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Critical Reviews™ in

Eukaryotic Gene Expression

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All articles are by invitation; however, the editors welcome suggestions of both topics and authors.

Critical Reviews™ in
Eukaryotic Gene Expression
Volume 20 / Issue 4 2010

TABLE OF CONTENTS

The cover shows schematic representation of the tentative neuro-inflammatory mechanism and its relationship to heterotopic ossification. See article in this issue by Salisbury et al, page 313.

Critical Reviews™ in Eukaryotic Gene Expression, 20(4):275–294 (2010)

Modulators of Androgen and Estrogen Receptor Activity

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ABSTRACT: This review focuses on significant recent findings regarding modulators of androgen and estrogen receptor activity. Selective androgen receptor modulators (SARMs) interact with androgen receptors (ARs), and selective estrogen receptor modulators (SERMs) interact with estrogen receptors (ERs), with variable tissue selectivity. SERMs, which interact with both ERα and ERβ in a tissue-specific manner to produce diverse outcomes in multiple tissues, continue to generate significant interest for clinical application. Development of SARMs for clinical application has been slower to date because of potential adverse effects, but these diverse compounds continue to be investigated for use in disorders in which modulation of the AR is important. SARMs have been investigated mostly at the basic and preclinical level to date, with few human clinical trials published. These compounds have been evaluated mostly for application in different stages of prostate cancer to date, but they hold promise for multiple other applications. Publication of the large STAR and RUTH clinical trials demonstrated that the SERMs tamoxifen and raloxifene have interesting similarities and differences in tissues that contain ERs. Lasofoxifene, bazedoxifene, and arzoxifene are newer SERMs that have been demonstrated in clinical trials to more potently increase bone mineral density and lower serum cholesterol values than tamoxifen or raloxifene. Both SARMs and SERMs hold great promise for therapeutic use in multiple disorders in which tissue-specific effects are mediated by their respective receptors.

KEY WORDS: SARM, SERM, tamoxifen, raloxifene, lasofoxifene, bazedoxifene, arzoxifene, osteoporosis, breast cancer, prostate cancer

I. INTRODUCTION

Selective androgen and estrogen receptor modulators have continued to show significant new benefits for management of human diseases in recent years. Selective androgen receptor (AR) modulators (SARMs) are used to prevent or treat prostate cancer in men.[1,2] Selective estrogen receptor (ER) modulators (SERMs) have been shown to prevent fractures and reduce loss of bone mineral density (BMD) in postmenopausal women, prevent or treat ER-positive breast cancer in postmenopausal women, and modulate ovulation in premenopausal women with infertility.[3,4] Therapeutic applications for SARMs and SERMs are still being evaluated, for potential additional uses.[5]

This review will first briefly review the physiology of the AR, and then discuss new basic and limited clinical trial information describing the use of SARMs in management of prostate cancer and other human disorders. The subsequent discussion of SERMs will include a brief overview of the current understanding of the physiology of ERα and ERβ, followed by a discussion of how SERMs have been used to differentially regulate these receptors in human disease. This section will conclude with a review of significant recent clinical trial information regarding newer SERMs.

II. SELECTIVE ANDROGEN RECEPTOR MODULATORS (SARMS)

Proposed mechanisms of action of SARMs reflect differential tissue distribution of the SARM ligands, potential interactions with 5α-reductase and/or aromatase enzymes at the tissue level, ligand-specific regulation of gene expression, and/or nongenomic actions at the molecular level.[6] Fundamental differences between androgen and estrogen physiology

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and signaling have been demonstrated. Only one AR has been identified, but the two endogenous ligands testosterone and dihydrotestosterone interact with this receptor differentially in different tissues.

The enzyme 5α-reductase is expressed in a tissue-specific distribution to convert testosterone to dihydrotestosterone.[7] Type II 5α-reductase is highly expressed in prostate tissue, making prostate an androgenic tissue, but at relatively low levels in bone and muscle, making these tissues anabolic tissues. The distribution of 5α-reductase ensures that dihydrotestosterone is the predominant androgen in prostate,[8] and that testosterone is the dominant form of androgen in the circulation and in bone and muscle. Use of 5α-reductase inhibitors results in testosterone becoming the dominant androgen in the prostate, despite testosterone having much lower potency for stimulation of prostate growth.

Existing preclinical evidence with BMS-564929 and other SARMs suggests that tissue distribution of 5α-reductase plays a significant role in determining tissue selectivity of SARMs. Recent studies with nonsteroidal AR ligands indicate that differential tissue distribution by itself is not likely to completely explain differences in pharmacological responses seen in prostate and muscle.[9] In addition, although AR binding involves ligand-induced conformational changes mediated via the ligand-binding domain, crystal structures of aryl propionamide and hydantoin SARMs have not shown the same magnitude of conformational changes as seen with SERMs binding to the ER ligand binding domain,[10,11] particularly in the AF2 region.[12,13] Crystal structures of the testosterone- and dihydrotestosterone-bound AR ligand binding domain are virtually identical, despite significant differences in their androgenic properties in the prostate.[11] Although these crystal structure determinations are helpful in determining AR ligand binding mechanisms, it has been difficult to model potential changes in receptor function occurring under endogenous physiological conditions.

AR coregulators, which regulate binding interactions between dimerized ARs and DNA, have recently been shown to also function as actin-binding proteins.[14,15] These coregulators establish a connection between actin cytoskeletal components and androgen signaling, particularly in skeletal muscle.[16] In cellular and animal models, androgen-activated AR modulates myoblast proliferation, promotes sexual dimorphic muscle development, and alters muscle fiber type. In the clinical setting, administration of anabolic androgens can decrease cachexia and speed wound healing. During myogenesis and regeneration of skeletal muscle in embryo and adult, the membranes of myoblasts fuse, and the actin cytoskeleton is rearranged to form an alignment with myosin that leads to formation of myotubes initially, and subsequently myofibrils. Contraction of skeletal muscle promotes growth of myocytes by relaying signals from the neuromuscular junction to intra-myofibrils through costameres, functional structures comprised of signal proteins closely associated with actin filaments, and involved in muscular dystrophy. The discovery of actin-binding proteins functioning as AR coregulators implies that androgen signaling is tightly regulated during the development and regeneration of skeletal muscle. SARMs that specifically target skeletal muscle, instead of other androgen-responsive tissues, could be utilized in engineered SARM-AR complexes to selectively recruit actin-binding coregulators.

Various agonists and antagonists have been developed that target the AR for prevention or treatment of male hypogonadism, prostate cancer, benign prostate hyperplasia, muscle wasting, or anemia.[17–19] None of these agents has yet been approved for prevention or treatment of male osteoporosis. Androgen receptor ligands are classified as agonists (androgens) or antagonists (antiandrogens), based on their ability to activate or inhibit the transcription of AR target genes. Both steroidal and nonsteroidal AR ligands have been identified.

Testosterone is the major circulating endogenous steroidal androgen. Testosterone is converted locally by 5α-reductase to dihydrotestosterone in prostate and skin, and by aromatase to estrogen in bone, adipose tissue, and the central nervous system. Androgen effects in reproductive tissues, including the prostate, seminal vesicles, testis, and accessory structures are considered to be androgenic effects, whereas the effects on bone and muscle are designated as anabolic effects.

Clinical application of testosterone preparations developed to date has been limited by virilizing androgenic side effects, such as acne or hirsutism, in women, hepatotoxicity, adverse lipid effects, and concerns regarding stimulation of prostate disease in men. A variety of testosterone formulations, including transdermal patches, injectable esters, and steroidal analogues, including 17α-alkylated androgens and 19-norandrogens, have been developed for clinical use. Oral nonsteroidal antiandrogens developed in the 1970s, including bicalutamide, flutamide, and nilutamide, continue to play a role in the treatment of prostate cancer. These antiandrogens have high specificity for the AR, but lack tissue selectivity, and therefore also block the AR in bone and skeletal muscle, as well as the hypothalamus-pituitary-gonadal axis.

The concept of SARMs was first proposed by Negro-Vilar in 1999.[20] The ideal SARM has high AR specificity, oral bioavailability, acceptable pharmacokinetics, and tissue-selective pharmacological effects. In recent years SARMs have been developed with greater tissue selectivity in order to minimize adverse effects in other tissues. Androgens or tissue-selective SARMs could potentially be used to prevent or treat osteoporosis, muscle wasting due to normal age-related frailty or burns, cancer, chronic kidney disease, or AIDS. These agents could be used for hormone replacement in men or women without concerns regarding their virilizing side effects. For example, tissue-selective antiandrogens could be used to prevent or treat benign prostate hypertrophy or prostate cancer without blocking anabolic androgen effects on bone, muscle, or the central nervous system (CNS).

In spite of their potential benefits, a number of regulatory issues have slowed the development of androgens as anabolic therapies.[21,22] Clinical trial evidence has shown that testosterone supplementation increases muscle mass and strength in men by inducing hypertrophy of type I and II muscle fibers and increasing myonuclear and satellite cell number. Androgens promote differentiation of mesenchymal multipotent cells into the myogenic lineage, and inhibit their adipogenic differentiation, by promoting association of ARs with beta-catenin and activating T-cell factor 4. Meta-analyses indicate that testosterone supplementation increases fat-free mass and muscle strength in HIV-positive men with weight loss, glucocorticoid-treated men, and older men with low or low-normal testosterone levels. However, poor oral bioavailability, pharmacokinetic properties, and receptor cross-reactivity of testosterone, coupled with adverse side effects, limit its clinical use. The effects of testosterone on physical function and outcomes important to patients, however, have not been studied in great detail. In older men, increased hemoglobin and increased risk of prostate hypertrophy or cancer are the most frequent testosterone-related adverse events, and concerns about long-term risks have restrained enthusiasm for use of testosterone as anabolic therapy. SARMs that are preferentially anabolic and that have minimal effect on the prostate hold great promise as anabolic therapies, particularly in treating the physical dysfunctions associated with chronic illness or aging.

SARMs largely remain in the discovery and early development stage, with a number of agents in preclinical development, and no SARMs yet approved for clinical use.[20] Most SARMs undergoing development currently are nonsteroidal anabolic agents derived from aryl propionamides[23] or quinolines[24] since 1998. SARMs of the aryl propionamide class were first shown to have tissue selectivity in 2003.[25] Later that same year, discovery of the tetrahydroquinoline class of SARMs was reported,[26] followed by discovery of the quinoline SARM class in 2006,[27] and hydantoin SARM class in 2007.[26] These anabolic SARMs show tissue selectivity in castrate animals, with stronger agonist effects in anabolic tissues, such as the levator ani muscle, than in androgenic tissues, including the prostate. Recently reported new SARMs include TFM-4AS-1 and FTBU-1,[28] ACP-105,[29] LGD2941[30] and LGD3303,[31,32] S-4[33] and S-22,[34] JNJ-37654032,[27] the class of oxazolidin-2-imines,[35] and the class of 5- or 6-oxachrysen-2-ones.[36]

Studies with the modified hydantoin SARM BMS-564929 indicate that this compound is among the most potent and highly tissue-selective SARMs reported to date.[37] However, this SARM and other SARMs potently suppress pituitary secretion of luteinizing hormone (LH), which results in decreased testicular production of testosterone in the dose range

associated with anabolic activity. Suppression of LH secretion by SARMs remains a barrier to further development of this category of compounds.

Although androgens are known to protect bone, side effects and poor oral bioavailability have limited their use in prevention or treatment of osteoporosis. S-3-(4-acetylamino-phenoxy)-2-hydroxy-2-methyl-N-(4-nitro-3-trifluoromethyl-phenyl)-propionamide (S-4) is a potent SARM. Kearbey et al. evaluated the skeletal effects of S-4 in an ovariectomized rat model.[33] Aged female rats were gonadectomized or sham operated on day 1 and assigned to treatment groups. S-4 treatment was started on day 90 and continued daily through day 210. Whole animal bone mineral density (BMD), body weight, and fat mass were determined by dual energy X-ray absorptiometry (DXA). Regional analysis of excised bones was performed using DXA or computed tomography, and femur strength evaluated by 3-point bending. The study showed that S-4 restored whole body and lumbar vertebrae (L5-L6) BMD to the same level as intact controls. Significant increases in cortical bone quality were observed at the femoral midshaft, which resulted in increased load bearing capacity. The investigators concluded that this SARM stimulated partial or complete recovery of various bone parameters to age-matched intact levels, and that the increased efficacy observed at cortical bone sites was consistent with reported androgen actions in bone.

Vajda et al. evaluated the effects of combination treatment with the SARM LGD-3303 and the bisphosphonate alendronate in a hypogonadal rat model.[31,32] *In vitro* competitive binding and transcriptional activity assays were initially used to characterize LGD-3303 as a potent nonsteroidal AR modulator with little or no cross-reactivity with related nuclear receptors. Orchidectomized male rats were treated with LGD-3303 orally for 14 days. LGD-3303 increased levator ani muscle weight above eugonadal levels, but had greatly reduced activity on the prostate, and did not increase ventral prostate weight to more than 50% of eugonadal levels, even at high doses. Ovariectomized female rats were treated with LGD-3303, alendronate, or both to evaluate their effects on bone. DXA scans, bone histomorphometry, and biomechanics were performed. LGD-3303 increased muscle weight in female rats, and increased BMD and BMC at both cortical and cancellous bone sites. At cortical sites, LGD-3303 effects were caused in part by anabolic activity on the periosteal surface. At every measured site, combination treatment was as effective as either single agent, and in some cases showed significant added benefit. The study concluded that the new SARM LGD-3303 has anabolic effects on muscle and cortical bone not seen with bisphosphonates, and that combination therapy with LGD-3303 and alendronate may have additive effects on bone, and potentially be a useful therapy for osteoporosis and frailty of old age.

In addition to their protective effects on the skeleton, SARMs could potentially prevent or treat low muscle mass associated with many medical conditions. JNJ-37654032 is a new nonsteroidal AR ligand with mixed agonist and antagonist activity in androgen-responsive cell-based assays.[27] It is orally active, with muscle selectivity in orchidectomized rat models. Studies have shown that it stimulates growth of the levator ani muscle with ED_{50} 0.8 mg/kg, and that it stimulates maximal growth at a dose of 3 mg/kg. In contrast, it also stimulates ventral prostate growth to 21% of its full size at 3 mg/kg, and reduces prostate weight in intact rats by 47% at 3 mg/kg, while having no inhibitory effects on muscle. Using magnetic resonance imaging to monitor body composition, JNJ-37654032 was shown to restore about 20% of the lean body mass that is lost following orchidectomy in aged rats. JNJ-37654032 also reduces follicle-stimulating hormone levels in orchidectomized rats, and reduces testis size in intact rats. The available data suggests that JNJ-37654032 is a potent prostate-sparing SARM with potential clinical applications in treatment of muscle-wasting diseases.

17β-hydroxyestra-4,9,11-trien-3-one (trenbolone; 17β-TBOH), is a new synthetic analog of testosterone that appears to act as a SARM while binding to the AR with approximately three times the affinity of testosterone.[38] This compound increases skeletal muscle mass, increases bone growth, and decreases adiposity in a variety of mammalian species. In addition to its direct actions via ARs, 17β-TBOH may also exert

anabolic effects by altering the action of endogenous growth factors or inhibiting the action of glucocorticoids. Compared to testosterone, 17β-TBOH appears to induce less growth in androgen-sensitive tissues that highly express the 5α-reductase enzyme (eg, prostate tissue and accessory sex organs). These reduced androgenic effects result from the fact that 17β-TBOH is metabolized to less potent androgens *in vivo*. Understanding the regulation of metabolism of 17β-TBOH may give insight into new ways to combat muscle- and bone-wasting conditions, obesity, or androgen insensitivity syndromes in humans.

Glucocorticoids are widely used for their anti-inflammatory effects in multiple diseases. However, prolonged use of glucocorticoids may cause adverse side effects such as muscle wasting, osteoporosis, or diabetes. Skeletal muscle wasting, for which there is currently no approved treatment, results from either reduced muscle protein synthesis or increased muscle protein degradation. An imbalance in protein synthesis may be the result of increased expression and function of muscle-specific ubiquitin ligases, muscle atrophy F-box (MAFbx)/atrogin-1 and muscle ring finger 1 (MuRF1), or decreased function of the IGF-I and phosphatidylinositol-3 kinase/Akt kinase pathways. Jones et al. evaluated the effects of a nonsteroidal SARM and testosterone on glucocorticoid-induced muscle atrophy and castration-induced muscle atrophy in a rat model.[39] The SARM and testosterone propionate both blocked dexamethasone-induced dephosphorylation of Akt and other proteins involved in protein synthesis, including Forkhead box O (FoxO). Dexamethasone caused significant upregulation of expression of ubiquitin ligases, but testosterone propionate and SARM administration both blocked this effect by phosphorylating FoxO. Castration-induced rapid myopathy of the levator ani muscle, accompanied by upregulation of MAFbx and MuRF1 and downregulation of IGF-I, were all attenuated by the SARM, but not testosterone propionate. These results demonstrate that levator ani atrophy, and therefore skeletal muscle atrophy, that is associated with hypogonadism may be the result of loss of IGF-I stimulation, whereas the muscle loss caused by glucocorticoid treatment appears to depend almost solely on upregulation of MAFbx and MuRF1. This study provides the first evidence that glucocorticoid- and hypogonadism-induced muscle atrophy may be mediated by distinct but overlapping mechanisms, and that SARMs may provide a more effective and selective pharmacological approach to prevent glucocorticoid-induced muscle loss than steroidal androgen therapy.

