laboratory findings and treatment investigations are often presented in separate sections of the annual report, the overall integrated nature of the FOP research should not escape anyone's attention.

Presently, we are exploring these new cell-based gene delivery options while at the same time collaborating with colleagues to acquire and modify an inducible promoter for the novel adeno-associated virus noggin gene delivery. We are currently working with attorneys and scientists at two pharmaceutical companies to facilitate the acquisition of an inducible promoter (a drug-regulated molecular switch) that can be used in the next phase of the noggin gene therapy studies.

Noggin Gene Therapy: The Future

While the possibility of using a form of noggin gene therapy to treat FOP remains promising, there are numerous challenges – some specific to FOP and some general to the field of gene therapy – that need to be solved before gene therapy can become a reality. The two most critical questions regarding FOP are the sensitivity of the pathway target and the specificity of the target cell. While BMP4 overexpression is a reasonable target for a gene therapy approach, better and more sensitive pathway targets will likely emerge as we learn more about the specific nature of the gene mutation that causes FOP and the resultant abnormalities in BMP receptor trafficking and signaling. Imagine, for example, that one is sailing along an unknown coastline for which there are no available

maps. From a distance, one spots what may look like a reasonable port to anchor the ship. However, as one more closely approaches the shore, better anchorages may come into sight that were not visible from a greater distance.

When one considers the possibility of gene therapy for FOP, one must always consider the specificity of the target cell. Is it better to distribute noggin throughout the whole body, or is it better to specifically target it to those cells either within the skeletal muscle or perhaps at a distant site in the bone marrow that harbor the stem cells responding to the renegade BMP4 pathway signals?

Another FOP-specific consideration in any potential gene therapy program is the selectivity of the molecule to be used. While recombinant native noggin may evade the body's immune system, it might not effectively reach the target sites in the stem cells. A noggin mutein, in contrast, might circulate and reach specific target cells that might be detected by the immune system and be inactivated over time. Only further testing will determine which may be the better approach. Another FOP-specific concern is the safety of the molecule to be delivered. While it is clear that too much BMP4 may lead to excessive bone formation, too much noggin may severely impair normal bone formation.

Since noggin is an antagonist to multiple BMPs, and BMPs subserve important roles outside of the skeletal system, too much noggin may impair the function of other organs. In essence, while too much BMP is bad for the body, too much noggin is also bad for the body. The body does not seem to like excessive amounts of anything, and likes its critical pathway-regulating molecules to be in balance. The challenge, therefore, will be to understand the exact cause of the imbalance in BMP trafficking and signaling, and restore it in the most safe and

effective manner without delivering any molecule in greater concentration than the body needs, wants, or can tolerate. Only with meticulous studies using an inducible promoter for noggin in conjunction with a more specific animal model for FOP, will we be able to determine the safest and most effective regimen for blocking FOP lesion formation.

In addition to FOP-specific concerns, such as sensitivity of the pathway target, specificity of the target cell, selectivity of the therapeutic molecule, and safety of the delivered molecule, there are gene therapy specific concerns regarding the safety of the delivery vehicle that must be addressed in the wider scientific community before gene therapy can be safely and effectively used in humans.

An editorial in the August 2003 edition of **Nature Medicine**, addresses the specific concerns of gene delivery. The editorial states: "The field of gene therapy has endured a roller-coaster ride of impending promise and grave setbacks. Despite the latter, experts remain optimistic that applied therapeutics for human disease are imminent. But to realize the full potential of gene therapy, there are some fundamental hurdles that must first be overcome. The limiting factor that hampers the application of all of these approaches is the pragmatic concern of gene delivery. The challenge of specifically and efficiently directing the therapeutic molecule to the site of action continues to impair effective translation from the laboratory to the clinic.

The critical importance of the vector system used in gene therapy is starkly illustrated by recent tragedies in clinical trials. The death of Jesse Gelsinger in 1990, was directly attributable to the viral vector used to deliver a functional gene. Gelsinger suffered a massive inflammatory response to the viral vector,

resulting in his death. More recently, two children who have undergone gene therapy to correct fatal x-linked severe combined immunodeficiency developed a leukemia-like disorder. The retroviral vector used to introduce a functional gene into the patient's T-lymphocytes had inserted into an oncogene (cancer-producing gene) giving the cells a huge selective growth advantage and leading to the leukemia-like disease.

New modalities for safe and efficient gene restoration are now under active development, but chromosomal integration of the therapeutic effector molecules to provide persistent gene expression remains a key focus of these efforts. Non-viral vectors are being developed and transposons (jumping genes) with site-specific enzymes that allow integration may refine chromosomal targeting. A wide variety of vectors that are inert and innocuous is needed; the ultimate choice will depend on the specific disease.

But, vector-host interactions will continue to be an overriding concern. Complication due to immune responses to the viral capsid proteins are among the biggest obstacles, but other problems must also be addressed. Given that human outcomes rarely correlate with animal model data, this will require validation in human systems. Currently, these essential toxicology studies compete poorly for funding. The U.S. National Institutes of Health and its counterparts elsewhere need to drive the research agenda, support innovated research into new vector systems, and dedicate funds for the more mundane pre-clinical trials that are crucial to investigating vector potential in humans. Not until safe, efficient, and specific delivery systems are in hand will gene therapy be able to realize all of its promise.

In summary, numerous major technical obstacles must be overcome before noggin gene therapy or any of its modifications could be considered for the treatment of patients who have FOP. These include the refinement of safe and effective viral vectors for use in humans, the successful development of inducible and regulated delivery of the noggin gene, complete toxicity studies on systemically-administered noggin and modified noggin, and ultimately the development of improved animal models based upon knowledge of the molecular genetics of FOP.

Molecular biologists will help to design vectors capable of cell-specific and tissue-specific expression of the noggin gene. Immunologists will continue to develop ways to prevent unwanted immunological consequences of the viral delivery vehicles and their modified noggin cargo. Cell biologists will devise ways to facilitate gene transfer to various tissues and will take the lead in identifying muscle or blood stem cells to which the vector can be introduced. Clinicians will carry out clinical trials on patients with FOP with the best vectors that the scientists can supply. To achieve successful gene therapy in patients, many branches of biology and medicine must contribute to this endeavor.

It is not yet possible to determine when or even if noggin gene therapy will become a practical clinical reality for children and adults who have FOP, but it is presently our best hope and we will continue to pursue it relentlessly until it either becomes a reality or until better solutions emerge. Whether or not noggin gene therapy will eventually be realized in patients with FOP depends on many major technical hurdles mentioned above and many scientific hurdles, which have been outlined earlier in this Annual Report. What is most important is that the FOP gene be determined, that all of the target stem cells be identified, and

that the molecular pathways be elucidated. Clearly then, the best treatment for FOP will emerge. For the meanwhile, we will move ahead with our experimentation on noggin gene therapy, as it is the best hope we now have.

Noggin: Crystal Clear

In related work, our colleague Dr. Jay Groppe, an x-ray crystallographer working at The Salk Institute in La Jolla, California published a major article in the journal **Nature** (December 2002) on the x-ray crystallographic (molecular 3D) structure of the noggin protein as it is bound to a BMP molecule. The work is an extraordinary technical tour-de-force. In the article entitled, **"Structural Basis of BMP Signaling Inhibition by Noggin, a Novel Cystine Knot Protein,"** Dr. Groppe and colleagues discuss how the interplay between bone morphogenetic proteins and their antagonists govern numerous developmental and cellular processes in the human body and throughout the animal kingdom. In the article, they show exactly and elegantly how the three-dimensional structure of noggin enables it to inhibit BMP signaling by blocking the interaction of BMP with both of its transmembrane receptors. They further demonstrate that the binding of BMP to noggin sequesters the BMP molecule in an inactive complex.

In a special visit to The Center for Research in FOP and Related Disorders in late November 2002, Dr. Groppe explained how noggin and various BMPs appeared to have evolved from a common ancestral gene. The proteins encoded by the BMP genes and the noggin gene are highly related not only in structure but in shape. In fact, when noggin binds to BMP, it binds in a back-to-back fashion with both molecules in a planar configuration. "It is as if two butterflies are linked back-to-back with the larger noggin molecule holding the smaller

BMP molecule in a full Nelson," Dr. Groppe explained.

Dr. Groppe went on to show how both BMP and noggin have heparin-binding domains – amino acid residues in their respective proteins that allow the molecules to bind to cell-surface heparan sulfate proteoglycans (HSPGs) that concentrate the molecules near the site of cell signaling at the membrane surface. Most importantly, he showed how the heparin- binding sites of both BMP and noggin are at opposite ends of the molecules from which the two molecules bind to each other, thus allowing the heparin-binding sites of each molecule to be attached to respective cell surfaces. This has enormous implications for the regulation of BMP signaling.

Even more importantly, Dr. Groppe showed graphically and dramatically how the molecular modification to remove the heparin-binding site from the noggin gene would not at all affect the ability of the molecule to bind and sequester BMPs, an important confirmation for our modified noggin gene therapy experiments described in the previous section. Experimental evidence at the biochemical level from our noggin gene therapy work has shown that Dr. Groppe's hypothesis was correct. The beauty and power of the x-ray crystallography studies showed why in fact that was true.

In an even more recent article on the structure of noggin entitled, "Structural Basis of BMP Signaling Inhibition by Noggin, a Novel Twelve-Membered Cysteine Knot Protein," published in October 2003, in the **Journal of Bone and Joint Surgery,** Dr. Groppe specifically noted the important relationship that his work has to the study of FOP. He noted that the structure of noggin provided the basis for engineering variants of noggin that may have therapeutic

applications in the treatment of FOP. Dr Groppe stated, "FOP is a rare genetic disorder of connective tissue, diagnosed in fewer than 150 patients in the United States, resulting from misexpression of BMPs by lymphocytes. This misexpression often occurs at sites of inflammation and leads to conversion of muscle to bone. Lack of noggin is not causative of FOP, as no mutations in the noggin locus of patients have been identified despite rigorous analyses at multiple laboratories. However, a variant of noggin lacking the heparin-binding site, constructed without the advantage of the three-dimensional structure of noggin, has shown great promise as a potential therapeutic agent against BMP-induced heterotopic ossification in a mouse model of FOP. A second generation of heparin-binding site deletion mutants can now be engineered on the basis of the crystal structure of noggin. These mutants should possess increased stability, which will enhance their potential as therapeutic agents by lowering the levels that must be administered to patients who have FOP."

Taken together, Dr. Groppe's work has profound implications for BMP4 signaling and for noggin gene therapy in FOP as well as for biologists in numerous related fields of molecular, developmental, and structural biology.

Anti-angiogenic Agents

Development and growth of the human embryo as well as growth and regression of many tumors is dependent on the control of new blood vessel formation (angiogenesis). Angiogenesis is also an absolute requirement for the formation and development of the skeleton, for the successful healing of fractures, and for the formation of heterotopic bone. The early stages of skeletal embryogenesis and fracture healing correspond to the highly vascularized pre-osseous fibroproliferative lesions seen in FOP. Angiogenesis, a

prominent histopathologic feature of pre-osseous FOP lesions, thus becomes a potential target for therapy.

Basic fibroblast growth factor (bFGF), a heparin-binding endothelial cell growth factor, is an extremely potent *in vivo* stimulator of angiogenesis, and has been implicated in the growth of solid tumors. bFGF has been investigated in FOP patients to determine if it is implicated in the pre-osseous lesions. Urinary bFGF levels are markedly elevated in patients who have FOP, especially during acute flare-ups of the disease process. In contrast, elevations of urinary bFGF were not detected during times of disease quiescence. These data suggested that urinary bFGF may be a biochemical marker for disease flare-ups in FOP patients and provided a biochemical basis for considering anti-angiogenic therapy at early stages of the disease process.

Squalamine, a new anti-angiogenic agent, with potential interest for FOP, was discovered in 1992 in the FOP laboratory by Dr. Michael Zasloff. Dr. Zasloff isolated squalamine from the body tissues of the dogfish shark, and discovered its anti-angiogenic properties by accident. Squalamine is a naturally occurring cholesterol-like molecule that inhibits the proliferation of endothelial cells (blood vessel cells) and exhibits potent anti-angiogenic activity in laboratory animals and humans. Squalamine modifies the response of endothelial cells to proteins that organize their shape and structure.