Postmenopausal women often experience decreased sexual desire and bone loss after undergoing natural or surgically induced menopause. Decreased sexual desire is most often attributed to loss of ovarian androgen secretion, and bone loss due primarily to loss of estrogen. Jones et al. synthesized a series of S-3-(phenoxy)-2-hydroxy-2-methyl-N-(4-cyano-3-trifluoromethyl-phenyl)-propionamide analogs to evaluate the effects of B-ring substitutions on *in vitro* and *in vivo* pharmacologic activity, especially female sexual motivation, using an ovariectomized rat model.[40] The AR relative binding affinities ranged from 0.1% to 26.5% relative to dihydrotestosterone, and demonstrated a range of agonist activity at 100 nM. *In vivo* pharmacologic activity was assessed in male rats. Structural modifications to the B-ring significantly affected the tissue selectivity of the SARMs, demonstrating that single atom substitutions may dramatically and unexpectedly influence activity in androgenic (eg, prostate) or anabolic (eg, muscle) tissues. The SARM S-23 displayed full agonist activity in androgenic and anabolic tissues. However, the remaining SARMs were more prostate-sparing, and selectively maintained the size of the levator ani muscle in castrated male rats. A partner preference paradigm was used to evaluate the effects of SARMs on ovariectomized female rat sexual motivation. With the exception of two 4-halo substituted analogs, these SARMs increased sexual motivation in ovariectomized rats, with potency and efficacy comparable to testosterone propionate. These results suggest that the AR is important in regulating female libido, given the non-aromatizable nature of SARMs. The study concluded that SARMs could potentially be a superior alternative to steroidal testosterone preparations in the treatment of human hypoactive sexual desire disorder.

SARMs potentially may also act non-genomically via membrane-bound AR, rather than inter-

actions with cytoplasmic or intranuclear ARs. The molecular mechanisms underlying the tissue selectivity of SARMs remain ambiguous. Narayanan et al. performed a variety of *in vitro* studies to compare and define the molecular mechanisms of a new aryl propionamide SARM, S-22, contrasted with dihydrotestosterone.[34] S-22 was shown to increase levator ani muscle weight, but to decrease prostate size in male rats. Analysis of the upstream intracellular signaling events indicated that S-22 and dihydrotestosterone mediated their actions through distinct pathways. Modulation of these pathways altered the recruitment of AR and its cofactors to the PSA enhancer in a ligand-dependent fashion. In addition, S-22 induced *Xenopus laevis* oocyte maturation and rapid phosphorylation of several kinases acting via pathways distinct from gonadal steroids. This study showed new differences in the molecular mechanisms by which S-22, a nonsteroidal SARM, and dihydrotestosterone mediate their pharmacological effects, with the findings implying that at least certain SARMs may exert nongenomic effects.

III. SELECTIVE ESTROGEN RECEPTOR MODULATORS (SERMS)

There are two forms of the ER, but estrogen is the only identified endogenous ligand for this type of receptor. ERα and ERβ have different structures, ligand affinities, tissue distributions, transcriptional properties, and biological roles. The presence of two ERs provides greater flexibility for regulation of estrogen action in different tissues.

SERMs directly bind to ERα and/or ERβ in target cell nuclei and exert estrogen- or antiestrogen-like actions in target tissues. These agents exert estrogenic benefits in certain tissues, and minimize estrogenic risks in other tissues. Once a SERM ligand binds to ERα and/or ERβ, it is believed to cause a conformational change in the ER molecule that results in dissociation of associated heat shock chaperone proteins, and release of the monomeric receptor from the apo-ER complex. The conformational change then results in altered interactions with complexed coactivator or corepressor proteins,[41] with subsequent monomeric ER translocation from the cytosol to the nucleus, followed by dimerization with a second monomeric ER before binding to specific DNA sequences in the regulatory promoter regions of target genes. Homodimeric binding of the ER to these promoter regions subsequently causes initiation or suppression of transcription of the genes.[42]

McDonnell et al. was among the first to show that a series of SERM ligands formed distinct ER-bound complexes, resulting from different induced conformational changes.[40] X-ray crystallography was subsequently used to quantitatively assess the conformational changes induced by agonist or antagonist binding to the ER ligand binding domain.[43] Initial structural evidence for the antagonist-bound ER conformation was obtained with the SERM tamoxifen,[45] showing that tamoxifen blocked ER access to nuclear receptor cofactor proteins.[44] Subsequent investigation showed that ER binding of many different SERMs caused development of the classical antagonist-bound ER conformation.[44] Evidence has also shown that SERMs may produce ER modulation through non-ER pathways, such as through androgen or progesterone receptors, when combined with SERM metabolites that have non-ER binding activities.[46]

Every ER ligand has SERM activity intrinsic to the ligand. Tissue-specific actions of SERMS are thought to be due to unique ER conformational changes caused by SERM ligand binding, resulting in a variety of specific interactions with other proteins within a cell. However, conformational change alone may not explain all actions of SERMs on target cells. Work in mice with targeted deletion of the ERα amino-terminal A/B domain has suggested that stimulation of ERα by SERMs with minimal activation of the amino-terminal activation domain AF-1 might preserve beneficial vascular effects, but minimize effects on sexual tissues.[47]

Human ERα and ERβ greatly differ in their target genes, transcriptional potency, and cofactor-binding capacity, and are differentially expressed in various tissues. In classical estrogen response element (ERE)-mediated transactivation, ERβ has a markedly reduced activation potential compared with ERα, but the mechanism underlying this difference

has been unclear. Zwart et al.[48] recently showed that the binding of steroid receptor coactivator-1 (SRC-1) to the AF-1 domain of ERα is essential, but not sufficient, to facilitate synergy between the AF-1 and AF-2 domains, which is required for a full agonistic response to 17β-estradiol. Complete synergy is achieved through the distinct hinge domain of ERα, which enables combined action of the AF-1 and AF-2 domains. The AF-1 domain of ERβ lacks the capacity to interact with SRC-1, which prevents hinge-mediated synergy between AF-1 and AF-2, thereby explaining the reduced 17β-estradiol-mediated transactivation of ERβ. Transactivation of ERβ by 17-estradiol requires only the AF-2 domain. A weak agonistic response to tamoxifen occurs for ERα, but not for ERβ, and depends on AF-1 and the hinge-region domain of ERα.

Functions of ERα and ERβ are best studied to date in bone, breast, uterine and genitourinary tissues, and brain. Because of the widely variable tissue effects of SERM ligands in different tissues, it is very difficult to reach conclusions about the complete clinical activity of a given SERM without conducting the appropriate clinical trials and assessing for adverse events in different tissues.

A variety of SERMs have been developed to date for different purposes, with raloxifene approved for prevention and treatment of postmenopausal osteoporosis and ER-positive breast cancer, tamoxifen for prevention and treatment of postmenopausal ER-positive breast cancer, and clomiphene for infertility treatment in premenopausal women. The initial SERMs were used as antiestrogens beginning about 50 years ago,[49] with the concept of selective estrogen receptor modulation introduced only about 15 years ago.[50] A variety of SERMs with special tissue selectivity remain under clinical investigation for prevention and treatment of these and other diseases.[51] SERMs may increase the risk of postmenopausal hot flashes, night sweats, leg cramps, deep venous thrombosis, or bone pain in some patients, particularly during the first few months of drug exposure.

Because currently available SERMs do not fully treat symptoms of the menopause, research continues to identify the optimal SERM for postmenopausal women, which would lessen hot flashes, reduce vaginal atrophy, and prevent bone loss and fractures, while protecting the uterus, mammary gland, and cardiovascular system. If an ideal SERM is not eventually found, as appears increasingly likely, SERMs may be used in postmenopausal women in tissue-selective estrogen complexes, in which a SERM is combined with an estrogen or estrogens, in order to obtain the beneficial effects of each component, with improved overall tolerability.[52] SERMs may also eventually be used in men to treat osteoporosis, syndromes associated with secondary hypogonadism, or possibly prostate cancer.

A number of SERMs have been clinically investigated since the first drug in this class was introduced in the form of clomiphene many years ago. Also, a number of SERMs have had their clinical investigation programs discontinued due to various adverse effects or lack of efficacy compared to available SERMs. Recently published clinical trials, over the last several years, have focused mostly on raloxifene, lasofoxifene, bazedoxifene, and arzoxifene.

III.A. Raloxifene

Raloxifene is a polyhydroxylated nonsteroidal compound with a benzothiophene core and high affinity for both ERα and ERβ,[53] which was originally investigated for breast cancer prevention in the early 1980s. It acts as a partial estrogen agonist in bone, thereby preventing vertebral fractures and loss of bone mineral density when given at the approved oral dose of 60 mg per day.[54,55] Raloxifene has also been shown to be more effective than a related SERM, tamoxifen, in reducing the risk of ER-positive breast cancers, but not ER-negative cancers, in postmenopausal women at high risk.[56] Neither drug reduced cardiovascular risk in this trial, however. Raloxifene is approved for prevention and treatment of postmenopausal osteoporosis, reduction in risk of invasive breast cancer in postmenopausal women with osteoporosis, and prevention of breast cancer in high-risk postmenopausal women.

The most recently published clinical trials with raloxifene include the Raloxifene Use in the

Heart (RUTH) study[57] and Study of Tamoxifen and Raloxifene (STAR) study.[58] The RUTH study evaluated the effects of raloxifene 60 mg per day vs. placebo in 10,101 postmenopausal women of mean age 67.5 years with coronary heart disease or multiple coronary heart disease risk factors over a follow-up period of 5.6 years. This study showed that raloxifene reduced the risk of invasive breast cancer, but not noninvasive breast cancer, in these women (Fig. 1). Raloxifene also reduced the risk of clinical vertebral fractures, but not nonvertebral or hip fractures. Unfortunately, raloxifene did not reduce the primary endpoint risk of coronary events (Fig. 1) or stroke, but it was associated with a statistically significant increased risk of stroke mortality and venous thromboembolism.

The STAR study evaluated the effects of raloxifene 60 mg per day vs. tamoxifen 20 mg per day in 19,747 postmenopausal women of mean age 58.5 years with high risk of breast cancer over a follow-up period of 5 years.[58] This study showed that raloxifene and tamoxifen caused similar reductions in the risk of invasive breast cancer, with the tamoxifen group having fewer cases of noninvasive breast cancer than the raloxifene group, although this difference was not statistically significant (Fig. 2). Neither drug reduced the risk of noninvasive breast cancer in postmenopausal women.

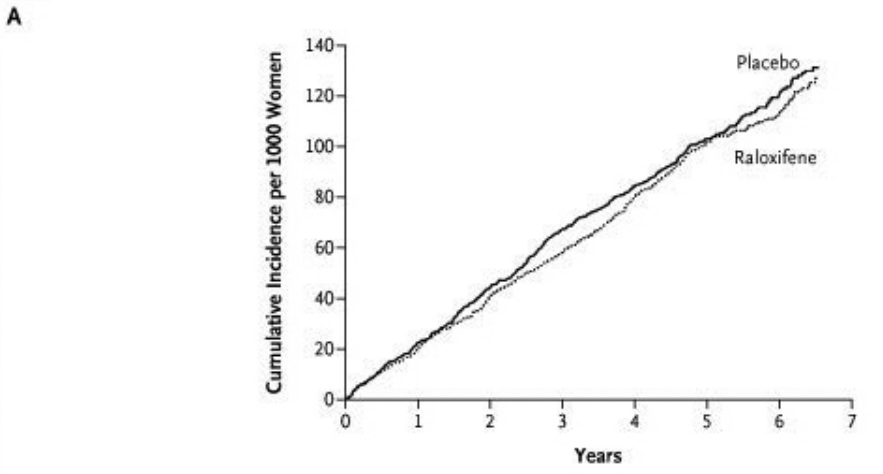
Gushima et al.[59] recently showed that raloxifene causes translocation of ERα into nucleoli in breast cancer cell lines, but not other cell types. Mutation analysis showed that helix 12 of ERα is essential to raloxifene-induced nucleolar translocation. This effect, which appears to be specific to raloxifene, may explain at least part of raloxifene's ability to suppress growth of breast cells.

Multiple studies have shown that raloxifene reduces serum total cholesterol and LDL cholesterol similar to estrogen, but that serum triglycerides and C-reactive protein are not affected. Raloxifene has not been shown to alter the risk of cardiovascular endpoints, cardiovascular death, or overall mortality in several studies, including the RUTH study.[57]

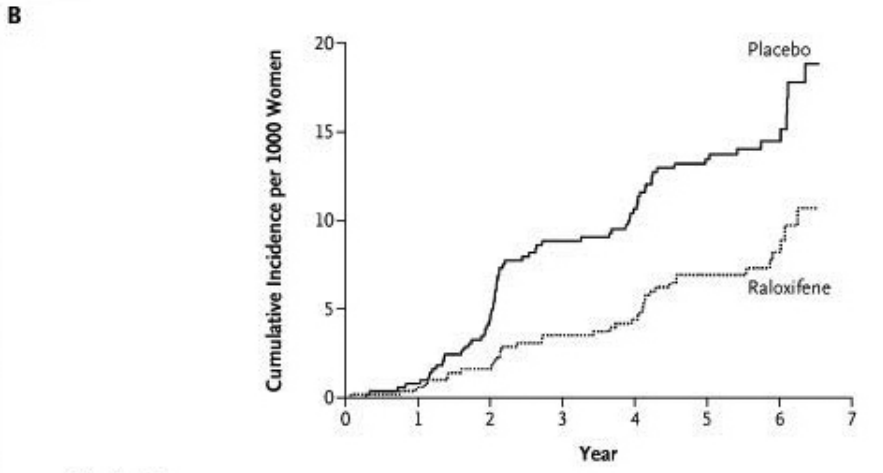
While raloxifene decreases the incidence of osteoporosis and invasive breast cancer, it also increases the risk of venous thromboembolism and fatal stroke in women with, or at high risk for, coronary heart disease. Grady et al.[60] assessed treatment effects of raloxifene on overall and cause-specific mortality by performing a pooled analysis of mortality data from large clinical trials of raloxifene (60 mg/day) vs. placebo. This study analyzed data from the Multiple Outcomes of Raloxifene Evaluation/Continuing Outcomes Relevant to Evista studies, with 7,705 postmenopausal osteoporotic women followed for 4 years, and a subset of 4,011 participants followed for an additional 4 years, with 110 deaths during follow-up. The analysis also included the RUTH trial, with 10,101 postmenopausal women with coronary disease or multiple risk factors for coronary disease followed for 5.6 years, with 1,149 deaths during follow-up. Cox proportional hazards regression models compared mortality by treatment assignment in a pooled analysis of the trial data. All-cause mortality was 10% lower among women assigned to raloxifene 60 mg/day vs. placebo (relative hazard, 0.90; 95% CI, 0.80–1.00; $P = 0.05$) (Fig. 3). This lower overall mortality was primarily due to lower rates of non-cardiovascular deaths, especially lower rates of non-cardiovascular, non-cancer deaths. The study did not identify mechanisms by which raloxifene reduced the risk of non-cardiovascular deaths.

Raloxifene has been shown to affect body composition.[61] In a randomized, double-blind, placebo-controlled trial involving 198 healthy women aged 70 years or older, participants were randomly assigned to receive raloxifene 60 mg or placebo daily for 12 months. At 12 months, fat-free mass (FFM) increased by a mean of 0.83 ± 2.4 kg in the raloxifene group, vs. 0.03 ± 1.5 kg in the placebo group ($P = 0.05$), and total body water increased by a mean of 0.6 ± 1.8 L in the raloxifene group vs. a decrease of 0.06 ± 1.1 L in the placebo group ($P = 0.02$). Muscle strength and power were not significantly different with raloxifene treatment. The study concluded that raloxifene significantly increased FFM and water content compared to placebo in postmenopausal women.

Raloxifene was shown to improve verbal memory in elderly postmenopausal women compared to placebo in a randomized, double-blind, placebo-controlled trial of 213 healthy Dutch women 70 years or older.[62] Participants were randomly assigned



Placebo Group							
Cumulative no. of events	0	112	219	327	405	488	541
No. at risk	5057	4842	4601	4349	4159	3647	1574
Raloxifene Group							
Cumulative no. of events	0	98	199	285	389	484	518
No. at risk	5044	4866	4669	4463	4225	3710	1633



Placebo Group							
Cumulative no. of events	0	4	21	42	50	62	65
No. at risk	5057	4910	4702	4488	4312	3813	1670
Raloxifene Group							
Cumulative no. of events	0	3	8	17	21	32	35
No. at risk	5044	4928	4775	4604	4404	3893	1724

FIGURE 1. Cumulative incidence of the primary outcomes of coronary events (death from coronary causes, nonfatal myocardial infarction, or hospitalization for an acute coronary syndrome other than myocardial infarction) (panel A) and invasive breast cancer (panel B). (By permission from Barrett-Connor E. et al. New Engl J Med. 2006;355:125–137.)

to receive raloxifene (60 mg) or placebo daily for 12 months. Measurements were taken at baseline and after 3, 6, and 12 months. The main outcome measures were direct and delayed verbal memory (Groningen 15 Words test), mental processing speed (Trails B test), mood/depression (Geriatric Depression Scale), anxiety (State-Trait Anxiety Inventory 1 and 2), and quality of life (Women's Health Questionnaire and

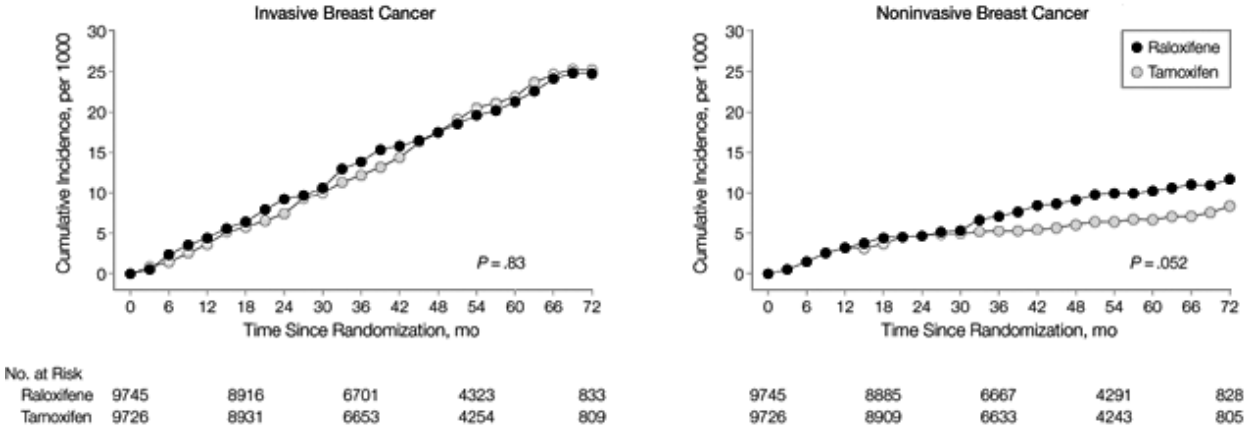


FIGURE 2. Cumulative incidence of invasive and noninvasive breast cancer. (By permission from Vogel VG, et al. JAMA. 2006;295:2727–2741.)