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. By directly blocking the angiogenic process, squalamine has the potential to slow the progression of the FOP lesions in muscle.

A phase I clinical trial of squalamine in FOP was established and targeted to a small group of adult FOP patients who were having severe pre-osseous flare-ups. The initial study was designed to evaluate the safety and efficacy of intravenous squalamine on the inhibition of angiogenesis, and permitted enrollment of no more than 10 adult patients with FOP. The study was fully approved in 2001 by The Food & Drug Administration of The United States, The Institutional Review Board of The University of Pennsylvania, The Clinical Research Center of the Hospital of the University of Pennsylvania, The Radiation Safety Board and The Clinical Studies Monitoring Unit of The University of Pennsylvania School of Medicine. However, due to lack of enrolment, the study was postponed by the pharmaceutical sponsor after two years. Factors included the complexity of the study design, the difficulty of patient travel to Philadelphia in the prescribed time period of seven days following the onset of a flare up, and the inability to enroll children in the study due to the lack of approval by the FDA to allow the drug to be studied in children who have FOP. The pharmaceutical sponsor is proceeding with its studies of squalamine in macular degeneration, a vascular disorder affecting vision, and will need to perform reproductive toxicity testing before squalamine could be studied in the pediatric FOP population.

Despite the postponement of the squalamine trial due to the lack of enrolment, angiogenesis may potentially be minimized with numerous other anti-angiogenic agents such as aminobisphosphonates, thalidomide, cycloxygenase-2 (cox-2) inhibitors, vascular endothelial growth factor traps, and humanized monoclonal antibodies directed against vascular endothelial growth factor (VEGF). At present, several of these agents are in pre-clinical development or early phase I clinical studies. Guidelines for the off-label use of several of these agents (aminobisphosphonates and cox-2 inhibitors) in patients with FOP can be found in the text and summary tables of the FOP Treatment Guidelines available on the IFOPA website at: www.ifopa.org.

Prostaglandins & the Cox-2 Inhibitors: Inflammation & FOP

During the past several years, an important new category of drugs has emerged with previously unexpected and important implications for the treatment of FOP. These are the cyclo-oxygenase-2 (cox-2) inhibitors, medications that specifically target pro-inflammatory prostaglandins.

The body essentially produces two types of prostaglandins: "physiological" prostaglandins and "inflammatory" prostaglandins. Physiological prostaglandins are normally produced in many of the body's tissues and protect organs, such as the stomach, from metabolic injury. Inflammatory prostaglandins are produced in response to injury, and play a major role in the inflammatory response to injury. Traditional non-steroidal anti-inflammatory drugs such as aspirin, ibuprofen and indomethacin inhibit the formation of both the physiological and inflammatory prostaglandins. The new cyclo-oxygenase 2 (cox-2) inhibitors primarily inhibit the inflammatory prostaglandins and leave the physiological prostaglandins relatively intact.

Inflammatory prostaglandins are potent co-stimulatory molecules along with BMPs in the induction of heterotopic bone. Studies in the orthopaedic literature have shown that lowering prostaglandin levels in experimental animals dramatically raises the threshold for heterotopic ossification, thus, making it more difficult for bone to form. Animals pretreated with prostaglandin inhibitors failed to form heterotopic bone following intramuscular injections of BMP-containing demineralized bone matrix. In contrast, animals treated with prostaglandin inhibitors co-incident with or after a demineralized bone matrix injection still formed heterotopic bone. These data suggest, that in order for prostaglandin inhibitors to be truly effective in preventing heterotopic ossification, the medication must be "in the system" (in other words circulating in the blood at the therapeutic levels) before a bone-induction signal occurred. In addition to their potent

anti-inflammatory properties, a recent study unexpectedly demonstrated that cox-2 inhibitors have potent anti-angiogenic properties as well as anti-inflammatory properties, a feature that makes them even more desirable for consideration in FOP.

An important paper published in 2002 by colleagues from The University of Rochester showed convincingly that animals genetically engineered to lack both copies of the gene encoding the cox-2 enzyme (cox-2 knockouts) failed to generate new bone formation at a fracture site, thus demonstrating the importance of the cox-2 enzyme in inflammatory bone formation. While pharmacologic doses of cox-2 inhibitors (medications that block the activity of the cox-2 enzyme) given to normal animals had a similar effect, the inhibition of bone formation in both sets of animals (cox-2 knockouts and animals treated with cox-2 inhibitors) could be overcome with massive amounts of recombinant BMP, indicating that cox-2 activity occurs upstream of BMP signaling and that intense overactivity of the BMP pathway (as can be seen in FOP) could plausibly overcome a cox-2 blockade. Similar results were reported in a separate study published in 2002 by a research group led by a former FOP Laboratory fellow who now works at The University of Medicine and Dentistry of New Jersey.

Inflammatory prostaglandin levels are dramatically elevated in the serum of patients who have FOP, especially during times of a disease flare-up. Inflammatory prostaglandins directly stimulate the induction of angiogenic peptides which can further promote the osteogenic process. These observations suggest the following hypothesis: lowering baseline prostaglandin levels in patients with FOP may raise the threshold for heterotopic ossification even in the presence of substantial endogenous levels of BMP4. This hypothesis is amenable to clinical testing and will be the focus of a placebo-controlled randomized double-blind study (the first of its kind) to assess the safety and efficacy of cox-2 inhibitors in the prevention of FOP flare-ups.

While the potential benefit of the new cox-2 inhibitors in preventing heterotopic ossification is no greater than the parent class of non-steroidal anti-inflammatory medications, the new cox-2 inhibitors offer the possibility of a lower gastrointestinal risk profile than the parent compounds. In addition, the half-life of some of the new cox-2 inhibitors is conducive to a once-daily dosage regimen, a factor which helps promote patient compliance.

While the cox-2 inhibitors are generally safe, their action must be carefully monitored, especially in those who are taking the medications for long periods of time, as rare but life-threatening side-effects and kidney-damaging effects can occur. As with any condition, the relative risks and benefits of potential therapies must be weighed against the potential risks of the underlying condition being treated.