EuroQol-5 dimensional questionnaire). Direct verbal memory improved significantly with raloxifene compared with placebo, with the women receiving raloxifene able to repeat more words in the words A + B test than did the women receiving placebo (P = 0.025). At 12 months, the change from baseline was 16 words in the raloxifene group and 10 words in the placebo group. In the words A test, direct repetition was also significantly better among women receiving raloxifene than among women receiving placebo (P = 0.023), with the change from baseline in the number of words repeated being nine words in the raloxifene group and six words in the placebo group at 12 months. The study concluded that raloxifene resulted in significantly improved verbal memory when compared with placebo in postmenopausal women.

Raloxifene has been shown to prevent estrogen-mediated suppression of autoreactive B cell elimination at the T1/T2 selection checkpoint, to reduce estrogen-induced CD40 overexpression on follicular B cells, making them less responsive to T cell costimulation, and to ameliorate estrogen-mediated CD22 downregulation on marginal zone B cells, thereby decreasing their responsiveness to B cell antigen receptor-mediated stimuli in the New Zealand Black/W F1 mouse model of systemic lupus erythematosus.[63] These findings indicate that raloxifene is able to suppress estrogen-mediated effects on the survival, maturation, and activation of autoreactive B cells in this strain of rodent.

III.B. Lasofoxifene

Lasofoxifene is a potent SERM that belongs to the naphthalene class of SERMs. Lasofoxifene improves lumbar spine bone mineral density more effectively than raloxifene, increases hip bone density similar to raloxifene, and reduces markers of bone turnover and LDL-cholesterol more effectively than raloxifene.[64,65]

The effects of lasofoxifene on the risk of fractures, breast cancer, and cardiovascular disease were demonstrated in the PEARL clinical randomized trial.[66] This trial randomized 8,556 women between the ages of 59 and 80 years, with bone mineral density T-score of -2.5 or less at the femoral neck or spine, to receive once-daily lasofoxifene at a dose of either 0.25 mg or 0.5 mg, or placebo, for 5 years. Primary end points of the study were vertebral fractures, estrogen receptor (ER)-positive breast cancer, and nonvertebral fractures, whereas secondary end points included major coronary heart disease events and stroke. Lasofoxifene at a dose of 0.5 mg per day, as compared with placebo, was associated with reduced risks of vertebral fracture (13.1 cases vs. 22.4 cases per 1,000 person-years; hazard ratio (HR), 0.58;

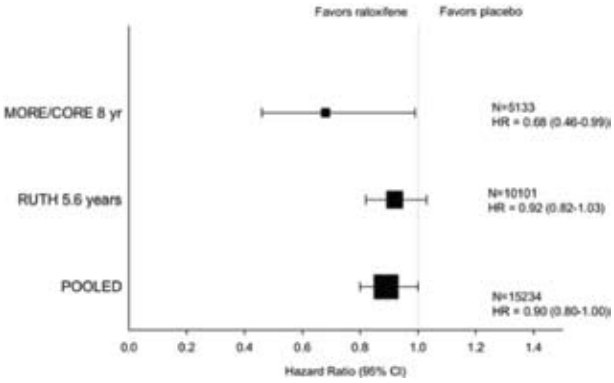


FIGURE 3. All-cause mortality in the MORE/CORE and RUTH studies. Error bars represent 95% confidence intervals. MORE = Multiple Outcomes Raloxifene Evaluation trial; CORE = Continuing Outcomes Relevant to Evista trial; RUTH = Raloxifene Use for the Heart trial. (By permission from Grady D, et al. Am J Med. 2010;123:469.e1–469.e7, Copyright © 2010 Elsevier.)

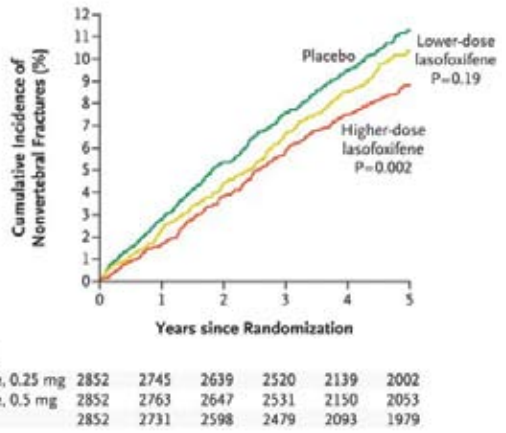


FIGURE 4. Cumulative incidence of nonvertebral fractures, according to study group. (By permission from Cummings SR, et al. New Engl J Med. 2010;362:686–696. Copyright © 2010 Massachusetts Medical Society. All rights reserved.)

95% CI, 0.47–0.70), nonvertebral fracture (18.7 vs. 24.5 cases per 1,000 person-years; HR, 0.76; 95% CI, 0.64–0.91) (Fig. 4), ER-positive breast cancer (0.3 vs. 1.7 cases per 1,000 person-years; HR, 0.19; 95% CI, 0.07–0.56), coronary heart disease events (5.1 vs. 7.5 cases per 1,000 person-years; HR, 0.68; 95% CI, 0.50–0.93), and stroke (2.5 vs. 3.9 cases per 1,000 person-years; HR, 0.64; 95% CI, 0.41–0.99) (Fig. 5). Lasofoxifene at a dose of 0.25 mg per day, as compared with placebo, was associated with reduced risks of vertebral fracture (16.0 vs. 22.4 cases per 1,000 person-years; HR, 0.69; 95% CI, 0.57–0.83) and stroke (2.4 vs. 3.9 cases per 1,000 person-years; HR, 0.61; 95% CI, 0.39–0.96). Lasofoxifene did not prevent hip fractures in this trial, however. Both the lower and higher doses of lasofoxifene, as compared with placebo, were associated with an increase in venous thromboembolic events (3.8 and 2.9 cases vs. 1.4 cases per 1,000 person-years; HRs, 2.67; 95% CI, 1.55–4.58 and HR, 2.06; 95% CI, 1.17–3.60, respectively). Endometrial cancer occurred in three women in the placebo group, two women in the lower-dose lasofoxifene group, and two women in the higher-dose lasofoxifene group. Rates of death per 1,000 person-years were 5.1 in the placebo group, 7.0 in the lower-dose lasofoxifene group, and 5.7 in the higher-dose lasofoxifene group. The authors concluded that, in postmenopausal women with osteoporosis, lasofoxifene at a dose of 0.5 mg per day was associated with reduced risks of nonvertebral and vertebral fractures, ER-positive breast cancer, coronary heart disease, and stroke, but increased risk of venous thromboembolic events.

III.C. Arzoxifene

Arzoxifene is a potent benzothiophene SERM being investigated for prevention and treatment of osteoporosis and chemoprevention of breast cancer. Arzoxifene was recently shown to be less effective than tamoxifen for progression-free survival and time to treatment failure in locally advanced and metastatic breast cancer, and to cause a similar tumor response rate, clinical benefit rate, and median response duration.[67]

In a 6-month, phase 2, randomized, double-blind, placebo-controlled study of 219 postmenopausal women with low bone density, mean age 59 years, arzoxifene significantly reduced bone turnover marker levels, and increased bone mineral density (BMD) compared to placebo.[68] Arzoxifene generally had greater effects on bone turnover and BMD than raloxifene, but its safety profile appeared similar to

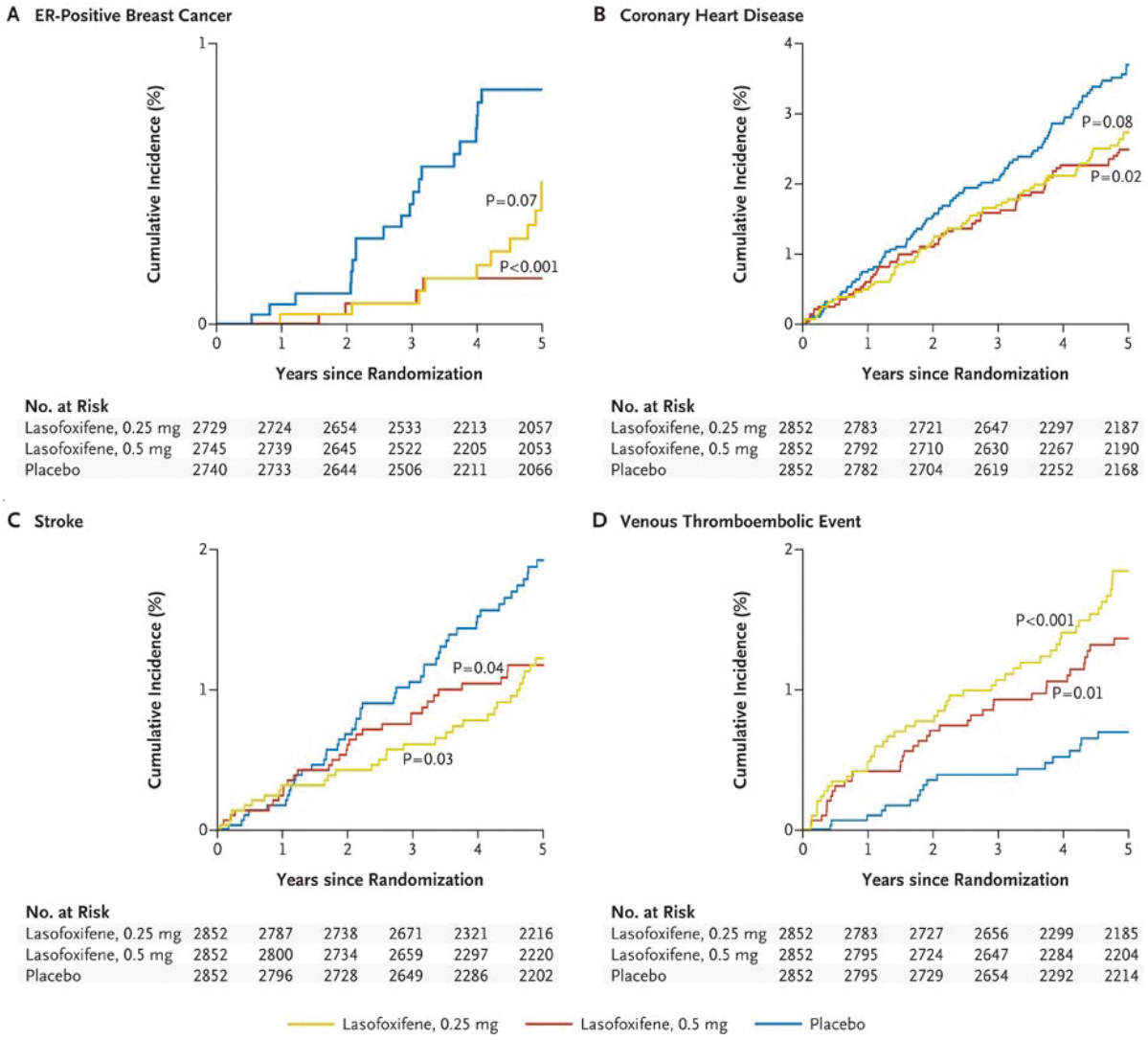


FIGURE 5. Cumulative incidence of events other than fracture, according to study group. The cumulative incidence of estrogen-receptor (ER)-positive breast cancer (Panel A), major coronary heart disease (Panel B), stroke (not including transient ischemic attacks) (Panel C), and venous thromboembolic events (Panel D) is shown. (By permission from Cummings SR, et al. New Engl J Med, 2010;362:686–696. Copyright © 2010 Massachusetts Medical Society. All rights reserved.)

raloxifene. Participants were randomized to receive arzoxifene 5, 10, 20, or 40 mg, raloxifene 60 mg, or placebo, and all received daily calcium. All arzoxifene doses significantly reduced osteocalcin (primary endpoint), type 1 collagen C-telopeptide, bone specific alkaline phosphatase, and procollagen type I amino-terminal propeptide compared to placebo, and increased lumbar spine BMD. Arzoxifene generally had greater effects on bone turnover and BMD than raloxifene. Arzoxifene decreased cholesterol, low-density lipoprotein cholesterol, and fibrinogen vs. placebo. Endometrial thickness change with arzoxifene was not significantly different from placebo or raloxifene, and no cases of endometrial hyperplasia or adenocarcinoma were observed. Adverse events with arzoxifene were similar to those with raloxifene, as

were hot flashes and night sweats. Arzoxifene suppressed bone turnover and increased BMD. Within the limitations of this study, the endometrial safety profile of arzoxifene appeared similar to that of raloxifene. While no clear dose effect was evident, arzoxifene 20 and 40 mg/day appeared to be the optimal doses for reducing bone turnover.

The effects of arzoxifene 20 mg/day on BMD, uterine safety, and overall safety were studied in the FOUNDATION study, a 2-year randomized, placebo-controlled trial including 331 postmenopausal women with normal-to-low bone mass.[69] Compared to placebo, arzoxifene significantly increased lumbar spine (+2.9%) and total hip (+2.2%) BMD. Arzoxifene decreased biochemical markers of bone metabolism compared to placebo. Changes in breast density were neutral or slightly decreased in the arzoxifene vs. placebo group. There was no evidence of endometrial hyperplasia or carcinoma in the arzoxifene group as assessed by central review of baseline and follow-up endometrial biopsies. There was no significant change between the groups in endometrial thickness assessed by transvaginal ultrasound. The incidence of uterine polyps and vaginal bleeding was not significantly different between the groups. Vulvovaginal mycotic infection was the only adverse event significantly increased in the arzoxifene vs. placebo group. Hot flashes were not significantly different between the groups. The study concluded that in postmenopausal women with normal-to-low bone mass, arzoxifene 20 mg/day increased BMD at the spine and hip, and had a neutral effect on the uterus and endometrium.

III.D. Bazedoxifene

Bazedoxifene is a new SERM recently approved in the European Union. This drug is currently undergoing FDA review in the United States for the prevention and treatment of postmenopausal osteoporosis.[70] The phase III pivotal fracture prevention clinical trial with bazedoxifene has not yet been published, but published reviews of this drug have indicated that in subgroup analysis of women at high risk for fracture, bazedoxifene significantly reduced the risk of new vertebral fracture compared to placebo, and improved bone mineral density and reduced bone turnover. Bazedoxifene also significantly reduced the risk of nonvertebral fracture compared to both placebo and raloxifene. The study showed that bazedoxifene was generally safe and well-tolerated in women with or at risk for osteoporosis, with no evidence of endometrial or breast stimulation.

Christiansen et al. recently reported the safety data for the phase III trial with bazedoxifene.[71] This study randomized 7,492 healthy postmenopausal osteoporotic women of mean age 66.4 years to receive bazedoxifene 20 or 40 mg, raloxifene 60 mg, or placebo daily for 3 years. The incidence of adverse events (AEs), serious AEs, and study discontinuations due to AEs in the bazedoxifene groups was not different from that in the placebo group. The incidence of hot flashes and leg cramps was higher with bazedoxifene or raloxifene compared to placebo. The rates of cardiac disorders and cerebrovascular events were low and evenly distributed among groups. Venous thromboembolic events (VTE), primarily deep vein thromboses, were more frequently reported in the bazedoxifene and raloxifene groups compared to the placebo group. Rates of VTE were similar with bazedoxifene and raloxifene. Bazedoxifene showed a neutral effect on the breast and did not cause endometrial stimulation. The incidence of fibrocystic breast disease was lower with bazedoxifene 20 and 40 mg vs. raloxifene or placebo. Reductions in total and low-density lipoprotein levels and increases in high-density lipoprotein levels were seen with bazedoxifene and raloxifene compared to placebo, and triglyceride levels were similar among groups. The study concluded that bazedoxifene showed a favorable safety and tolerability profile in women with postmenopausal osteoporosis. The 5-year follow-up safety data from this trial were also recently published, with findings consistent with the 3-year safety data.[72]

III.E. Tamoxifen

The SERMs toremifene and tamoxifen have been shown to be therapeutically equivalent treatments for metastatic breast cancer. The North American

Fareston versus Tamoxifen Adjuvant trial assigned 1,813 perimenopausal or postmenopausal women with hormone receptor (HR)-positive invasive breast cancer to adjuvant treatment with either tamoxifen or toremifene.[73] On the basis of intent-to-treat analysis, 5-year actuarial disease-free survival was not significantly different between tamoxifen and toremifene (91.2% ± 1.2% vs. 91.2% ± 1.1%, respectively). Similarly, 5-year actuarial overall survival was not significantly different between tamoxifen and toremifene (92.7% ± 1.1% vs. 93.7% ± 1.0%, respectively). Controlling for patient age, tumor size, and tumor grade, Cox multivariate survival analysis found no difference between patients randomized to toremifene vs. tamoxifen in terms of overall survival (OR = 0.951; 95% CI, 0.623–1.451, P = 0.951) or disease-free survival (OR = 1.037; 95% CI, 0.721–1.491, P = 0.846). Adverse events were similar in the 2 groups. The study concluded that women treated with adjuvant hormonal therapy enjoyed excellent disease-free survival and overall survival, but no significant differences were found between treatment with either tamoxifen or toremifene.

Even though ERα is a marker used to identify postmenopausal breast cancer patients most likely to benefit from endocrine therapy, approximately 50% of ERα-positive breast cancers are resistant to tamoxifen. Preclinical studies have shown that phosphorylation of ERα at serine-118 (ERα S118-P) is required for tamoxifen to mediate inhibition of ERα-induced gene expression. In a study of 239 premenopausal patients with breast cancer who participated in a randomized trial of two years of adjuvant tamoxifen treatment vs. no systemic treatment, ER-α S118-P expression in breast tissue assessed by immunohistochemistry was shown to be associated with response to tamoxifen.[74]

It has been shown that inherited variants in the CYP2D6 gene, involved in tamoxifen metabolism to its major metabolites 4-hydroxytamoxifen and endoxifen, affect breast cancer outcomes related to tamoxifen. A retrospective analysis of German and US patient cohorts treated with adjuvant tamoxifen for early stage breast cancer included 1,325 patients diagnosed with stages I through III breast cancer between 1986 and 2005.[75] Subjects included were mainly postmenopausal (95.4%). Time to recurrence, event-free survival, disease-free survival, and overall survival were assessed with median follow-up over 6.3 years. At 9 years of follow-up, recurrence rates were 14.9% for extensive metabolizers of tamoxifen, 20.9% for heterozygous extensive/intermediate metabolizers, and 29.0% for poor metabolizers, and all-cause mortality rates were 16.7%, 18.0%, and 22.8%, respectively. Compared with extensive metabolizers, there was a significantly increased risk of recurrence for heterozygous extensive/intermediate metabolizers (time to recurrence adjusted HR, 1.40; 95% CI, 1.04–1.90) and for poor metabolizers (time to recurrence HR, 1.90; 95% CI, 1.10–3.28). Compared with extensive metabolizers, those with decreased CYP2D6 activity (heterozygous extensive/intermediate and poor metabolism) had worse event-free survival (HR, 1.33; 95% CI, 1.06–1.68) and disease-free survival (HR, 1.29; 95% CI, 1.03–1.61), but there was no significant difference in overall survival (HR, 1.15; 95% CI, 0.88–1.51). The study concluded that there is an association between CYP2D6 variation and clinical outcomes among women with breast cancer treated with tamoxifen. It appears that the presence of two functional CYP2D6 alleles is associated with better clinical outcomes, and that the presence of nonfunctional or reduced-function alleles predicts worse outcomes.

If inherited variants in candidate genes involved in tamoxifen metabolism predict clinical outcomes of treatment of breast cancer with tamoxifen, then it is possible that genes involved in ER signaling or tamoxifen metabolism could also affect tamoxifen effects on bone. In a prospective multicenter clinical trial, 297 women starting tamoxifen therapy for the first time had their lumbar spine and total hip BMD values assessed by dual-energy X-ray absorptiometry (DXA) at baseline and after 12 months of tamoxifen therapy.[76] Single-nucleotide polymorphisms (SNPs) in the genes for ERα, ERβ, and cytochrome P450 2D6 were tested for associations with menopausal status, previous chemotherapy, and mean percentage change in BMD over 12 months. The percentage increase in BMD was greater in postmenopausal women and in subjects who had previously been treated with chemotherapy. No significant associa-

tions were found between the tested SNPs and either baseline BMD or change in BMD with 1 year of tamoxifen therapy. The study concluded that the evaluated SNPs in these genes did not influence BMD response in tamoxifen-treated subjects.

Unlike raloxifene in postmenopausal women, tamoxifen has been shown to have adverse neuropsychological effects on postmenopausal women with breast cancer. Assessments performed before and after one year of adjuvant tamoxifen or exemestane treatment in Dutch postmenopausal patients with breast cancer who were not treated with chemotherapy showed adverse effects on verbal memory and executive functioning.[77] Study subjects were participants in the international Tamoxifen and Exemestane Adjuvant Multinational trial, a prospective randomized study investigating tamoxifen vs. exemestane as adjuvant therapy for hormone-sensitive breast cancer. After one year of adjuvant therapy, tamoxifen use was associated with statistically significant lower functioning in verbal memory and executive functioning, whereas exemestane use was not. These results accentuate the need to include assessments of cognitive effects of adjuvant endocrine treatment in long-term safety studies.

III.F. Other SERMs

Small clinical trials of several other SERMs, including ospemifene, pipendoxifene, HMR-3339, and fulvestrant are at various stages of development or are underway for prevention and treatment of breast cancer and postmenopausal osteoporosis. Each of these SERMs has unique features which endow them with specific characteristics potentially useful for various clinical applications.

Fulvestrant is currently approved for use in postmenopausal women with hormone receptor positive advanced breast cancer that has progressed on treatment with endocrine therapy.[78] Fulvestrant is a pure estrogen antagonist that avoids the risk of detrimental side effects of selective ER modulators such as tamoxifen, which has partial agonist activity. Fulvestrant is the only parenteral agent available for treatment of breast cancer, and thus far appears to have a good side effect profile and to be well tolerated. Due to its unique mode of action, fulvestrant lacks cross-resistance with existing SERMs.

III.G. Non-Nuclear ERα Signaling

SERMs may potentially also act via cell membrane-bound ER, rather than via classical intranuclear ER. Non-nuclear estrogen receptor-α signaling has recently been reported. Chambliss et al. showed that an estrogen-dendrimer conjugate that is excluded from the nucleus is able to stimulate endothelial cell proliferation and migration via ERα, direct ERα-Gαi interaction, and endothelial NOS (eNOS) activation, but not stimulate uterine or breast cancer growth, in mice.[79] This estrogen-dendrimer conjugate therefore serves as a non-nuclear selective ER modulator *in vivo*, and in mice, is able to promote cardiovascular protection but not stimulate uterine or breast cancer growth.

III.H. Non-ER Mediated Effects

Recent studies have indicated that SERMs may also have additional non-ER-mediated effects on cells. It has been suggested that induction of oxidative stress by SERMs could be one non-ER-mediated mechanism by which SERMs exert a proapoptotic effect in ER-negative cells. Tumor cells have a high requirement for glutamine that serves multiple functions within the cells, including as a nutritional and energy source, as well as serving as one of the precursors for the synthesis of natural antioxidant glutathione. Tamorova et al.[80] showed that tamoxifen and raloxifene inhibited glutamine uptake in a dose-dependent manner by inhibition of the ASCT2 glutamine transporter in MDA-MB231 breast cancer cells. This effect was associated with inhibition of glutathione production and apoptosis. Treatment of these cells with N-acetyl-L-cysteine and 17beta-estradiol 2 reversed the effects of raloxifene and tamoxifen. These results indicate that one of the mechanisms of action, and possibly some of the side effects, of tamoxifen and raloxifene is inhibition of

cellular glutamine uptake leading to oxidative stress and induction of apoptosis.

SERMs have been shown to regulate aspects of calcium signaling and apoptosis in an ER-independent manner in some systems, suggesting that some of the activity of drugs within this class may be due to their ability to interact with targets other than ER. DuSell et al.[81] showed that 4-hydroxytamoxifen directly binds to and modulates transcriptional activity of the aryl hydrocarbon receptor. In the absence of ER, 4-hydroxytamoxifen was shown to induce the expression of aryl hydrocarbon receptor target genes involved in estradiol metabolism, cellular proliferation, and metastasis in cellular models of breast cancer. The potential role of the aryl hydrocarbon receptor in SERM pharmacology was underscored by the ability of 4-hydroxytamoxifen to suppress osteoclast differentiation *in vitro* in part through aryl hydrocarbon receptors. These findings suggest that it may be necessary to reevaluate the relative roles of the ER and aryl hydrocarbon receptor in mediating the pharmacological actions and therapeutic efficacy of tamoxifen and other SERMs.

IV. CONCLUSION

Available data suggests that individual SARMs and SERMs have unique tissue-specific activities that require elucidation in clinical trials. The tissue-specific effects of SARMs are not yet as well established as those of SERMs, but future clinical trials will eventually provide this information. The clinical profiles of different SERMs are strikingly variable. The largest head-to-head comparison trial of SERMs to date was the STAR trial, which showed that raloxifene and tamoxifen had similar beneficial effects on invasive breast cancer and clinical fractures, and similar noneffects on ischemic heart disease and stroke. Raloxifene, however, had lower risk of venous thromboembolism, cataracts, and cataract surgery, while tamoxifen had a nonsignificantly lower risk of noninvasive breast cancer. The future of SARMs and SERMs remains rich with possibilities, but their successful clinical application has been slowed by their variable tissue-specific effects.

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FGF Signaling in Craniofacial Biological Control and Pathological Craniofacial Development

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ABSTRACT: Fibroblast growth factor receptors comprise a family of four evolutionarily conserved transmembrane proteins (FGFR1, FGFR2, FGFR3 and FGFR4) known to be critical for the normal development of multiple organ systems. In this review we will primarily focus upon the role of FGF/FGFR signaling as it influences the development of the craniofacial skeleton. Signaling by FGF receptors is regulated by the tissue-specific expression of FGFR isoforms, receptor subtype specific fibroblast growth factors and heparin sulfate proteoglycans. Signaling can also be limited by the expression of endogenous inhibitors. Gain-of-function mutations in FGFRs are associated with a series of congenital abnormality syndromes referred to as the craniosynostosis syndromes. Craniosynostosis is the clinical condition of premature cranial bone fusion and patients who carry craniosynostosis syndrome-associated mutations in FGFRs commonly have abnormalities of the skull vault in the form of craniosynostosis. Patients may also have abnormalities in the facial skeleton, vertebrae and digits. In this review we will discuss recent *in vitro* and *in vivo* studies investigating biologic mechanisms by which signaling through FGFRs influences skeletal development and can lead to craniosynostosis.

KEY WORDS: craniofacial development, craniosynostosis, heparin sulfate proteoglycan, Frs2, PLCγ, MAPK

I. INTRODUCTION

Fibroblast growth factors (FGFs) and their receptors (FGFRs) regulate a variety of fundamental processes such as placental, limb, lung, neural, skin, long bone and craniofacial development. FGF signaling also plays significant roles in adult organisms, including roles in wound healing, angiogenesis and cancer.[1,2] In this review we will primarily focus upon the role of FGF/FGFR signaling as it influences the development of the craniofacial skeleton.

II. FGFR STRUCTURE

Fibroblast growth factor receptors (FGFRs) comprise a family of evolutionarily conserved receptor tyrosine kinases.[3] The FGFR family consists of four distinct but highly homologous transmembrane proteins (FGFR1, FGFR2, FGFR3, FGFR4), which act as high affinity receptors for the FGF ligands. Each full-length FGFR contains a signal peptide, three extracellular immunoglobulin-like domains (Ig1, Ig2, Ig3), an acid box domain (a contiguous box of acidic residues within the linker domain between Ig1 and Ig2), a transmembrane domain, an intracellular juxtamembrane domain and an intracellular split tyrosine kinase domain (Fig. 1). The third immunoglobulin domain provides for FGF ligand specificity, while the acid box confers the ability for glycosaminoglycan modification of the receptor at a serine residue immediately N terminal to this domain. Presence of the first immunoglobulin domain (Ig1) can prevent receptor glycosaminoglycan modification through steric hindrance and inhibit signaling.[4–6] Splicing involving exons encoding the first immunoglobulin domain and the acid box determines if these domains will be present in the mature FGFR protein.

Fibroblast growth factors (FGFs) are secreted glycoproteins that are commonly sequestered in the extracellular matrix by heparin sulfate proteoglycans (HSPGs).[7] Heparinase or protease liberated FGFs stimulate a diverse array of biologic responses by binding and activating cell surface FGFRs. The majority of FGFs bind with high affinity to FGFRs to stimulate downstream signaling only in the presence of heparin or heparin-like moieties, such as cell surface-bound heparin sulfate glycoproteins (HSPGs) or addition

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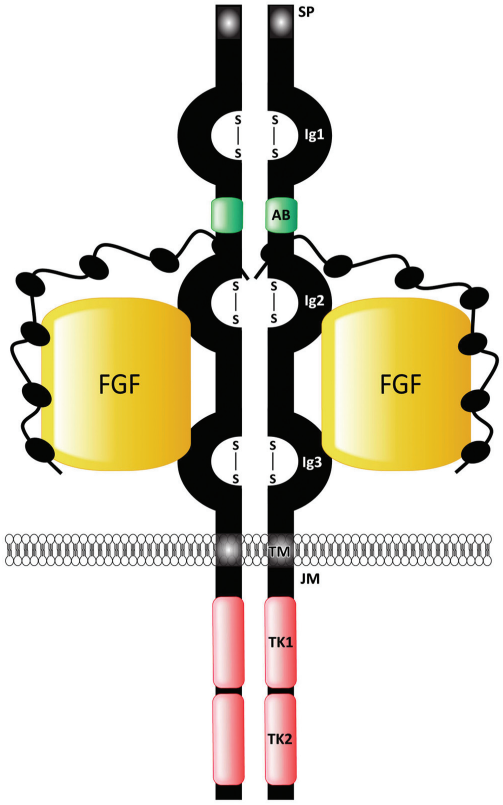

FIGURE 1. Schematic diagram of FGFR/FGF/Heparin Sulfate Proteoglycan binding complex. Each full-length FGFR contains an extracellular region, a single transmembrane region and an intracellular region. The extracellular region is composed of a signal peptide (SP), three immunoglobulin-like domains (Ig1, Ig2, Ig3), and an acid box domain (AB). Cysteine residues present within each Ig domain maintain the tertiary structure of the receptor via intramolecular disulfide bonding (S-S). Structural and biochemical studies indicate that FGFRs bind with high affinity to FGFs only in the presence of heparin sulfate proteoglycans (HS), which may be soluble, membrane-bound or matrix-bound. Presence of the acid box is required for HS incorporation into the binding complex, while presence of the Ig1 domain can sterically inhibit binding to HS. FGF binding involves both Ig2 and Ig3 domains, and specificity of ligand binding is determined by alternative splicing of the latter half of Ig3, as indicated by the lightning bolt. The intracellular region is composed of a juxtamembrane region (JM) and a split tyrosine kinase domain (TK1, TK2). Frs2 binds to the JM region in a phosphorylation independent manner. Upon ligand binding, FGFRs autophosphorylate at several residues to then phosphorylate multiple signaling proteins, including Frs2 and PLCγ.

of glycosaminoglycan moieties to the receptor.[4,6,8] Crystallographic and biochemical studies support a structural model that incorporates two FGFs with two heparin moieties and two FGFRs in a symmetric complex (Fig. 1). This structure may explain why distinct heparin sulfate motifs are required to elicit the activation of different FGF/FGFR pairs.[9] It is also important to note here that while the vast majority of FGFs bind with high affinity to FGFRs in the presence of HSPGs, the endocrine family of FGFs (FGF19, FGF21 and FGF23) lack an HSPG binding site and bind with high affinity to FGFRs in the presence of the coreceptors Klotho or βKlotho.[10]

FGF receptors are N-glycosylated and can be expressed as unglycosylated, partially glycosylated or fully glycosylated receptor forms.[11] Protein glycosylation sites are commonly found at sites of change in the secondary structure of a protein and have been suggested to stabilize the overall structure of a protein.[12] Glycosylation is also well known for its role in protein folding and quality control in the ER via the ER resident lectins, calnexin and calreticulin.[13] More recent data suggests a role for N-glycosylation in controlling FGF receptor subcellular localization and signaling. N-glycan branching allows for receptor interaction with the galactin-3 lattice, which inhibits endocytosis, promotes cell surface expression and hinders the downregulation of receptor signaling.[14,15] Receptor N-glycosylation is regulated by intracellular N-acetylglucosamine (GlcNAc) levels. UDPGlcNAc serves as a substrate for the transfer of N-acetylglucosamine residues to substrates. High cellular levels of UDP-GlcNAc, as occurs upon elevated glucose intake, promote an immediate increase in receptor N-glycosylation within the Golgi compartment. This enhances association of the receptor to galactin-3, thereby promoting cell surface expression and the ability to respond to ligands.[14] FGFR glycosylation can also regulate ligand binding. Mature FGFRs are heavily N-glycosylated (have many N-glycan branches). Removal of N-glycan branches increases receptor binding to FGFs and to heparin-like moieties, indicating that N-glycan branches may interfere with the binding of FGFR to FGF, and FGFR to the heparin or heparin-like coreceptor.[16] Finally, FGFR2 that is deficient in its

ability to mature into a fully glycosylated receptor exhibits retention in ER and Golgi compartments and increased proteosomal degradation.[17]

III. FGFR ISOFORMS

The diversity of this group of four receptors is further enhanced by the existence of multiple alternative splice sites that can result in the generation of numerous isoforms. Each FGFR isoform has its own distinct spectrum of affinities for the various FGF ligands and heparin or heparin-like coreceptors.[18,19] For example, alternative splicing involving exons encoding the latter portion of the third immunoglobulin-like domain results in expression of the epithelial cell lineage receptor, FGFR2IIIb, or the mesenchymal cell lineage receptor, FGFR2IIIc.[20] FGFR2IIIb exhibits receptor affinity for FGFs that are expressed by mesenchymal lineage cells while FGFR2IIIc preferentially binds FGFs that are expressed by epithelial lineage cells.[5] This form of alternative splicing facilitates epithelial-mesenchymal communication during development and homeostasis. As previously stated, splicing of exons encoding Ig1 and the acid box domains controls posttranslational glycosaminoglycan modification of the receptor, which also influences ligand binding and signaling.[6] Alternative splicing of the acid box domain within FGFR3 also influences receptor affinity for FGFs.[21] Specificity of downstream signaling upon receptor activation can be regulated by alternative splicing of valine and threonine residues within the intracellular juxtamembrane domain that are critical for Frs2 binding and MAPK signaling.[22] Tissue-specific expression of the various FGFR isoforms provides for precise control of signaling during development.[23]

IV. FGF/FGFR SIGNALING

Upon activation, FGFRs elicit downstream signaling via receptor dimerization, autophosphorylation and recruitment of docking and signaling proteins at the plasma membrane (Fig. 2). FGFR1 and FGFR2 can directly bind to activate PLCγ1, and can indirectly activate the Ras/Raf/MEK/MAPK signaling pathway.[24] Receptor autophosphorylation of a tyrosine at position 766 in FGFR1, 769 in FGFR2 and 760 in FGFR3 creates a specific binding site for the SH2 domain of PLCγ1.[24–26] Activated PLCγ1 hydrolyzes phosphatidyl inositol to form diacylglycerol (DAG) and inositol triphosphate (IP3), which, in turn, stimulate intracellular calcium release and the activation of protein kinase C (PKC).[27] Ras activation is achieved through recruitment and tyrosine phosphorylation of the docking protein Frs2, followed by binding and activation of adaptor proteins Grb2, Shp2 and Sos1.[28] MAPK activation can also be stimulated via Frs2-bound, atypical PKCs (PKCλ and PKCζ).[29,30] In either scenario, MAPK activation is dependent upon the binding and tyrosine phosphorylation of Frs2 by the FGF receptor. Upon phosphorylation, MAPK translocates to the nucleus where it functions to regulate gene expression by phosphorylating transcription factors.[31] FGF receptor activation can also lead to PI3 kinase activity resulting in Akt cell survival/antiapoptosis signaling via formation of an Frs2/Grb2/Gab1 complex.[32] Downstream cell type-specific proliferative and differentiation effects of FGFR1 and FGFR2 signaling can also be mediated by p38, PKCα and PKCδ signaling.[33–36] FGFR3 is distinct from FGFR1 and FGFR2 in its additional ability to signal through Stat proteins, although all four FGF receptors contain the conserved tyrosine residue that is critical for signaling through Stats.[37–40]