Cox-2 inhibitors are available by prescription. They are currently being tested in children with rheumatoid arthritis, and are being used sporadically by pediatric specialists for the treatment of severe inflammatory conditions such as FOP where few other treatment options exist. Presently, a placebo-controlled study of one of the cox-2 inhibitors has been designed and is awaiting final approval. The study is designed to determine whether this new class of medications may be truly beneficial in preventing FOP flare-ups. The work on the cox-2 inhibitors integrates important findings from the FOP laboratory on prostaglandin production, mast cell recruitment, and angiogenic factor release with the pathologic findings of severe inflammatory pre-osseous lesions of FOP.

Aminobisphosphonates: A 2004 Update

Bisphosphonates are a potent class of medications that have profound effects on

bone remodeling and exert their primary effect by decreasing the life span of osteoclasts. Bisphosphonates are thus widely used in the treatment of numerous bone diseases where bone resorption exceeds bone formation -- disorders such as osteoporosis, osteogenesis imperfecta, Paget's disease, fibrous dysplasia, and bone cancer.

The first clinically used bisphosphonate, Etidronate, when administered at high doses, also potently inhibits mineralization of newly formed cartilage and bone protein and had been proposed as a possible treatment for FOP and other disorders of heterotopic ossification as far back as 30 years ago.

Etidronate has been studied for FOP because of its inhibitory effect on bone mineralization and its potential to impair ossification at high dosages. Unfortunately, at high doses, it also causes osteomalacia (soft bones) and impairs ossification of the entire skeletal system, not just the heterotopic bone of the "second skeleton." Its utility is therefore extremely limited.

In a published study, the effects of intravenously administered Etidronate and oral corticosteroids were evaluated. Thirty-one FOP flare-ups were observed in seven patients during a mean follow-up of 6 years. In 29 flare-ups, the authors observed a rapid diminution of local inflammation, swelling, and pain during the first 7 days of treatment. However, despite the Etidronate treatment, 10 new ossifications were observed, causing severe deterioration of joint mobility in all

affected patients. In 21 flare-ups, no new ectopic ossification appeared. The radiologic pattern of pre-existing ossifications did not change during the treatment. The results suggest the possibility that intravenous administration of Etidronate and oral corticosteroids may be helpful, but more control data on the spontaneous resolution of early flare-ups are needed. While high-dose Etidronate temporarily inhibits mineralization, the newer bisphosphonates do not possess this activity. At the present time, we do not use Etidronate regularly for the treatment of FOP.

While its effectiveness in FOP is uncertain, Etidronate has enjoyed limited use in the treatment of more focal disorders of heterotopic ossification such as those that arise following soft tissue trauma or injuries to the central nervous system. Unlike Etidronate, the newer bisphosphonates (including the aminobisphosphonates) have no appreciable affect on inhibiting mineralization, but are hundreds to thousands of times more potent than Etidronate in inhibiting bone resorption, a property that dictates their current utility in a wide range of bone diseases characterized by excessive bone resorption.

So, why would the newer aminobisphosphonates, which act primarily to inhibit bone resorption, even be considered in the context of FOP, a condition where decreased bone resorption (at least in the heterotopic skeleton) would not be desirable? At first glance, there would appear to be little rational use for compounds such as the newer aminobisphosphonates in the treatment of FOP.

However, the story is not that simple.

All medications have side-effects, but it is an interesting sidelight of medical practice that, on occasion, medications have been used either mistakenly or coincidentally with unanticipated beneficial effects. Very often, a new use for an old medication is discovered serendipitously or accidentally only after a medication has been released for a specific use.

Such a scenario occurred recently with the use of the aminobisphosphonates in the treatment of FOP. Several credible and anecdotal reports (to FSK & DLG) from physicians and FOP patients worldwide highlighted the response of FOP flare-ups to Pamidronate, one of the newer aminobisphosphonates. One of these reports from Dr. Mordechai Weiss, M.D., Chief of The Endocrine Institute of Assaf Harofeh Medical Center in Zerifin, Israel and his FOP patient, Dr. Orly Doron-Goldstein, a molecular biologist at The Weizmann Institute of Science in Rehovot, Israel, documented a particularly poignant and brilliant set of clinical observations on the use of Pamidronate in FOP flare-ups. But, why would Pamidronate even be considered for the treatment of FOP flare-ups? Ironically, in all three cases reported to us, the medication had been used with the mistaken belief that Pamidronate was more potent than Etidronate in inhibiting mineralization. It is not. None of the newer bisphosphonates including Pamidronate have any effect on inhibiting mineralization. Nevertheless, all three patients and their physicians independently reported substantially

decreased swelling, redness, and pain following high dose intravenous Pamidronate administration during a new flare-up. In one patient, the Pamidronate was administered alone, while in the other two patients, it was administered along with an oral steroid (such as Prednisone) for several days during the early phases of a new FOP flare-up.

All of us in the FOP community know that such anecdotal observations could be purely coincidental - that is, that the flare-ups might have receded spontaneously without treatment and that the Pamidronate might have had nothing to do whatsoever with the reported improvement, especially since oral glucocorticoids were used intercurrently in two of the three FOP patients. Also, one cannot discount a potent placebo effect in any uncontrolled observation. Nevertheless, we also know that such observations of potential improvement in an FOP flare-up cannot be ignored. It is entirely possible to stumble on something worthwhile even for the wrong reason!

As word of this Pamidronate-associated response (with or without steroids) spread rapidly throughout the FOP community in the past year (generally by internet communications among patients and families), 21 patients (in consultation with us and their local physicians), have used Pamidronate empirically (either alone or with steroids) for the treatment of acute flare-ups, especially those involving major joints. In 16 of the 21 patients (76%), there was reported improvement in the symptoms and signs of an FOP flare-up. In five of

the 21 patients (24%), there was no reported improvement in the symptoms or signs of the flare-up by either the physician or the patient. Interestingly, there seemed to be no protective effect whatsoever on the occurrence of subsequent flare-ups in any of the patients treated with either a single dose or a 3-day course of intravenous Pamidronate. Therefore, whatever improvement there may have been was transient and affected only the lesion present at the time of the flare-up. While these patient reports are not scientifically valid, they constitute an important set of anecdotal observations that compel further stringent scientific inquiry in controlled laboratory and clinical studies.

The treatment protocols varied slightly between the patients (depending on age, body weight, and site of involvement) but in general were similar. Treatment protocols were based upon published guidelines for children and adolescents with osteogenesis imperfecta as that group of patients constitutes the largest known group of children and adolescents in whom intravenous Pamidronate has been used. The three-day cycle of intravenous Pamidronate treatment should be repeated only during flare-ups involving the major joints and no more than 4 times annually. The Pamidronate should be administered as early following the appearance of the flare-up as possible and preferably within the first 48 hours.

Oral corticosteroids (prednisone) can be added to the treatment regimen according to the guidelines listed in the **FOP Treatment Guidelines** (www.ifopa.org). In general, oral corticosteroids are administered concurrently

for four days for the treatment of flare-ups involving major peripheral joints, the jaw, or the submandibular region. Corticosteroids are generally not used in conjunction with Pamidronate for flare-ups involving the neck, back, or chest as the timing of the onset of flare-ups in those areas is generally more difficult to determine and the reported success of prednisone for flare-ups in those regions has been more equivocal than for flare-ups in the major peripheral joints. The combined use of prednisone and Pamidronate for flare-ups in the trunk and back has therefore not been systematically assessed and has been tried in only one child with some anecdotal success.

For treatment of acute flare-ups involving major peripheral joints, most of the patients reported treatment with a 4-day course of oral prednisone in conjunction with a 3-day cycle of IV Pamidronate. Side-effects of the intravenous Pamidronate infusions in the FOP patients included flu-like symptoms of fever, chills, and muscle aches. These symptoms can often be lessened by pre-treatment with acetaminophen. One patient developed tetany (uncontrolled muscle contractions due to a low vitamin D level in the blood prior to Pamidronate therapy), and one patient developed peripheral phlebitis (inflammation of the vein) at the intravenous infusion site, which required inpatient intravenous antibiotic treatment. A recent case report from 2003 published in **The New England Journal of Medicine** documented the development of osteopetrosis (marble bone disease) in a child treated with 60 mgs. of IV Pamidronate every three weeks for two years. The child did not

have FOP.

Insight and support for the use of Pamidronate in FOP was provided in 2003 by a study in children and adolescents with osteogenesis imperfecta (O.I). Treatment with cyclical intravenous Pamidronate infusions (3-4 cycles annually) has led to substantial improvements in the clinical management of children and adolescents with O.I., with generalized increases in bone density and dramatically fewer resultant fractures throughout the skeleton. Despite its well-known beneficial effects on skeletal remodeling and bone strength, the effects of Pamidronate on the new endochondral skeletogenesis of the type that would occur at a fracture site, have not been well characterized. In an extensive study, Dr. Francis Glorieux and colleagues at the Shriner's Hospital for Children and McGill University in Montreal showed that incomplete fracture healing in patients with O.I. was more than twice as frequent when Pamidronate therapy had been started before the fracture occurred. Furthermore, delayed osteotomy healing was almost four times more frequent when Pamidronate had been started before surgery. The study demonstrated that cyclical intravenous Pamidronate therapy was associated with a significant delay in fracture healing and osteotomy healing in children and adolescents with O.I. Although the study was conducted for entirely different reasons and in a different patient population than FOP, the study provides support for the hypothesis that Pamidronate can increase bone density and decrease fracture incidence in the normotopic skeleton through its effect on bone remodeling, while simultaneously inhibiting

endochondral skeletogenesis at orthotopic sites.

An important question that these observations from routine clinical care of FOP patients raises is: What might be the physiologic basis for any potential beneficial effect of aminobisphosphonates in the treatment of FOP flare-ups? As a consequence of their potent inhibition of bone resorption, the aminobisphosphonates effectively inhibit the release of growth factors and morphogens (such as BMPs) which are stored in the extracellular bone matrix of the skeleton. The action of the bisphosphonates on the suppression of bone resorption is exceedingly long, longer than for any other class of medications, and is on the order of months to years. Therefore, if aminobisphosphonates inhibited FOP lesions by decreasing the release of BMPs sequestered in the skeleton, one would expect a more pronounced effect on the prevention of subsequent flare-ups which was not seen in the patients treated. Clearly, if the aminobisphosphonates are truly beneficial in the treatment of FOP flare-ups, there must be a mechanism of action that is very brief and substantially different from that of osteoclast inhibition from which the medication derives its beneficial effects in the normotopic skeleton.

All bisphosphonates have an affinity for sites of normal and pathological mineralization. The latter effect plausibly explains the avid uptake of bisphosphonates at sites of severe skeletal muscle injury where calcium is released from the mitochondria and sarcoplasmic reticulum of dying muscle cells. This seminal property of all bisphosphonates to home to

areas of normal and pathological mineralization like molecular stealth missiles suggests plausible mechanism of bisphosphonate sequestration at sites of early FOP lesions where muscle cells are dying. If bisphosphonates are in fact sequestered at sites of early FOP flare-ups as suggested by radionucleide bone scans, the bisphosphonates would be biologically available to a wide variety of target cells (lymphocytes, mast cells, fibroproliferative cells, angiogenic cells) that compose the early developmental stages of an FOP lesion. Once internalized by a target cell (not yet determined for FOP lesional cells), the potent aminobisphosphonates such as Pamidronate will disrupt the mevalonate pathway by specifically inhibiting the activity of the farnesyl diphosphate synthase enzyme within the cell. As a result of this enzymatic inhibition, the target cell is rendered incapable of post-translational prenylation (a type of protein modification) of small GTPases such as Ras, Raf, and Rac which are essential for cellular activity. Consequently, target cells are rendered functionally inactive and undergo apoptotic cell death.