FGF signaling is tightly regulated at a cellular level. Threonine phosphorylation of Frs2 after FGF stimulation of the receptor leads to the subsequent downregulation of MAPK signaling.[41] FGF stimulated FGFR1 and FGFR2 are themselves downregulated via Frs2-mediated recruitment of the E3 ubiquitin ligase, Cbl, which leads to receptor degradation.[42] The magnitude and duration of downstream signaling is also controlled by Sprouty (Spry) proteins. Sprouty was initially identified in *Drosophila* as an inhibitor of FGF signaling and controller of airway branching during development.[43] Studies in vertebrates indicated that Spry proteins are upregulated by FGF signaling and function as inhibitors of FGF

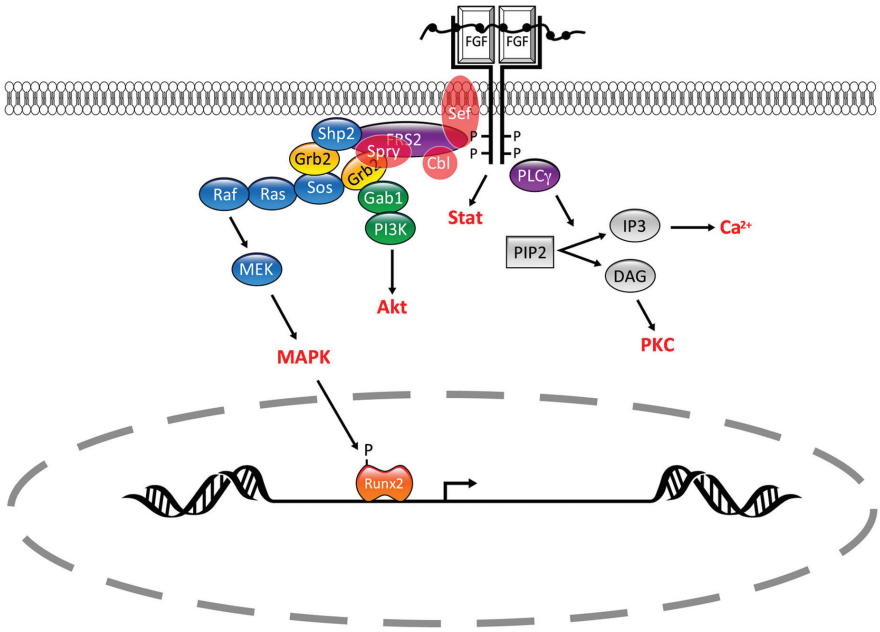

FIGURE 2. Schematic diagram of FGFR signaling. High affinity ligand binding induces receptor dimerization and intracellular tyrosine autophosphorylation. PLCγ is recruited directly to the phosphorylated receptor and, through the formation of diacylglycerol (DAG) and inositol triphosphate (IP3) from phosphatidylinositol-4,5-biphosphate (PIP2), stimulates intracellular calcium release and the activation of protein kinase C (PKC). The FRS2 docking protein mediates downstream Akt and MAPK signaling via complex formation with Grb2 and Gab1 or Grb2 and Sos1, respectively. Complex formation between FRS2, Grb2 and Sos1 can also be mediated by Shp2. Upon phosphorylation, MAPK translocates to the nucleus where it functions to regulate gene expression by phosphorylating transcription factors, including the osteoblast specific transcription factor, Runx2. FGFR activation also stimulates signaling through Stat proteins. Inhibitory proteins (indicated in translucent red) bind to FGFRs and FRS2 to diminish signaling and biologic effects of the activated FGF receptor.

signaling and long bone outgrowth.[44] Subsequent biochemical studies have demonstrated that Spry proteins translocate to the plasma membrane and are tyrosine phosphorylated upon cellular stimulation with growth factor.[45] Tyrosine phosphorylated Spry1 and Spry2 inhibit downstream MAPK signaling by binding with high affinity to Cbl and by inhibiting complex formation between FRS2, Grb2 and Sos1.[45,46] FGFR signaling is also mediated by Sef, a transmembrane protein that can bind to the receptor to inhibit autophosphorylation, phosphorylation of Frs2 and downstream signaling.[47] Together these mechanisms provide multiple regulatory layers and distinct negative feedback loops that are critical for the spatial and temporal control of FGF receptor signaling during development and homeostasis.

V. FGFR KNOCKOUT MOUSE MODELS

That FGF receptor expression is essential for normal development is evidenced by numerous reports investigating the effects of specific FGFR gene deletions (Table 1). Early studies indicated that both FGFR1 and FGFR2 are essential for early development because both FGFR1 and FGFR2 knockout mice are not viable.[48–52] Creation of isoform specific FGFR1 and 2 knockouts has enabled us to better elucidate distinct receptor isoform roles in development. FGFR1IIIc-specific knockout mice are defective in cell migration through the primitive streak and patterning of the mesodermal anterior-posterior axis while FGFR1IIIb mice appear viable and fertile with no overt phenotype.[53] Conditional Cre-mediated

TABLE 1. FGFR Knockout Mice Reveal Critical Functions During Development

Receptor	Model	Phenotype	Reference
FGFR1	Gene targeted FGFR1 knockout	Embryonic lethal. Defective mesodermal patterning during gastrulation.	Deng et al., 1994 Yamaguchi et al., 1994
FGFR1	Chimera made from $FGFR1^{-/-}$ and $FGFR1^{+/+}$ ES cells	Embryonic lethal. Failure of cell migration through primitive streak. Defective limb and neural tube formation.	Ciruna et al., 1997 Deng et al., 1997
FGFR1IIIb	Gene targeted FGFR1IIIb knockout	Viable and fertile.	Partanen et al., 1998
FGFR1IIIc	Gene targeted FGFR1IIIc knockout	Embryonic lethal. Failure of cell migration through primitive streak. Defective anterior-posterior mesodermal patterning.	Partanen et al., 1998
FGFR1 conditional knockout	Cre-LoxP mediated FGFR1 knockout in limb progression zone at E10.5 and in lateral plate mesoderm at E9.5	Limb bud and patterning defects. Defective autopod formation and digit patterning.	Li et al., 2005
FGFR2	Gene targeted FGFR2 knockout	Embryonic lethal. Trophoblast defects. Abnormal placental formation. Defective limb bud formation.	Xu et al., 1998
FGFR2	Chimera made from $FGFR2^{-/-}$ and $FGFR2^{+/+}$ ES cells	Lethal at postnatal day 1 (P0). Defective limb outgrowth. Defective lung formation.	Armad et al., 1999
FGFR2IIIb	Gene targeted FGFR2IIIb knockout	Lethal at P0. Defective limb, lung, pituitary gland, salivary gland, inner ear, teeth, skin and skull development.	De Moerlooze et al., 2000
FGFR2IIIc	Gene targeted FGFR2IIIc knockout	Viable. Delayed ossification with synostosis of skull base sutures, craniosynostosis and dwarfism.	Eswarakumar et al., 2002
FGFR3	Gene targeted FGFR3 knockout	Viable. Prolonged endochondral bone growth and inner ear defects.	Colvin et al., 1996 Deng et al., 1996
FGFR3IIIb	Gene targeted FGFR3IIIb knockout	Viable. No overt phenotype.	Eswarakumar et al., 2007
FGFR3IIIc	Gene targeted FGFR3IIIc knockout	Viable. Endochondral bone overgrowth and diminished bone density.	Eswarakumar et al., 2007
FGFR4	Gene targeted FGFR4 knockout	Viable. Defective cholesterol metabolism and bile acid synthesis. Hyperlipidemia, glucose intolerance, insulin resistance, and cholesterolemia.	Weinstein et al., 1998 Yu et al., 2000 Huang et al., 2007

deletion of FGFR1 has since revealed that FGFR1 is also critical for limb development. Cre-mediated deletion of FGFR1 after initial limb budding primarily affects development of the first two digits while Cre-mediated deletion of FGFR1 prior to the condensation of limb mesenchyme leads to excessive cell death, defective autopod formation and abnormal digit patterning.[54] FGFR2IIIb knockout mice exhibit abnormalities in development of the lungs, anterior pituitary, thyroid, teeth and limbs, while FGFR2IIIc null mice exhibit primarily defective long bone and craniofacial skeletal development and mineralization, indicating that this receptor variant may be critical for both endochondral and intramembraneous bone formation.[55,56] Although the appendicular skeleton and skull were reported to be proportionate in shape, the FGFR2IIIc deficient mice were reduced in size by 40% to 50% and delayed ossification was evident. Skulls of homozygous FGFR2IIIc null mice were smaller and had mineralized to a significantly lower extent than wild-type littermates. BrdU assays of the coronal suture showed evidence of diminished proliferation. Evidence of diminished expression of the osteoblast markers, cbfa1/runx2 and osteopontin, was also noted. These findings suggest that FGFR2IIIc functions to positively regulate bone growth and mineralization. Notably, these mice were also found to exhibit premature fusion of the cranial and skull base sutures. A mouse with conditional elimination of FGFR2IIIc in pre-skeletal tissue again supports the idea that this receptor isoform plays a positive role in normal skeletal development.[57] Mice homozygous null for FGFR2IIIc in osteoblast precursor-specific mesenchymal tissues were found to exhibit skeletal dwarfism with both diminished bone density and bone size. Vertebral abnormalities included non-ossification of the vertebral midline and absence of the spinous process. Osteoblasts were found to express normal levels of osteoblast differentiation markers at embryonic day 16.5 while there was evidence of diminished osteoblast marker expression by postnatal day 7. Diminished mineral apposition rate and diminished proliferation rates with no apparent differences in apoptosis were also evident. While these findings again suggest that FGFR2 signaling functions to enhance skeletal development, it is striking that these mice also exhibited dome-shaped skulls and tarsal bone fusions, characteristic of FGFR-associated syndromic craniosynostosis.

FGFR3 null mice exhibit inner ear and long bone defects.[58,59] FGFR3IIIb null mice have no obvious skeletal phenotype while FGFR3IIIc null mice show increased chondrocyte proliferation and skeletal overgrowth, indicating that FGFR3IIIc functions as a negative regulator of endochondral bone formation.[60] Finally, while initial studies indicated that FGFR4 was not essential for development (FGFR4 null mice have no gross abnormalities), more recent studies have demonstrated that hepatocytic FGFR4 expression is critical for cholesterol metabolism and maintenance of plasma lipid levels, as well as for regulating glucose tolerance and insulin sensitivity.[61–63] As expected from their developmental expression patterns, these studies together indicate that FGFR1IIIc, FGFR2IIIc and FGFR3IIIc all play an essential role in skeletal development.

VI. FGFRs AND CRANIOSYNOSTOSIS

Craniosynostosis is a clinical condition in which one or more of the sutures between cranial bones becomes prematurely fused. This fusion results in increased intracranial pressure as a result of limited growth at the fused sutures, and an abnormal craniofacial shape as a result of limited growth at fused sutures with compensating overgrowth at non-fused sutures. Severity of the phenotype depends upon the timing of synostosis and on the number of sutures affected.[64] Craniosynostosis has a relatively high incidence of approximately 1 in 2500 live births. Current treatment is limited to genetic counseling, surgery, orthodontics, and medical and social support. Craniosynostosis carries high morbidity, with many patients requiring multiple craniofacial surgeries throughout childhood. Craniosynostosis can occur sporadically or as part of a genetic syndrome.

Several theories regarding the etiology of craniosynostosis have been proposed. These include concepts that are biological or mechanical in nature. Mechanical theories involve the contribution of tensile and expansive forces upon cranial bone and

suture development while biological theories more typically propose a role for abnormal cell function in the cranial suture environment.[64–66] An important first step towards understanding the pathogenesis of craniosynostosis is to define the process of normal cranial bone and suture development. Development of the cranial bones occurs via intramembraneous ossification. Ossification begins at sites of mesenchymal condensations that, with continued proliferation, differentiation and mineralization, become the central zones of ossification for each cranial bone. With continued ossification, the cranial bones increase in size and ultimately grow into a close proximal relationship. At this point, ossification continues to occur via bony apposition along the osteogenic fronts, while suture patency is maintained. Cranial suture patency is more commonly maintained until adulthood, and is believed to allow for distortion of the cranium during birth, dampening of mechanically transmitted forces (to decrease injury) and/or compensation for the expansive forces generated by the growing brain.

It has been known for over a decade that mutations in FGF receptors promote abnormal craniofacial development and craniosynostosis in humans. Mutations in FGFR2 cause Apert, Crouzon, Jackson-Weiss and Pfeiffer syndromes, while mutations in FGFR1 cause Pfeiffer syndrome and mutations in FGFR3 cause Muenke craniosynostosis syndrome and Crouzon syndrome with acanthosis nigricans (Fig. 3). Craniosynostosis syndrome-associated mutations in FGFRs are autosomal dominant and exhibit complete penetrance with variable expression. It is a commonly held belief that the craniosynostosis associated FGFR mutations act as gain-of- function mutations in terms of FGF/FGFR signaling. More specifically, S252W and P253R Apert syndrome-associated mutations in FGFR2 result in loss of ligand binding specificity (the S252W mutation allows the mesenchymal splice form of FGFR2 (FGFR2IIIc) to bind and be activated by the mesenchymally expressed ligands, FGF7 and FGF10) and in increase of ligand binding affinity, such that these receptors exhibit increased ligand-dependent signaling.[67,68] Crouzon syndrome associated C332Y, Y340H, C342Y, C342R, C342S, S35C, W290G, T341P, C278F mutations and a Crouzon syndrome-associated 345–361 amino acid deletion in FGFR2 result in ligand independent autophosphorylation, dimerization and tyrosine kinase activity, such that these mutations are thought to promote ligand independent signaling.[17,69–71] The P252R mutation in FGFR1 and the analogous P250R mutation in FGFR3 (causative for Pfeiffer and Muenke syndromes, respectively) increase ligand binding affinity without altering ligand binding specificity.[72] Finally, the Crouzon syndrome with acanthosis nigricans-associated A391E mutation in FGFR3 lowers the free energy of FGFR3 dimerization, which may enhance ligand independent and/or ligand dependent signaling.[73] *In vivo* studies also support the idea that the pathogenesis of FGFR-associated craniosynostosis is mediated by increased receptor signaling. Chemical inhibition of tyrosine kinase signaling was shown to prevent coronal suture fusion in $FGFR2^{C342Y/+}$, $FGFR2^{S252W}$ and $FGFR2^{P253R/+}$ calvaria in organ

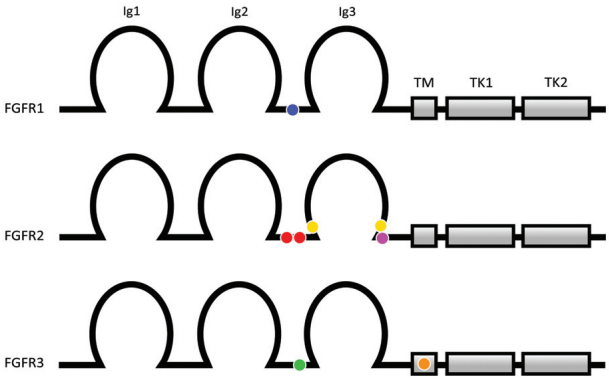

FIGURE 3. Location of FGFR mutations associated with syndromic craniosynostosis. Classic mutations in FGFR1, FGFR2 and FGFR3 genes associated with Apert, Crouzon, Crouzon with acanthosis nigricans, Peiffer and Muenke craniosynostosis are mapped onto the protein structure. Extracellular immunoglobulin-like domains 1, 2 and 3 are denoted Ig1, Ig2 and Ig3. Transmembrane and tyrosine kinase domains are labeled TM, TK1 and TK2, respectively. Classic mutations associated with the various FGFR-associated craniosynostosis syndromes are indicated with dots (red = Apert, yellow = Crouzon, blue = Pfeiffer, green = Muenke, orange = Crouzon with acanthosis nigricans, pink = Jackson-Weiss). Note that both Pfeiffer and Crouzon syndromes are also associated with numerous other mutations in FGFR2 (see Fig. 4).

culture.[74–76] Additionally, uncoupling of FGFR2$^{C342Y/+}$ from Frs2 signaling via mutation of the Frs2 binding site prevented craniosynostosis in mice that carry this mutation.[77] Importantly though, more recent studies have indicated that at least some of the craniofacial defects seen in FGFR2$^{C342Y/C342Y}$ mice (cleft palate) may be mediated by diminished receptor signaling.[78] This finding, in combination with the fact that craniosynostosis is evident in FGFR2IIIc null mice, suggests that FGFR associated craniofacial abnormalities such as craniosynostosis may result from either too much or too little FGF receptor signaling.[56]

It is likely that craniosynostosis is both multifactorial and polygenetic in nature such that even those forms that are presently believed to be sporadic may have some genetic component. The literature suggests that the craniosynostosis syndromes are in fact genetically "plastic" in that the same FGFR mutation can result in more than one syndromic phenotype and that different mutations can result in the same syndromic phenotype.[79–81] Existence of "plasticity" supports the idea that these syndromes are polygenetic and multifactorial in nature, with likely environmental influences, although it is also possible that much of this plasticity is actually the result of the inconsistent diagnosis of these craniosynostosis syndromes. These clinical syndromes typically involve the premature fusion of specific sutures with a resultant characteristic skull shape, midface deficiency, hypertelorism and ocular proptosis. Clinical diagnosis of a given syndrome is also often based upon the existence (or lack thereof) of associated hand and foot abnormalities such as large and deviated broad toes and/or syndactyly.