While the potential mechanism of action of the aminobisphosphonates on early FOP lesions or BMP-induced FOP-like lesions remains speculative, recently published papers provide some additional tantalizing clues. These papers, published in the peer-reviewed cancer literature, document the extremely potent antiangiogenic effects (decreased new blood vessel formation) of Pamidronate and zoledronic acid (Zoledronate) *in vitro* and *in vivo.* In one paper, Pamidronate administered intravenously was shown to dramatically decrease vascular endothelial growth factor (VEGF) levels and basic fibroblast growth

factor (bFGF) levels in cancer patients with bone metastasis. Both VEGF and basic FGF are potent tumor–associated angiogenesis factors. In another paper published in the basic science literature in October 2003, zoledronic acid was reported to impair the adhesion, migration, and survival of endothelial cells, and sensitized endothelial cells to tumor necrosis factor-induced programmed cell death. Tumor necrosis factor is, in fact, one of the molecules stimulated by the aminobisphosphonates and one of the primary culprits of the flu-like symptoms experienced following intravenous administration of the bisphosphonates. Furthermore, the study showed that zoledronic acid impaired endothelial cell activity by suppressing the sustained activation of focal adhesion kinase and protein kinase B/Akt, an important inter-cellular signaling pathway not previously implicated in aminobisphosphonate activity.

The effect of Pamidronate and other aminobisphosphonates on inhibiting angiogenesis in mice was totally unanticipated but not surprising in light of the extraordinarily potent effects of these medications as adjuvant therapies in the treatment of various cancers. Potential anti-angiogenic effects of Pamidronate in FOP are also compatible with the known brief half-life of the medication in the circulation prior to its long-term stable deposition in the skeleton and could explain why the medication may have an effect on active lesions but not on the prevention of new lesions.

Intravenous Pamidronate has also been shown to modulate the gamma-delta lymphocyte subpopulation in the circulation, which is responsible for the medication's dose-related side-effects of causing flu-like symptoms. We cannot yet rule-out the possibility that Pamidronate may affect the early lymphocytic infiltration into skeletal muscle seen in both BMP4-induced FOP-like lesions and in FOP lesions themselves.

Other possible mechanisms by which Pamidronate might affect FOP lesions include a direct inhibition on the proliferation of a rapidly dividing population of cells. Such an effect was noted recently in a study investigating the effects of aminobisphosphonates on cancer cells *in vitro*. It is certainly possible that Pamidronate may affect one or more cell types including mesenchymal progenitor cells, endothelial cells, or even BdACs in an early FOP lesion.

While the mechanism for Pamidronate's action on fracture healing remains to be determined, the potent inhibition of matrix metalloproteinase (MMP) activity by the bisphosphonates is a likely contributing factor. Studies published in 2003 showed that aminobisphosphonates are potent inhibitors of nearly all of the matrix metalloproteinases (MMPs). Bone formation and skeletal regeneration is severely impaired in the absence of specific MMPs. It remains to be seen in FOP and in appropriate animal models of BMP-induced heterotopic ossification, whether cyclical infusions of Pamidronate or the more potent aminobisphosphonate zoledronic acid (Zoledronate) can impair endochondral skeletogenesis at heterotopic sites. Pre-clinical laboratory studies investigating

the role of aminobisphosphonates in inhibiting BMP-induced heterotopic ossification were begun in January 2004 following approval by The Institutional Animal Care and Use Committee.

Despite hopes to the contrary, one must consider the stark possibility there may be no positive effects whatsoever of the Pamidronate on FOP lesions and that the reports to date are the results of observational bias and/or coincidence. Only rigorous controlled laboratory investigations *in vitro* and *in vivo*, as well as placebo–controlled clinical trials will be able to definitively decipher these possibilities and provide a solid rational basis for determining whether or not one or more of the aminobisphosphonates may have a beneficial role in the treatment of FOP.

Finally, apart from their postulated and observed effect on endochondral skeletogenesis, the use of the aminobisphosphonates could be considered in any FOP patient who is being treated chronically and intermittently with high dose glucocorticoids for new FOP flare-ups. The aminobisphosphonates generally have an excellent safety and efficacy profile in protecting the normotopic skeleton from the profound osteopenic effects of intermittent high-dose glucocorticoids in the type of regimen that is frequently used to manage acute flare-ups of FOP.

Laboratory studies to assess both the potential therapeutic benefit and potential

mechanism of action of aminobisphosphonates in the model of BMP-induced heterotopic ossification as well as in the model of lymphoblastoid cell implantation (discussed earlier in this report) are being conducted in the FOP laboratory. Simultaneously, a controlled clinical study is being designed to assess the potential benefit of intravenous aminobisphosphonates in the treatment of acute FOP flare-ups. The study will then be subject to rigorous approval by numerous regulatory Investigational Review Boards at The University of Pennsylvania, The Children's Hospital of Philadelphia, the pharmaceutical company co-sponsoring the study, and the FDA, as well as numerous local review boards associated with the private practices where a child or an adult may be seen and treated. We would like to design the study so that it could be carried-out at multiple sites and so that transportation to Philadelphia will not be an issue. While the logistics of this may be difficult, we will work on it.

The results of such a carefully controlled clinical study would almost immediately give us better insight into the potential efficacy of the aminobisphosphonates in the treatment of FOP flare-ups. However, as mentioned above, it is extremely important to have rigorous controlled observations in order to understand the potential use of this medication (and other aminobisphosphonates) for the long-term.

Will Pamidronate or the newer generation of the aminobisphosphonates (such as

Zoledronate, not yet approved for use in children) show efficacy for the treatment of FOP? Only time and rigorous experimental approaches will provide clear answers to that question. While Noggin gene therapy and related approaches such as the development of BMP receptor antagonists, BMP pathway antagonists, BMP signal transduction inhibitors, and monoclonal antibodies directed against activated lymphocytes or components of the BMP signaling pathway may eventually prove to be more definitive in the ultimate treatment and prevention of FOP, we hope that the use of more immediately available medications such as glucocorticoids, leukotriene inhibitors, mast cell inhibitors, cox-2 inhibitors, and perhaps the aminobisphosphonates will allow us to buy time for FOP patients. As Jeri Licht, the mother of Daniel Licht stated so eloquently and passionately in the **BBC** documentary, ***The Skeleton Key***, "They need to slow down the progression of this condition and slow down or stop the formation of the bone once the flare-up starts. Then they'll have the time, and we'll have the luxury to have them look for a cure for the condition completely."

VII. PRESENTATIONS, MEETINGS, REPORTS, AND PUBLICATIONS

During 2003, we were privileged to present major lectures on FOP at:

- Baylor College of Medicine; Houston, Texas
- Beth Israel Deaconess Medical Center; Boston, Massachusetts
- Campbell Clinic; Memphis, Tennessee
- Dallas Children's Hospital; Dallas, Texas
- Harvard University; Cambridge, Massachusetts
- Indiana University – Purdue University School of Medicine; Indianapolis, Indiana
- Johns Hopkins University School of Medicine; Baltimore, Maryland
- Johnson & Johnson Research Foundation; New Brunswick, New Jersey

- KinderKlinik & RheumaKinderKlinik; Garmisch-PartenKirchen, Germany
- Loyola University; Stritch School of Medicine; Chicago, Illinois
- Mayo Clinic; Rochester, Minnesota
- Northwestern University; Feinberg School of Medicine; Chicago, Illinois
- Orthopaedic Research Society; New Orleans, Louisiana
- Rush University Medical Center; Chicago, Illinois
- Stanford University School of Medicine; Stanford, California
- Texas Scottish Rite Hospital for Children; Dallas, Texas
- University of Alabama School of Medicine; Birmingham, Alabama
- University of Chicago, Pritzker School of Medicine; Chicago, Illinois
- University of Iowa, Roy & Lucille Carver College of Medicine; Iowa City, Iowa
- University of Medicine & Dentistry of New Jersey, Robert Wood Johnson Medical School; Piscataway, New Jersey
- University of Minnesota School of Medicine; Minneapolis, Minnesota
- University of Santa Casa de Misericordia School of Medicine; Sao Paulo, Brazil
- University of Saskatchewan College of Medicine; Saskatoon, Saskatchewan, Canada
- University of Tennessee Health Science Center; Memphis, Tennessee
- University of Texas Southwestern Medical Center; Dallas, Texas
- University of Western Ontario Faculty of Medicine and Dentistry; London, Ontario, Canada
- University of Winnipeg School of Medicine; Winnipeg, Manitoba, Canada

During 2003, we were honored to present highlights of FOP research at major regional, national, and international FOP family meetings and gatherings in:

- Aberdeen, Scotland
- Fontenay-aux-Roses, France

- Montclair, New Jersey
- Orlando, Florida
- Philadelphia, Pennsylvania
- Santa Maria, California
- Sao Paulo, Brazil
- Sausalito, California
- Valbert, Germany

Six major presentations on FOP were made by members of the FOP Laboratory at The American Society for Bone and Mineral Research (ASBMR) in Minneapolis, Minnesota in September 2003. Several of these papers were presented at Plenary Sessions of the meeting which were attended by over 6,000 physicians and scientists worldwide. As always, it was a wonderful opportunity to present major highlights and discoveries from the FOP and POH laboratory to an extraordinarily large and distinguished group of scientists and physicians worldwide.

Last year, for the first time, Harvard University experimented with a novel program in undergraduate education. Under the direction of Dr. William Gelbart, Professor of Molecular and Developmental Biology at Harvard University, a major new freshman biology class was introduced into the curriculum. Unlike most introductory biology courses taught throughout the world, which follow a similar formula of starting with molecules and cells and building in complexity, Dr. Gelbart's course entitled, "Genes and Genomes" embarked on a stunning new educational adventure. The course adopted a novel approach of teaching biology and genetics through the example of a small selection of human genetic diseases. The entire curriculum of the class was centered around six genetic disorders. One of the six chosen for intense study was FOP.

We are extremely honored and grateful to have FOP as a major focus of this novel freshman biology course at Harvard University and we were honored to present a major lecture on FOP to Dr. Gelbart's freshman biology class at Harvard College. The enthusiasm of the students was extraordinary and several have already approached us about the opportunity for summer research positions in the FOP laboratory.

Dr. Gelbart is the discoverer of the decapentaplegic (dpp) gene, the Drosophila homologue of BMP2 and BMP4, as well as the discoverer of major downstream signaling molecules in the BMP signaling pathway. Dr. Gelbart has been an important collaborator in FOP research from its very inception and is a wonderful friend of the FOP community. Dr. Gelbart appeared in the award-winning **BBC *Horizon*** documentary of FOP research in 1999.

We are grateful to Dr. Gelbart for his inclusion of FOP in the curriculum of the introductory freshman biology course at Harvard University, and we are excited about the opportunity of introducing the principles of FOP research to the minds of young, brilliant, and enthusiastic students at all levels of the academic program.

Center for Research in FOP & Related Disorders

Writing in the **Scientist**, John Trojanowski, M.D, Ph.D., Director of The Institute on Aging at The University of Pennsylvania, stated: "Most of today's biological problems require multidisciplinary research strategies that entail the use of multiple, diverse methodologies that no single investigator can master. The most tangible benefit of Center and program grants may be the rapid and economical acquisition of insights into complex biological questions that result from the research conducted by dedicated investigators working synergistically." The Center for Research in FOP & Related Disorders was established several years ago by the Cali Family Endowment with the

same spirit in mind.

Since its inception, the Development Grants program of the Center for Research in FOP and Related Disorders has supported 12 novel projects relevant to our long-term mission. Already six major peer-reviewed papers have been published as a result of this support, and several other major papers are in preparation.

During the past year, the Developmental Grants Program of the Center for Research In FOP and Related Disorders has sponsored four major projects in affiliated laboratories at The University of Pennsylvania. Several of these projects have been highlighted in previous sections of this report (or in previous reports), and have already produced important results and insights for FOP research. Work sponsored by the Developmental Grants Program over the past several years has included studies to identify target cells in BMP-induced heterotopic ossification, studies to characterize the role of angiogenesis at two critical phases of skeletogenesis, work to develop new adeno-associated viral vectors for use in the noggin gene therapy experiments, studies to define the role of the fibroblast growth factor pathway in BMP antagonist signaling, work to clarify the role of neural crest stem cells in osteogenesis, studies to define the role of glucocorticoids in modulating the BMP-antagonist response to ambient BMP4 levels, and efforts to use microarray gene expression studies to determine the response of chondrocytes to fluoroquinolone-stimulated apoptotic signals in chondrocytes. The Center is presently sponsoring work to explore the use of zebrafish as a viable vertebrate animal model for FOP studies, and the extremely exciting work to define the role of blood derived adherent stem cells from FOP patients in heterotopic ossification.