Pfeiffer syndrome (OMIMN ID# 101600) can be caused by a P252R mutation in the linker region between the Ig2 and Ig3 domains of FGFR1 or by mutations in FGFR2.[79,82–86] Classic Pfeiffer syndrome is characterized by craniosynostosis, hyperteloric or wide-set eyes, midface hypoplasia and characteristic anomalies of hands and feet consisting of broad thumbs, broad and short great toes, mesial deviation of great toes, brachydactyly and variable syndactyly.

Jackson-Weiss syndrome (OMIM ID #123150) is most commonly associated with an A344G mutation within the Ig3 domain of FGFR2.[87] This syndrome is characterized by variable craniosynostosis, frontal bossing, hypertelorism, strabismus and foot abnormalities, including broad great toes with medial deviation, and tarsal-metatarsal coalescence. Hand anomalies are rare. Most patients are of normal intelligence. Some patients have foot but no craniofacial anomalies. Ocular proptosis is usually mild. The syndrome is considered to exhibit much phenotypic variability.[88]

Muenke syndrome (OMIM ID #602849) is caused by a P250R mutation in the linker region between the Ig2 and Ig3 domains of FGFR3.[89] This syndrome is commonly characterized by unicoronal or bicoronal craniosynostosis, midface hypoplasia, hypertelorism and hand and foot abnormalities, including brachydactyly, thimble-like middle phalanges, coned epiphyses, carpal and tarsal bone fusions. Sensorineural hearing loss and developmental delay is evident in a minority of cases. While the radiologic findings of hands and feet can be helpful in the recognition of this syndrome, identification of the P250R mutation in FGFR3 provides for a definitive diagnosis.[90]

Crouzon syndrome is the most common of the FGFR-associated craniosynostosis syndromes. Common features of Crouzon syndrome (OMIM ID #123500) include bicoronal suture craniosynostosis with occasional pansynostosis, hypertelorism, severe ocular proptosis, strabismus, hypoplastic maxilla and relative mandibular prognathism.[91] In-depth radiographic analyses of Crouzon syndrome patients has also revealed a high percentage of patients to have conductive hearing loss, joint stiffness, calcification of the stylohyoid ligament and vertebral fusions.[92] Hydrocephalus is also not uncommon. Crouzon syndrome is associated with mutations in FGFR2. While mutations that cause Crouzon are distributed across multiple domains of the protein, the vast majority of mutations map to C278 or C342, two residues that are critical for formation of the intramolecular disulfide bridge within the Ig3 domain (Fig. 4).

Crouzon syndrome with acanthosis nigricans (OMIM ID #612247) is distinct from Crouzon syndrome both in genotype and phenotype. While Crouzon syndrome is consistently associated with mutations in FGFR2, Crouzon with acanothosis

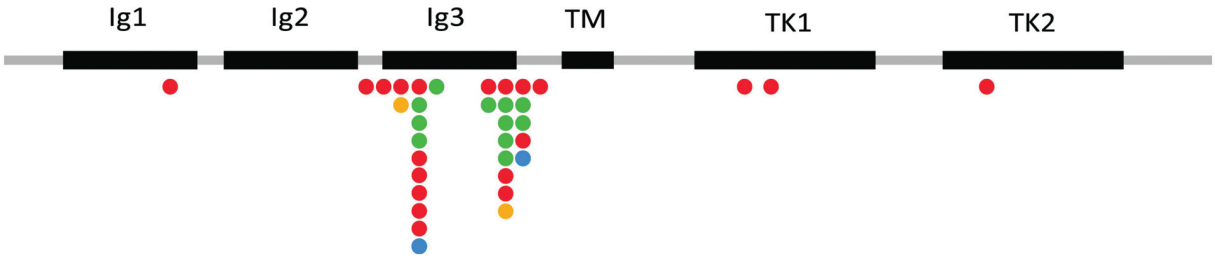

FIGURE 4. Location of Crouzon syndrome-associated mutations in FGFR2. Mutations in the FGFR2 gene associated with Crouzon syndrome are mapped onto the protein structure. Extracellular immunoglobulin-like domains 1, 2 and 3 are denoted Ig1, Ig2, Ig3. Transmembrane, juxtamembrane and tyrosine kinase domains are labeled TM, JM and TK, respectively. Red symbols denote missense mutations. Missense mutations creating or eliminating a cysteinyl residue are indicated as red symbols with black outline. Blue symbols denote small deletions. Purple symbols denote small insertions. The most common mutations resulting in Crouzon syndrome occur at residues C278 and C342, which form the intramolecular disulfide bridge within the Ig3 domain.

nigricans is associated with an A391E mutation in the transmembrane domain of FGFR3.[93,94] Patients with this syndrome have the clinical characteristic of Crouzon syndrome plus acanthosis nigricans. The skin abnormalities are of early onset and include velvety hyperplasia, accentuation of skin markings and hyperpigmentation in flexural regions including the neck, axillae, elbow and groin. Additional associated features include choanal atresia and shortened vertebral bodies.[95]

Apert syndrome is the most severe of the craniosynostosis syndromes (OMIM ID #101200) and is caused by an S252W or P253R mutation in the linker region between the Ig2 and Ig3 domains of FGFR2.[96] This syndrome is characterized by craniosynostosis with hypertelorism, down-slanting palpebral fissures, midface hypoplasia and widely patent fontanelles. Bony syndactyly of hands and feet with sparing of the thumbs results in a "mitten-like" appearance. Multiple central nervous system abnormalities have also been noted including hydrocephalus, ventriculomegaly, megalencephaly and gyral malformations. Defects in the corpus callosum, septum pellucidum, hippocampus and cerebral cortex may also be present. Cleft palate or a bifid uvula and hearing loss due to fused ossicles may also be observed. Mental retardation is not uncommon. Fused cervical vertebrae and elbow ankylosis may also be present.[97,98] Most cases of Apert syndrome are sporadic and the syndrome is associated with advanced paternal age.[99] More recent studies indicate that the high frequency of *de novo* Apert syndrome-associated FGFR2 mutations may be due to an increase in the clonal expansion of spermatogonia which carry these mutations.[100,101]

VII. MOUSE MODELS OF FGFR-ASSOCIATED CRANIOSYNOSTOSIS

Despite our knowledge in the genetics of craniosynostosis (Fig. 3), the biologic pathogenesis by which mutations in FGF receptors lead to the craniosynostosis phenotype remains elusive. FGFR2 is expressed in proliferating and differentiating osteoprogenitor cells and FGFR1 is expressed in differentiating osteogenic cells along and within the osteogenic fronts of growing cranial bones, indicating that FGFR activity could control cranial suture development by altering precursor cell survival, proliferation, differentiation, cell fate, matrix deposition, and/or mineralization.[102–105] Unfortunately, important studies that have attempted to link expression of mutant FGFRs with these cellular phenotypes *in vitro* have revealed conflicting and inconsistent results.[35,106–115] The development of mouse models of FGFR-associated craniosynostosis has allowed for an *in vivo* approach to investigating the molecular mechanism(s) that lead to craniosynostosis. Analyses of these mutant mice do provide some insight into the pathogenesis of craniosynostosis (Table 2).

TABLE 2. Mouse Models of FGFR-associated Craniosynostosis

Receptor	Mutation	Human Syndrome	Phenotype	Reference
FGFR1	FGFR1$^{P250R/+}$	Pfeiffer syndrome, human mutation FGFR1$^{P252R/+}$	Viable and fertile with normal long bones. Craniosynostosis of frontal, sagittal and coronal sutures. Dome-shaped skull, facial asymmetry, midface hypoplasia and hypertelorism. Increased calvarial cellular proliferation and osteoblast differentiation. Increased calvarial bone mineralization.	Zhou et al., 2000
FGFR2	FGFR2$^{C342Y/+}$	Crouzon syndrome, human mutation FGFR2$^{C342Y/+}$	Heterozygotes are viable and fertile with midface hypoplasia, ocular proptosis and craniosynostosis (coronal sutures). Homozygotes are lethal at P0 with cleft palate, tracheal and severe lung defects. Vertebral joint fusions with diminished vertebral body ossification. Increased coronal suture cell proliferation. Increased coronal suture cell Runx2 & osteopontin expression. Increased bone marrow osteoprogenitor numbers. No difference in bone mineral density.	Eswarakumar et al., 2004
FGFR2	FGFR2^{S250W}	Apert syndrome, human mutation FGFR2$^{S252W/+}$	Viable with decreased fertility of females. Small body size, dome-shaped skull, craniosynostosis (coronal sutures), hypertelorism, midface hypoplasia. No premature fusion of cranial base synchondroses. No difference in cellular proliferation. No differences in osteoblast differentiation. Increased coronal bone & suture cell apoptosis. Diminished cranial bone thickness and formation.	Chen et al., 2003
FGFR2	FGFR2^{P253R}	Apert syndrome, human mutation FGFR2$^{P253R/+}$	Viable and fertile. Growth retardation, dome-shaped skull, hypertelorism, midface hypoplasia, craniosynostosis (coronal sutures), variable syndactyly and delayed fusion of posterior frontal suture. Ectopic cartilage detected in sagittal suture. Increased osteopontin expression in coronal suture. Retarded endochondral growth and ossification.	Yin et al., 2008
FGFR3	FGFR3$^{P244R/+}$	Muenke syndrome, human mutation FGFR3$^{P250R/+}$	Viable and fertile. Dome-shaped skull, facial bone synostosis, variable craniosynostosis and sensorineural hearing loss. Delayed calvarial ossification. Diminished long bone mineral density. Genetic strain dependent phenotype.	Twigg et al., 2009

Genetic knockin mice heterozygous for the C342Y Crouzon syndrome-associated FGFR2 mutation have a phenotype quite similar to that of Crouzon syndrome patients.[116] Heterozygous mice are viable and exhibit a dome-shaped skull, wide-set and proptotic eyes, craniosynostosis, vertebral joint fusions and diminished vertebral body ossification. Increased expression of the bone markers, osteopontin and Cbfa1/Runx2, was evident around the coronal suture at P1 but no differences in bone density or

mineralization were evident. Osteoblast number and BrdU incorporation were increased in heterozygotes, as compared to wild-type littermates. Homozygous mice were nonviable. Close inspection of homozygous embryos revealed lung defects, cleft palate, severely shortened nasomaxilla, lack of ossification of vertebral bodies, absence of tracheal rings, synarthrosis of multiple joints and fused sternebrae.

Mice carrying one of the most common FGFR2 mutations linked to Apert syndrome, S250W, also exhibit phenotypic abnormalities similar to those of Apert syndrome patients.[117] Mice heterozygous for the S250W mutation had diminished body size, an abnormal skull shape, wide-set eyes, coronal suture fusion and no fusion of cranial base sutures. BrdU and immunohistochemistry revealed no evident differences in proliferation or differentiation of calvarial osteogenic cells around the coronal suture. TUNEL staining showed evidence of increased apoptosis in the coronal suture, suggesting that a dysregulation of apoptosis may play a role in the pathogenesis of Apert craniosynostosis. Strikingly, the $FGFR2^{S250W/+}$ mice exhibited decreased cranial bone formation, and cranial bone thickness was found to be significantly diminished in the mutant mice. Subsequent analysis of the $FGFR2^{P253R/+}$ mouse model of Apert syndrome demonstrated precocious coronal suture fusion, a shortened cranial base and shortened long bones in the mutant mice. *In situ* hybridization and real time PCR revealed increased bone marker expression around the coronal suture and in primary calvarial osteoblasts *in vitro*, while chondrocytes exhibited diminished proliferation and diminished chondrogenesis.[118] These results indicate that Apert syndrome may result from a combination of defective osteo- and chondrogenesis. More recent analysis of the $FGFR2^{P253R}$ mutation in a mouse model revealed diminished osteoblastic proliferation and differentiation around the coronal suture and in the long bones of $FGFR2^{P253R/+}$ mutant mice.[119]

It is striking that the $FGFR2^{C342Y/+}$ mouse, the $FGFR2^{S250W/+}$ mouse, and the $FGFR2^{P253R/+}$ mouse all exhibit craniosynostosis but do not exhibit increased bone formation or mineralization. The mouse model of Muenke craniosynostosis exhibits a phenotype similar to human patients and also does not show enhanced calvarial bone formation. These $FGFR3^{P244R/+}$ mice have a dome-shaped skull with consistent facial bone synostosis and variable craniosynostosis. Remarkably, these mice also show delayed calvarial ossification and diminished bone mineral density compared to wild-type littermates.[120] In contrast to this, mice carrying the P250R mutation in FGFR1 associated with Pfeiffer syndrome exhibited a dome-shaped skull, midfacial hypoplasia, hypertelorism and craniosynostosis of multiple cranial sutures, with advanced calvarial bone mineralization, increased proliferation and osteoblastic differentiation of cells within and around the sagittal suture.[121] Taken together, these studies indicate that while increased calvarial bone formation and mineralization may be a component of some forms of craniosynostosis, it is certainly not central to the pathogenesis of all forms of FGFR-associated craniosynostosis.

In support of the idea that FGFR-associated craniosynostosis does not necessarily result from increases in bone formation and/or mineralization is the finding that human activating mutations in FGFR1 that cause osteoglophonic dysplasia lead to dwarfism, craniosynostosis, hypophosphatemia and severe demineralization of both endochondral and intramembraneous bones (OMIM ID #166250).[122] Similarly, craniosynostosis is also seen in humans and mice with X-linked hypophosphatemic rickets due to mutations in the phosphate regulating protein, Phex (OMIM ID #307800).[123,124] Patients and mice with Phex mutations have low serum phosphate and defective bone mineralization due to high circulating FGF23 levels and diminished renal phosphate reabsorption. It is unknown how these defects result in craniosynostosis, but similar to studies of human patients with FGFR2-associated craniosynostosis, these patients also commonly have paradoxical heterotopic calcification of normally non-mineralizing tissues, such as tendons and ligaments.[125] Additionally, craniosynostosis is also seen in up to 78% of infants with hypophosphatasia (OMIM ID #171760) due to inactivating mutations in the enzyme, tissue non-specific alkaline phosphatase (TNAP).[126] These patients have severely deficient bone mineralization.[127] TNAP is a local generator of inorganic phosphate and an established critical mediator of tissue mineralization,

but it is again unknown how diminished TNAP activity leads to craniosynostosis.[128,129] It is worthy to note here that several reports have previously demonstrated that FGF signaling suppresses TNAP expression but a role for diminished TNAP expression in FGFR-associated craniosynostosis has yet to be established.[17,110,130–132] That craniosynostosis occurs in multiple mouse models and human syndromes involving dysregulated phosphate homeostasis does make it tempting to hypothesize that craniosynostosis may be promoted by abnormal tissue levels of inorganic phosphate.

VIII. CONCLUSIONS

For more than a decade we have known that mutations in the genes encoding fibroblast growth factor receptors promote craniosynostosis, yet a central hypothesis regarding the etiology of this disorder has not yet emerged. This is not entirely surprising considering the fact that FGF/FGFR signaling has many roles during development and has potentially compound effects upon craniofacial growth and tissue mineralization. Future studies involving the use of established mouse models of craniosynostosis should further illuminate molecular mechanisms by which mutations in FGF receptors lead to abnormal craniofacial development and craniosynostosis, and provide for the development of biologically rational therapeutics to treat infants and children with this debilitating condition.

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Heterotopic Ossification Has Some Nerve

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ABSTRACT: Heterotopic ossification, defined as the formation of bone in abnormal anatomic locations, can be clinically insignificant or devastating and debilitating, depending on the site and duration of new bone formation. There are many causes of heterotopic ossification (HO), including soft tissue trauma, central nervous system injury, vasculopathies, arthropathies, and inheritance. One of the least understood components of HO is the interaction of the peripheral nervous system with the induction of this process. Recent work has shown that, upon traumatic injury, a cascade of events termed neurogenic inflammation is initiated, which involves the release of neuropeptides, such as substance P and calcitonin gene related peptide. Release of these peptides ultimately leads to the recruitment of activated platelets, mast cells, and neutrophils to the injury site. These cells appear to be involved with both remodeling of the nerve, as well as potentially recruiting additional cells from the bone marrow to the injury site. Further, sensory neurons stimulated at the injury site relay local information to the brain, which can then redirect neuroendocrine signaling in the hypothalamus towards repair of the injured site. While numerous studies have highlighted the important role of nerve-derived signals, both central and peripheral, in the regulation of normal bone remodeling of the skeleton,[1] this review focuses on the role of the local, peripheral nerves in the formation of heterotopic bone. We concentrate on the manner in which local changes in bone morphogenetic protein (BMP) expression contribute to a cascade of events within the peripheral nerves, both sensory and sympathetic, in the immediate area of HO formation.

KEY WORDS: heterotopic ossification, peripheral nervous system, BMP2, neurogenic inflammation, sensory, sympathetic

I. HETEROTOPIC OSSIFICATION

I.A. Stages of Heterotopic Ossification

Heterotopic ossification (HO) appears to form *de novo* within tissues, presumably through the recruitment of stem cells and progenitors, which then undergo all stages of endochondral bone formation. Much speculation has suggested that injury to the tissue, through trauma, may lead to the recruitment of stem and progenitor cells to the injury site. Upon arrival, these progenitors are then exposed to osteoinductive factors that direct their differentiation towards the chondro-osseous fate. The newly formed ectopic bone is similar to skeletal bone, possesses a bone marrow cavity, and can often fuse the normal skeleton.