We are fortunate to have an extraordinarily talented group of research collaborators working with us at The University of Pennsylvania, and we are delighted with the

progress and promise that this unique program supports. In May 2003, we held our second annual one-day scientific symposium at Penn for scientists who have received support through the Developmental Grants Program. In 2004, we will begin to explore opportunities to expand this work to investigators and potential collaborators at outside institutions.

The Medical Management of FOP - Online

In September, 2003, we published the second edition of **The Medical Management of Fibrodysplasia Ossificans Progressiva (FOP): Current Treatment Considerations.** The document, also known as **The FOP Treatment Guidelines**, is available on the IFOPA website (www.ifopa.org), can be easily downloaded, and is available to all interested individuals. Due to the gracious help of friends and translators, the document has been translated to French and Portuguese, and its translation to Spanish and Chinese is currently being worked on. We urge all of you who have not seen this document to read it and download it from the IFOPA website (www.ifopa.org). A hard copy can be obtained by contacting Dr. Kaplan's assistant, Kamlesh Rai at: kamlesh.rai@uphs.upenn.edu.

Papers in Press

In 2003, there were eleven major peer-reviewed publications on FOP and POH. Several papers are currently in press, many others are in preparation, and there are more to come!

VIII. YOUR FOP LABORATORY

FOP research is slowly becoming a Research Center with expandable walls. While the core FOP laboratory occupies approximately 2000 square feet of space, our walls have expanded during the past four years with the establishment of the Developmental Grants Program and the wonderful collaborations with colleagues in many departments and at several schools throughout The University of Pennsylvania. The pictures of our FOP and POH children adorn the hallways of our Core Laboratory and are a constant reminder of our goals and our mission. As we tell the children and adults who visit the FOP Center and Laboratory, "This is really ***your*** Center and Laboratory." We love when you come and visit.

During 2003, the staff of the FOP Core Research Laboratory included as many as 17 researchers: three principal investigators, five research specialists, four post-doctoral fellows, two graduate students, one medical student, and two pre-medical students. In addition, four undergraduate college students worked on FOP projects in the laboratory during the summer of 2003.

IX. ACKNOWLEDGEMENTS

In the war against FOP, all of us find ourselves involved in a great battle. The battlefield itself is small, but the consequences for our community are large, and the consequences for the larger world of those who suffer more common afflictions of the skeleton should not be underestimated.

These are hopeful times in our war against FOP. We are thankful that steady progress continues to be made. We are proud that important discoveries that are being made in the FOP core and collaborative laboratories are now beginning to be translated into clinical protocols and into pilot studies that can be tested in a stringent scientific manner. When a

cure is found, the war will be won. Until then, we will fight the war on all fronts until it is won. We will not tire; we will not yield; we will not relent.

As we have mentioned many times before, *cause* and *cure* are the two words that motivate us and provide the guiding principle for all that we do: to discover the exact genetic and molecular cause of FOP and to use that knowledge to develop effective treatments and eventually a cure. Our work continues to be highly focused and we believe that a great deal of our success to date has been due to an unrelenting focus on our goals. The FOP Center and core laboratory continues to be a unique world-wide resource for both patients and the medical community. We strive for excellence and leadership in all areas vital to our mission: patient care, education, and the generation of new knowledge.

In summary, 2003 was a year of major developments for FOP research and was highlighted by the addition of three critically-important multigenerational FOP families to the genome-wide linkage studies, by seminal discoveries on the dysregulation of BMP receptor signaling and trafficking in FOP cells, by important insight from the powerful FOP microarray experiments, by the identification of blood-derived adherent stem cells in FOP patients, by the validation and publication of new FOP animal model systems, by the identification of potential new drugs and drug targets and by the development of safer and potentially more effective viral delivery systems for noggin gene therapy in FOP. As before, we have much work yet to do. We are hopeful that 2004 will be a year of even greater milestones in FOP research and that exciting discoveries will highlight the year ahead.

The FOP community has charted a long and difficult journey over the past 13 years but it is amazing how far we have come. We continue to be, in fact, a robust and vibrant

community that spans the globe. We are united in our effort and we possess a momentum and verve to accomplish the goals we have set for ourselves. We are reminded each day that we have a long way yet to go to achieve those goals, but we are encouraged by our accomplishments and we are energized by our challenges.

As always, our heartfelt thanks go to the children, adults, and families who live with FOP every moment of their lives. Their equanimity and nobility provide the perpetual inspiration that dignifies this work and all who are privileged to participate in it.

The FOP Collaborative Research project arose out of a mutual desire to establish the cause and to find a cure for this disabling condition. We will accomplish that goal and we will prevail. As always, finding a cure for FOP is not a job, it is a mission.

All of us in the core FOP Laboratory, 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:

- The International FOP Association (IFOPA)
- The National Institutes of Health (The People of the United States of America)
- The Center for Research in FOP & Related Disorders
- The Cali Family Endowment for FOP Research
- The Weldon Family Endowment for FOP Research
- The Isaac and Rose Nassau Professorship of Orthopaedic Molecular Medicine
- The Allison Weiss Fellowship in Orthopaedic Molecular Medicine
- The Born-Lotke Zasloff Fellowship in Orthopaedic Molecular Medicine
- The Whitney Weldon - Stephen Roach Fellowship

- The Roemex Fellowship
- The Grampian Fellowship
- The Progressive Osseous Heteroplasia Association
- The Hartford Foundation
- The Medical Research Council and Oxford University (United Kingdom)
- The Association Française Contre Les Myopathies (France)
- Members of the FOP International Research Consortium
- Johnson & Johnson, Inc.
- Regeneron Pharmaceuticals
- The People of Santa Maria (11 years of extraordinary service)
- And the many individuals, families, and friends throughout the world who contribute generously and tirelessly to the FOP effort.

FOP continues to be one of the most obstacle-ridden and perplexing quandries of the human condition. We have often said that if it were as common a condition as muscular dystrophy or cancer, thousands of scientists and thousands of laboratories around the world would be working to solve the mystery of FOP. Despite its rarity, we truly believe that FOP research is the key to understanding not only FOP, but also many other more common conditions that affect the skeleton. It is fundamentally the skeleton key.

Thank you, as always, for your continued generous and heartfelt support of this vital effort.