Studies from mouse models of HO,[2,3] where bone formation is induced through delivery of bone morphogenetic protein 2 (BMP2), show a series of changes within the soft tissues, including nerves, vessels, and muscle. One of the initial changes observed at the site of new bone formation is the appearance of brown adipocytes. These cells are capable of utilizing their uncoupled aerobic respiration to reduce localized oxygen tension and effectively pattern the newly forming cartilage condensations.[4] These unique cells are also able to express angiogenic factors, such as VEGF-A and -D, which can enhance rapid, new vessel formation.[5] This vascular ingrowth must occur for the transition of avascular cartilage to bone.[6] Therefore, it is not surprising that a mechanism exists for regulating both local oxygen tension and vessel growth as a component of HO. Interestingly, just prior to chondrogenesis, expression of markers of endothelial adhesion (E-selectin, SDF-1, CXCR4, VCAM) and vascular remodeling are elevated, simultaneous to the appearance of proliferating

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inflammatory-like cells (CD68+, SMA+, SMMHC+, Lysozyme M+) within the tissues (personal communication[7]). These inflammatory-like cells lose these more primitive markers upon expression of the chondrocyte/Schwann cell marker Sox9, forming a sharply demarcated perichondral region delineated by the Sox 9 expression.[7] Further, we find that these cells appear to be adjacent to the oxygen reducing brown adipose, and thus, form a three dimensional architecture where the brown adipose may be regulating chondrogenic differentiation through hypoxia.[4,7] As the cartilage and bone form, they appear to surround and engulf the muscle tissues, with significant muscle hypertrophy, and death. It is unclear what governs the inflammatory response after induction of bone morphogenetic protein (BMP) signaling, but recent studies in using these mouse models suggest a regulatory role for peripheral nerve signaling. In these models, BMP2 appears to lead to neuroinflammation, which involves recruitment of mast cells and neutrophils, activation of platelets, and significant expansion of myeloid progenitors (Salisbury et al., in preparation), suggesting that peripheral nerve stimulation by BMP2 may be involved in the induction of HO.

I.B. Clinical Scenarios of HO

Although many have speculated that HO is a heterogeneous disorder stemming from a number of different causes, study of the literature, across the different fields, reveals striking similarities to animal models. One commonality appears to be the enhanced expression or release of BMP2, at a time when stem cells and progenitors are identified within the tissues. Clearly, traumatic injury to skeletal bone and muscle leads to the increased expression of BMPs at the injury site, and recent reports suggest not only a role for this protein in bone formation, but also a critical role in muscle regeneration and repair.[8] Clever et al. showed that many components of the BMP signaling pathway were activated within 24 hours of injury, and played a critical role in myoblast progenitor expansion.[8] It is unclear how BMPs can be involved in muscle stem cell expansion and repair, and still induce bone formation. One possibility is that the levels of BMP within the tissue may be a defining factor. Often, in cases of significant muscle injury, the adjacent bone is also disrupted, which could lead to the substantial release of BMPs within the local environment. Since BMP expression within the muscle must be turned off in order for the muscle progenitors to differentiate, perhaps lower levels of BMPs are required for limited times. When these higher levels of BMP are found, the results are shifted towards endochondral bone formation. Alternatively, there could be a secondary mechanism evoked, beyond the alteration in BMP expression. It is intriguing that in certain types of traumatic injury, such as myositis ossificans traumatica, which appears to result from muscle trauma, HO occurs without injury to the skeletal bone.[9] Beiner et al.[9] demonstrated that the inflammatory response evoked appears to rapidly destroy the muscle fibers, which are replaced with heterotopic bone. Clinically, it is unclear what the inductive components are that lead to HO, but the data suggests that BMP expression upon injury may play a key role.

One of the most common causes of heterotopic ossification in the general population is central nervous system injury. Heterotopic ossification is an especially challenging problem for spinal cord injury patients who have an elevated risk for HO. While such patients often lose all motor and sensory function below the level of the spinal cord injury, the distal nerves themselves remain viable and functional, although they no longer communicate with the brain. Indeed, neuronal activity in the lower extremity of spinal cord injured patients can be abnormally high, frequently causing spasticity.

Surprisingly, studies of HO in cardiovascular tissues have striking similarities to HO at other sites. Two primary sites within cardiac tissues appear to form bone: cardiac valves and within atherosclerotic plaques. Although the mechanisms that govern aortic valve (AV) degeneration are largely unknown, many of the pathways involved in embryonic formation of the valve appear to be disrupted in AV degeneration.[10] Sucosky et al. recently demonstrated that shear stress within the valve, from alterations in blood flow, appeared to rapidly enhance BMP2/4 signal-

ing. The authors further suggested that the increase in BMP2/4 signaling led to localized inflammation and degeneration of the valve tissue.[11] Intriguingly, the valve contains peripheral nerves, which undergo neuroinflammatory remodeling during AV degeneration.[10]

Like the valves, shear stress and changes in hemodynamics have been suggested to be responsible for HO formation in atherosclerotic plaques.[12] Several BMPs have been detected in atherosclerotic plaques, including BMP2.[13] Elevation in BMP signaling through shear stress and a reduction in blood flow is thought to be responsible for early vascular inflammation.[14,15] Interestingly, Yao et al. demonstrated that increased BMP signaling led to the elevation of the endothelial adhesion molecules CD68, E-selectin, VCAM and ICAM-1.[14] They speculated that induction of BMP signaling in cardiac tissues induces monocyte infiltration through elevation in these endothelial adhesion molecules.[14] Further, the authors speculate that the elevated BMP signaling could also lead to osteochondrogenic lineage reprogramming of smooth muscle cells.[16] Intriguingly, the mechanisms evoked in cardiovascular HO are very similar to what is observed in the BMP2 mouse model, again suggesting that this disorder may follow a common mechanism, regardless of the location of onset.

One of the best examples of the direct correlation between heterotopic bone formation and enhanced BMP signaling is the genetic disease fibrodysplasia ossificans progressiva (FOP).[17] Recently, Shore et al.[18] identified an activating mutation in the activin receptor type 1, a bone morphogenetic protein type 1 receptor, in patients with FOP, presumably leading to the formation of HO in skeletal muscle, tendons, and ligaments. This activating mutation leads to BMP signaling. However, the receptor activity can still be enhanced upon addition of BMP protein,[18] suggesting that there is a threshold level of BMP required for induction of bone formation. Interestingly, in patients that possess the mutation, even minor trauma to the muscle appears to rapidly induce HO, presumably by the rapid elevation in BMP expression in muscle after injury.[8] Perhaps this trauma releases BMPs within the muscle itself,[17] providing the small amount of additional stimulus to form the bone. Alternatively, Kitterman et al. showed the formation of HO along the needle track after childhood vaccinations in patients with FOP, suggesting that peripheral nerves, such as sensory neurons, may also contribute to induction of the bone formation.[19] BMPs have been shown to be expressed in normal peripheral nerves regulating neuronal function, and BMP signaling appears activated upon peripheral nerve damage, suggesting that BMPs play a role in the peripheral nerve's response to injury.[20]

We have highlighted the most common areas for heterotopic ossification to occur. However, the risk of HO within the general population is fairly low, approximately 5%, suggesting that it is still a very rare event. This most likely contributes to our lack of mechanistic knowledge of the subject. However, recent statistics from the military suggest that as many as 60% of all military casualties[21] are reported to have some form of HO. These numbers are staggering and have led researchers to question what is behind the significant increase in incidence. One possible reason is the type of injuries sustained in the military population. Approximately 60% to 70% of traumatic injuries are a direct result of blast or burn injuries associated with improvised explosive devices (IEDs), which can have dramatic effects on peripheral and central nervous system signaling, but can sometimes, paradoxically, leave the body's tissues with undetected or minimal damage.[21] One commonality among these types of injuries appears to be trauma to the peripheral nervous system. Here we examine the potential link between the peripheral nervous system and induction of heterotopic bone formation.

II. HETEROTOPIC OSSIFICATION AND THE SENSORY NERVOUS SYSTEM

II.A. TRPV1 Sensory Neurons and Heterotopic Bone

Little is known about sensory nerves and bone. Studies in our own laboratory suggest a functional role for these nerves in HO. Recent studies in mice

lacking TRPV1 (transient receptor potential cation channel V1) sensory neurons have shown these mice develop significantly less heterotopic bone after induction with BMP2, as compared to the normal counterpart (Salisbury et al., in preparation). Dissection of this sensory pathway after BMP2 induction showed a significant elevation in both substance P (SP) and calcitonin gene-related peptide (CGRP), which was absent in mice lacking TRPV1 sensory neuron function.

The small diameter, afferent sensory fibers of the peripheral nervous system (PNS) are of major importance in the release of SP and CGRP, and subsequent inflammatory effects. Within the tissues, these nociceptive primary afferent neurons respond to noxious mechanical, thermal, or chemical stimuli, providing feedback on pain and temperature.[22] Upon injury or inflammation, noxious stimuli activate these nociceptive, sensory fibers, which release neuropeptides both in the periphery, leading to neurogenic responses, and centrally to transmit the nociception to the central nervous system. The vanilloid (capsaicin) receptor TRPV1 is a nociceptive, ion channel located on sensory nerve endings that is activated by some of these noxious stimuli and involved in the mediation of pain sensation.[23,24] Capsaicin, the compound in hot chili peppers which gives them "heat," is one chemical stimuli that can activate TRPV1, causing the ion channel to open, leading to an influx of calcium and sodium ions into the sensory neuron and triggering depolarization of the neuron. At normal levels, capsaicin binding transmits the sensation of pain. However, high doses of capsaicin lead to a massive influx of ions, resulting in cell death of sensory neurons expressing TRPV1.

II.B. Neurogenic Inflammation and Heterotopic Bone

While TRPV1 activation sends afferent signals to the central nervous system for the communication of pain, it also leads to neurogenic inflammation by the release of SP and CGRP within the tissue.[25] Indeed, TRPV1 is highly coexpressed with the substance P-positive and CGRP-positive neurons of the dorsal root ganglion.[26] This neurogenic inflammatory process is mediated by the release of neuropeptides from sensory nerves, which in turn act on target cells in the periphery, such as mast cells, to produce inflammation[22,27] (Fig. 1).

Intriguingly, BMP has been shown to upregulate CGRP, as well as SP, expression in sensory neurons cultured from dorsal root ganglia,[28] suggesting this molecule plays a role in producing these neuroinflammatory responses. Therefore, release of BMP2, such as during the induction of HO in soft tissue, initiates neurogenic inflammation within the local environment (Fig. 1). It is important to note that the small diameter, capsaicin-sensitive sensory neurons, which are critical in generation of neurogenic inflammation, are themselves activated upon injury and trauma, consequently augmenting the inflammatory response produced by the sensory nerves in scenarios of HO involving traumatic injury. The ability of BMP signaling to evoke this mechanism may, in part, explain why patients with an inherited form of HO, FOP, exhibit an increase in mast cell density within the lesional area of heterotopic bone, as compared to unaffected tissues.[29]

These pro-inflammatory neuropeptides bind to receptors expressed on mast cells, stimulating their activation and subsequent release of a variety of enzymes and inflammatory factors from intracellular granules within the mast cell, a process referred to as degranulation[30,31] (Fig. 1). Upon degranulation, mast cells release a variety of mediators, including serine proteases, such as chymase and tryptase, histamines, and cathepsins, which are associated with many types of tissue remodeling.[30] In addition, many sensory nerve terminals are lined with receptors for the various mast cell mediators, which, upon activation, can lead to further release of SP and CGRP, creating a positive feedback loop for the perpetuation of neurogenic inflammation.[30] Studies in our BMP2-induced mouse model of HO support a role for mast cell degranulation in the progression of HO (Salisbury et al., in preparation). Mice treated with cromolyn, which is known to inhibit mast cell degranulation, prior to BMP2-induction, develop a significantly smaller heterotopic bone lesion than untreated animals (Salisbury et al., in preparation).

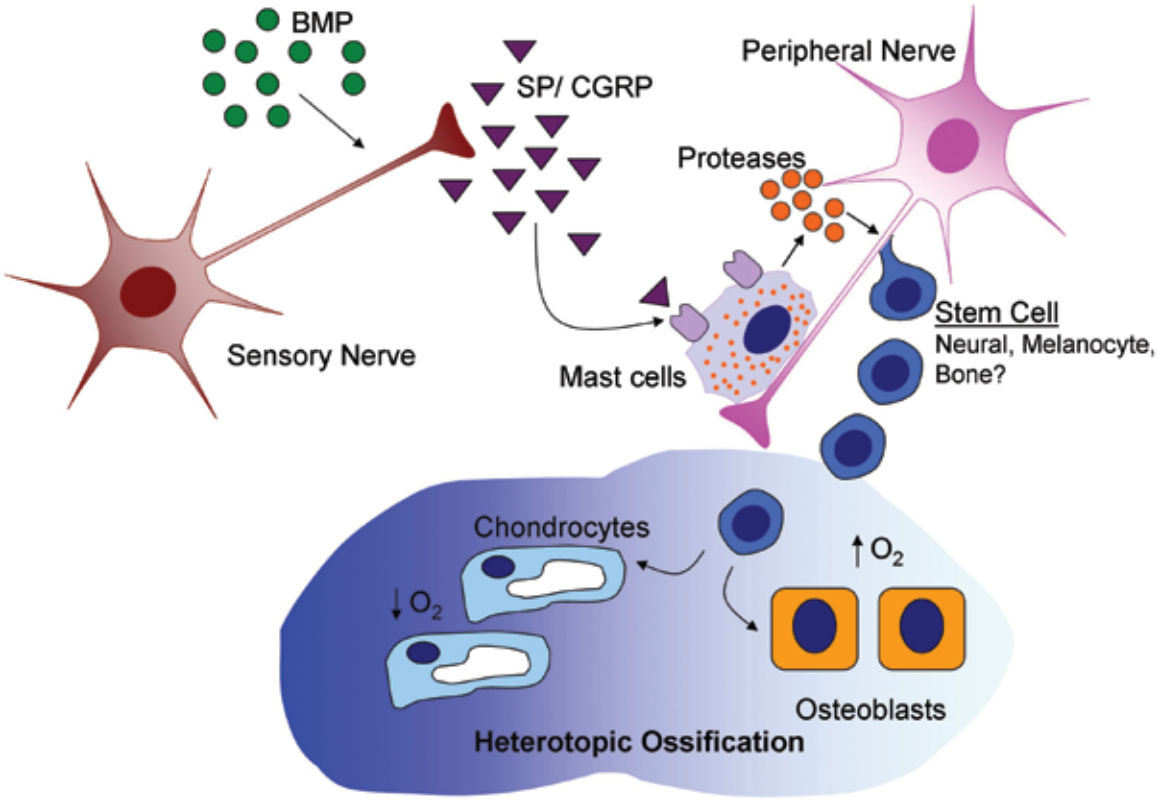

FIGURE 1. Schematic representation of the tentative neuroinflammatory mechanism and its relationship to heterotopic ossification. BMP2 can induce recruitment of mast cells and nerve tissue remodeling, through activation of sensory neurons and release of Substance P (SP) and calcitonin gene-related peptide (CGRP).

Mast cell proteases released upon degranulation are also linked to remodeling of the peripheral nerve.[32] Upon injury to the nerve, Schwann cells associated with the nerve start to repair the damaged nerve sheath.[33] This phenomenon holds greater significance when given the current findings that stem cells, which contribute to other tissues, are stored within the nerve sheath. Recently, Adameyko et al.[34] demonstrated the presence of a primitive stem cell within the nerve that contributed to melanocytes within the skin. Additionally, in patients with the complex disease neurofibromatosis, cells cannot migrate from the nerve; therefore, they remain within the nerve sheath and form the characteristic nerve-associated tumors of the disease.[35] These patients also display skeletal and skin abnormalities, including partial, early closure of the growth plate, bone loss, and café au lait spots within the skin. These phenotypes hint at a mechanism where stem cells for bone and melanocytes also reside in the nerve and become trapped in this disorder, leading to improper bone formation and skin pigmentation. Finally, studies in the developing sciatic nerve isolated from rats revealed three distinct stem cell populations within the nerve: one, a population of multipotent, self-renewing progenitors, presumably derived from the neural crest,[36] which contribute to the generation of peripheral nerves;[37] two, a population that appeared to generate Schwann (glial) cell precursors, which express glial fibrillary acidic protein (GFAP); three, a population of smooth-muscle like cells, which appeared to be absent from other nerve structures, but the authors speculate could contribute to more mesenchymal lineages. Interestingly, these cells were

SMA+, SMMHC+ similar to the cells identified as tentative chondrocytes[13,38–40] and the prechondrocytes we observed in our model of HO. All of these studies point to a pool of stem cells within the nerve, with the potential to contribute to the structures of bone, including chondrocytes and osteoblasts.

In addition to nerve remodeling, the mediators released by mast cells can elicit a variety of pro-inflammatory effects within the tissues. In concert, SP and CGRP, along with activating mast cells, can induce other immune cells, including monocytes, macrophages, lymphocytes, and platelets.[22,41,42] Both neuropeptides are potent vasodilators.[43] We have observed an elevation of platelets in the blood early after injection of BMP2-producing cells, and at later times, we have observed an elevation of neutrophils (Salisbury et al., in preparation). Platelets play a critical role in wound healing and hemostasis, as well as in repairing bone fracture.[44] Induction of the sensory neuropeptides, whether by injury, BMP, or a combination of the two, modulates the local immune response, thus promoting the progression of HO.

II.C. Sensory Neuropeptides and Skeletal Bone

Intriguingly, capsaicin-sensitive sensory neurons and sensory neuropeptides have been implicated in the maintenance of the normal skeleton as well.[45] Capsaicin-induced denervation of the sensory neurons results in a loss of trabecular bone volume, decreased osteoblast activity, and impaired bone formation. Additionally, there is evidence that CGRP plays a fundamental role in osteoclast formation and function. Several studies showed that CGRP inhibits the formation of osteoclasts and that capsaicin-induced denervation leads to impaired recruitment of osteoclast precursors.[45] Both SP and CGRP have also been identified to promote osteogenesis *in vitro*.[46,47] Consequently, these neuropeptides appear to have the potential to interact with some of the principal cells, osteoblasts and osteoclasts, involved in bone formation and remodeling of the normal skeleton. This may suggest a similar potential in the formation of heterotopic bone formation, although these mechanisms have not currently been examined.

Additional evidence for the role of the peripheral nervous system, in particular the sensory nerves, in *de novo* bone formation comes from a number of clinical observations and basic science studies on the healing of fractured bone. Several animal studies have shown that transection or denervation of the complete peripheral nerve leads to an impaired healing of fractures.[48–50] While these studies examined the effects of combined motor, sensory, and autonomic denervation, a more recent study by Apel et al. further demonstrated that sensory denervation alone impairs fracture healing.[51] Using a model of capsaicin-denervated animals, which impairs the CGRP- and SP-positive nerve fibers of the PNS, the authors showed that sensory denervated animals displayed a fracture callus that is significantly larger and less ossified, with reduced mechanical strength, compared to fractures in animals with intact sensory nerves. In line with these results, clinical studies have revealed that the levels of the sensory peptides, such as CGRP and substance P, are significantly increased in patients within 24 h of bone fracture.[52] Following fracture of the rat tibia, studies have also shown a substantial increase in CGRP-expressing neurons that colocalize with new bone formation,[53] and a significant increase in the number of SP-positive nerve fibers.[54] In addition to fracture models, studies examining the repair of an experimental bone defect model in the rat tibia also demonstrated an increase in the number of nerve fibers expressing substance P and CGRP within the first few days following the defect, which returned to normal by 3 weeks.[55] All of these observations suggest that peripheral nerves, particularly the sensory component, are closely involved in fracture healing and bone repair following injury. Further, the data supports a global mechanism for bone formation involving the sensory neurons and neuroinflammation. Neuroinflammation mediated by the sensory nerves can lead to not only vasodilation, extravasation, and the recruitment of potential progenitors, but also potential nerve remodeling and the release of progenitors that contribute to bone formation.

III. HETEROTOPIC OSSIFICATION AND THE SYMPATHETIC NERVOUS SYSTEM

BMPs have been demonstrated, *in vitro*, to induce development of sympathetic neurons from neural crest cell cultures. Additionally, *in vivo* studies revealed that delivery of the BMP antagonist, noggin, to the chick embryo during the time of sympathetic neuron differentiation prevented expression of noradrenergic marker genes and generation of sympathetic nerves.[56] More recent studies, using conditional knockout embryos, have further defined the mechanisms by which BMP signaling regulates sympathetic nervous system (SNS) development, including a role for BMP signaling in survival of SNS precursors and SNS differentiation and proliferation.[57] Moreover, BMP2 has been shown to induce neurotransmitter and neuropeptide expression in rat neonatal sympathetic neurons.[58] Given the defined and important role of BMP during these key developmental events, it would not be surprising to observe BMP involvement in regulating SNS function during heterotopic bone formation within the adult organism.

III.A. Sympathetic Nerve Regulation of HO

As mentioned, one of the earliest steps in our mouse model of HO is the biogenesis of brown fat, approximately two days following injection of BMP2-producing cells.[4] These brown fat cells are critical for patterning of the local oxygen environment necessary for further cartilage and bone formation. While the exact mechanism by which BMP2 induces the rapid production and expansion of brown fat is currently under investigation, the induction of brown adipose tissue (BAT) has been shown to involve the SNS. Interestingly, heterotopic ossification in *Misty Grey Lean* mice, which lack functional brown adipose,[59] led to enhanced bone formation.[4] In these studies, the white adipose appeared to compensate for the loss of brown adipose, by utilization of its lipid to induce a hypoxic environment. Thus, the contribution of BAT in this model could be considered inhibitory, since we obtained a greater response in bone formation. However, the utilization of the white adipose, which is unable to uncouple, in this model, is at the expense of creating considerably reactive oxygen.[60] Recently, the mutation in *Misty Grey Lean* mice was identified to be in a protein known as *dock 7*,[61] which is known to be involved in axonal migration. This suggests a possible relationship between the potential nerve cell migration and expansion from the sensory neurons and the production of brown adipose through SNS stimulation.

Noradrenaline release from sympathetic neurons stimulates β_3-adrenergic receptors abundantly expressed on brown fat cells, ultimately directing a number of proteins involved in the upregulation of a brown fat phenotype.[62] In support of sympathetic regulation of BAT, administration of β_3-adrenergic receptor agonists increases BAT in mice, dogs, and primates[63]; adult humans with enhanced noradrenaline release, due to rare tumors of the adrenal glands, also develop more abundant brown fat deposits. Therefore, the SNS likely has a role in controlling the induction of BAT during HO (Fig. 2).

Interestingly, the production of BAT through sensory nerve stimulation during the initial stages of HO leads to further stimulation of sensory neurons within the local environment. Since sensory neurons, particularly the small diameter, afferent sensory fibers of the PNS, respond to thermal stimuli, heat produced by the brown adipose will continue to induce signaling and resultant neuroinflammation. Brown adipocytes, in addition to their ability to generate hypoxic stress within the tissue, are known for their function in heat generation, or thermogenesis.[62] Brown adipocytes exclusively express UCP1 (uncoupling protein 1), which is capable of uncoupling the electron transport chain from the generation of ATP to the generation of heat.[62] Therefore, an additional outcome of BAT activation is the release of heat within the local environment. Thus, the initial pulse of BMP ultimately sets in motion a cascade of neuronal signaling events that propagate and reinforce each other to lead to heterotopic bone formation.

Finally, one of the other factors released by mast cell degranulation is serotonin in lipid vesicles,[64] although its function is unknown. It is conceivable that the serotonin released from mast cells leads to

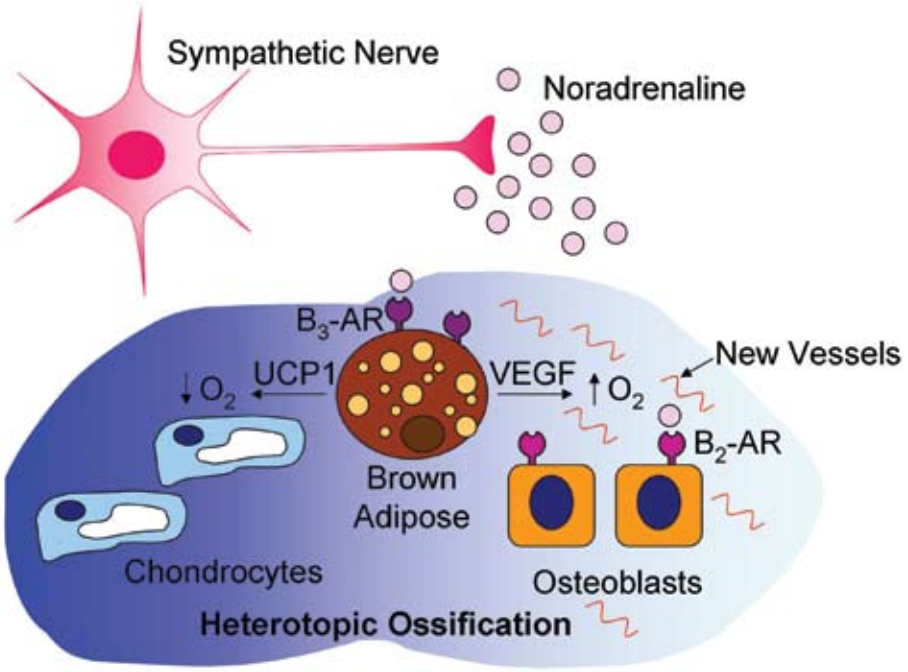

FIGURE 2. Schematic representation of the tentative interaction of the sympathetic nervous system and heterotopic ossification. Activation of the sympathetic nervous system, through sensory stimulation, leads to regulation of adipose, particularly the rapid appearance of brown adipose within the area of HO. The brown adipose appears to be critical to bone patterning and formation.

the stimulation of sympathetic neurons at the site of injury. Surprisingly, serotonin has been reported to have two opposing actions on bone remodeling. When released outside the hypothalamus, the hormone appears to inhibit bone formation, but when used as a neurotransmitter, it exerts positive effects on bone mass, by enhancing formation and limiting desorption.[65]

III.B. SNS Regulation of Osteoblasts

The SNS has also been linked to the regulation of orthotopic bone mass.[66] Inhibitors of sympathetic signaling, such as the β-blocker propranolol, have been shown to increase bone mass in wild-type mice, and reduce bone loss in ovariectomized mice and rats.[67] This sympathetic regulation of bone mass was further attributed to signaling mechanisms activated through β_2-adrenergic receptors expressed on osteoblasts. While in the normal skeleton this sympathetic signaling mechanism appears to inhibit the formation of bone, the potential effect on heterotopic bone is currently unknown. However, these studies provide further evidence for an additional cell type involved in bone formation and potentially under the control of sympathetic signaling (Fig. 2).

The SNS may be regulating osteoblasts directly, or regulating progenitors of osteoblasts. We[68] and others[69] have shown that the hematopoietic stem cell (HSC) is the precursor for the osteoblast. Intriguingly, the SNS has been implicated in the recruitment and mobilization of HSCs.[70,71] Sympathetic signaling has been demonstrated to regulate the release of stem cells from the bone marrow.[72] Activation of β_3-adrenergic receptors expressed on stromal cells within the bone marrow niche leads to the downregulation of Cxcl12, a chemokine critical for stem cell attraction within the marrow.[70] Consequently, decreased expression of Cxcl12 within the bone marrow microenvironment encourages stem cell mobilization from the marrow to the peripheral circulation. Upon mobilization,

these stem cells could then recruit to the area of new bone formation for further differentiation. This may suggest another pool of potential progenitor cells, in addition to the primitive stem cells within the local, peripheral nerves. It is also possible that the nerve-associated stem cells are progenitors to the HSC. Indeed, the large numbers of neural markers on HSCs has been noted before.[73] Additionally, it has been previously reported that such neural stem cells can rescue lethally irradiated animals.[74,75] Future studies aimed at further understanding and identification of these various progenitor sources, under the control of neuronal signals, will provide a new area for potential treatment and prevention of HO.

IV. CONCLUSIONS

As we have outlined, a number of recent studies are beginning to shed light on the role of the peripheral nerves in the production of HO. Sensory stimulation, by injury and BMP release, can evoke local, neuroinflammatory processes, which ultimately enable the recruitment of progenitors for chondro-osseous differentiation. Neuroinflammation within the local environment may lead to the activation of the sympathetic nervous system, through the release of mast cell serotonin. Intriguingly, stimulation of the SNS then continues to trigger the sensory nervous system, through generation and thermogenesis of the brown adipose. Sensory neurons also transmit information regarding the local environment to the CNS and hypothalamus, potentially regulating both heterotopic bone formation and skeletal remodeling. This relationship is unclear, but, often, in clinical scenarios that favor HO, it appears to be at the expense of the adjacent skeletal bone, suggesting the production of HO is perhaps a response to replace the skeletal bone.

While heterotopic ossification is considered an aberrant process, its origins may stem from the critical need to maintain an intact skeleton for survival. In fact, it may be the peripheral nervous system that plays a key role in the surveillance required to preserve normal, functional bone. On one hand, the PNS may relay information to the CNS, to regulate the everyday remodeling of the normal skeleton, critical for maintaining homeostasis within the organism. This information may arise from mechanosensors on osteocytes, which provide additional signaling between the PNS and skeletal bone.[76] However, when the body sustains a traumatic injury, and the normal environment becomes altered through trauma and BMP release, the sensory nerves may be the first in line to detect any damage to the bone itself. Once these nerves "sense" these alterations within the local environment, they may initiate a program of regeneration of the bone and soft tissues, over the normal, remodeling mechanisms. The sensory nerves signal to the CNS to override the remodeling program, to set in motion the mechanisms to rebuild *de novo* bone. In certain instances, this mechanism may generate new bone in incorrect places, and result in HO. Thus, knowledge of peripheral nerve regulation of HO may be translatable to other repair mechanisms and may provide invaluable insight into the body's ability to detect and regenerate those tissues most valuable for survival, including the bone.

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Cancer Metabolism and the Warburg Effect As Anabolic Process Outcomes of Oncogene Operation

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ABSTRACT: Having considered the results of early works by other authors, their assumption was developed using modern knowledge that made it possible to propose the concept of a Warburg effect mechanism in cancer metabolism. This concept accents the high prevalence of anabolic processes in cancer metabolism, taking into account oncogene operation. The explanation of the Warburg effect mechanism alone has been provided considering the mechanism of energy shift to an anabolic pathway of cancer development. On the basis of previous experiments, the concept has also detected the point of bifurcation of the pathways of anabolic and catabolic processes, which have made it possible to ascertain the consumption of acetyl-CoA for anabolic processes and the lack of acetyl-CoA for catabolic processes. Thus, the results of other authors' experiments were explained using the proposed concept. The mechanism of surviving cancer cells (apoptosis resistance) was also explained using this concept. In addition to using the Theorell formula and the proposed concept, an explanation has also been given from the physical chemistry point of view of the mechanism of the phenomenon regarding the absence of contact inhibition of proliferating cells in malignant tumors. Thus, this made it possible to explain other cancer phenomena mechanisms, including metastasis, nonhealing ulcers, and irrepressible growth mechanisms. In addition, this concept has made it possible to also explain experimental results that early and modern authors could not explain. Lastly, the distinction between mechanisms of the Pasteur effect and the Warburg effect was explained using the proposed concept.

KEY WORDS: pyruvate dehydrogenase, pyruvate kinase, Akt oncogene, HIF, reactive oxygen species, apoptosis resistance, Pasteur effect

1. INTRODUCTION

In 1924, Warburg found that aerobic glycolysis is shown in the characteristic metabolism of tumor tissue, unlike in healthy tissue.[1] This feature of tumor tissue metabolism has been termed the "Warburg effect." In studies of the Warburg effect, results of separate experiments did not make it possible to explain the effect's blanket mechanism. For example, research by Dickens, Greville, and Simer on the respiratory coefficient in cancer tissue states that the respiratory coefficient is higher in cancer tissue than aerobic oxidation of respiration in normal tissue.[1–4] By causing the attachment of excess intermediate products of tricarboxylic acid in the Krebs cycle, research by Craig et al.[5] and Elliott et al.[6] stated that the absence of increased oxygen consumption takes place in cancer tissue, as compared with normal tissue. Calculation of the Meyerhof index shows that oxygen consumption is approximately identical in malignant tumor tissue and normal tissue, despite a high level of glycolysis in malignant tumor tissue.[7] Busch, Potter, and Le Page created a high level of glycolysis in their experiments, which did not result in the increase of subsequent products of catabolic anaerobic exergonic processes of tricarboxylic acids in the Krebs cycle; thus, it was impossible to take up the increase of these products experimentally in cancer tissue as opposed to healthy tissue.[7–10] By blocking some links of tricarboxylic acid in the Krebs cycle, research by Potter and Busch showed the absence of the accumulation of the metabolism of previous products in cancer tissue, as compared with normal tissue metabolism.[9,10] Potter, Le Page, and Busch suggested an assumption to explain these experimental results.[5–8] The proposed concept develops this assumption from the point of view of modern knowledge.

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II. THE CONCEPT OF THE WARBURG EFFECT MECHANISM AS A RESULT OF DEVELOPING THE POTTER, LE PAGE, AND BUSCH ASSUMPTION

In explaining their research, Potter, Le Page, and Busch assumed that there was a "bottleneck" in the metabolism of cancer tissue at the introduction level of tricarboxylic acids in the Krebs cycle (acetyl-coenzyme A [acetyl-CoA]).[7–10] However, this assumption could not provide a complete explanation of the mechanism of the Warburg effect. In developing on the fundamental Potter, Le Page, and Busch[7–10] assumption from the point of view of the current knowledge, the concept of a Warburg effect mechanism has been proposed.

II.A. The Concept of the Warburg Effect Mechanism

As a result of the operation of oncogenes that cause enormous anabolic processes in cancer tissue and the enormous consumption of energy and acetyl-CoA for anabolic (biosynthetic) processes, an overload of the nodal point of bifurcation of anabolic and catabolic processes (NPBac) occurs because of the remaining lack of acetyl-CoA for catabolic oxidative processes. This shift in anabolic processes, and the lack of acetyl-CoA, causes suppression of the development of catabolic processes in cancer tissue. Increased lactic acid production is the necessary endergonic mechanism for the accumulation of energy for many anabolic processes in glycolytic metabolism and the high consumption of energy for anabolic processes in cancer tissue. An outcome of oncogene operation is that the anabolic processes cause high consumption of energy and acetyl-CoA and suppress the catabolic processes in cancer tissue. Lactic acids accumulate energy for anabolic processes in glycolytic metabolism in cancer tissue. This concept makes it possible to explain the Warburg effect mechanism and to distinguish between mechanisms of the Pasteur effect and the Warburg effect.

The proposed concept aids in explaining the results of experiments by early and modern authors, the mechanisms of which they could not previously explain. Two mechanisms, contact inhibition of propagating cells in normal tissue and the absence of contact inhibition of propagating cells in malignant tumors, were explained by using the Theorell formula and the proposed concept. Thus, this makes it possible to explain the mechanisms of irrepressible tumor growth, nonhealing cancer ulcers, and mechanisms of metastases formation. Accepting all of the modern explanations of the Warburg effect mechanism, the proposed concept further develops them. Thus, the tendered concept does not contradict the modern theories; rather, it complements them.

II.B. Other Situations That Prove the Significance of Lactic Acids in Anabolic Processes

The intensive work of transversal striated muscle fibers in skeletal muscle ($W_{str.m.}$) via contractility promotes their accumulation in lactic acids. The decrease of a muscle fatigue necessitates a rest phase for the elimination of excess lactic acids. In addition to the regular work of transversal striated muscle fibers in skeletal muscle ($W_{str.m}$), increased mass of these muscles (muscles hypertrophies) along with an increased quantity of muscle fibers result in anabolic processes. Thus, lactic acid participates in the mechanism of anabolic processes. Unlike transversal striated muscle fiber in skeletal muscle, the intensive and regular work of a cardiac smooth muscle ($W_{csm.m}$) does not result in the accumulation of lactic acids in cardiac myocytes of smooth muscle, nor does it result in the increased mass of these muscles (hypertrophy of cardiac muscle) or in anabolic processes. The intensive and regular work of a cardiac smooth muscle ($W_{csm.m}$) decreases only in aerobic conditions of the catabolic metabolism of pulmonary oxidative respiration in normal tissue. Therefore, the anabolic processes in cardiac smooth muscle ($W_{csm.m}$) do not happen or occur in minimal quantity for renewal. However, a circulatory deficiency causes anaerobic conditions that result in the accumulation of lactic acid, causing hypertrophies in cardiac muscle. All of these situations also confirm the participation of lactic

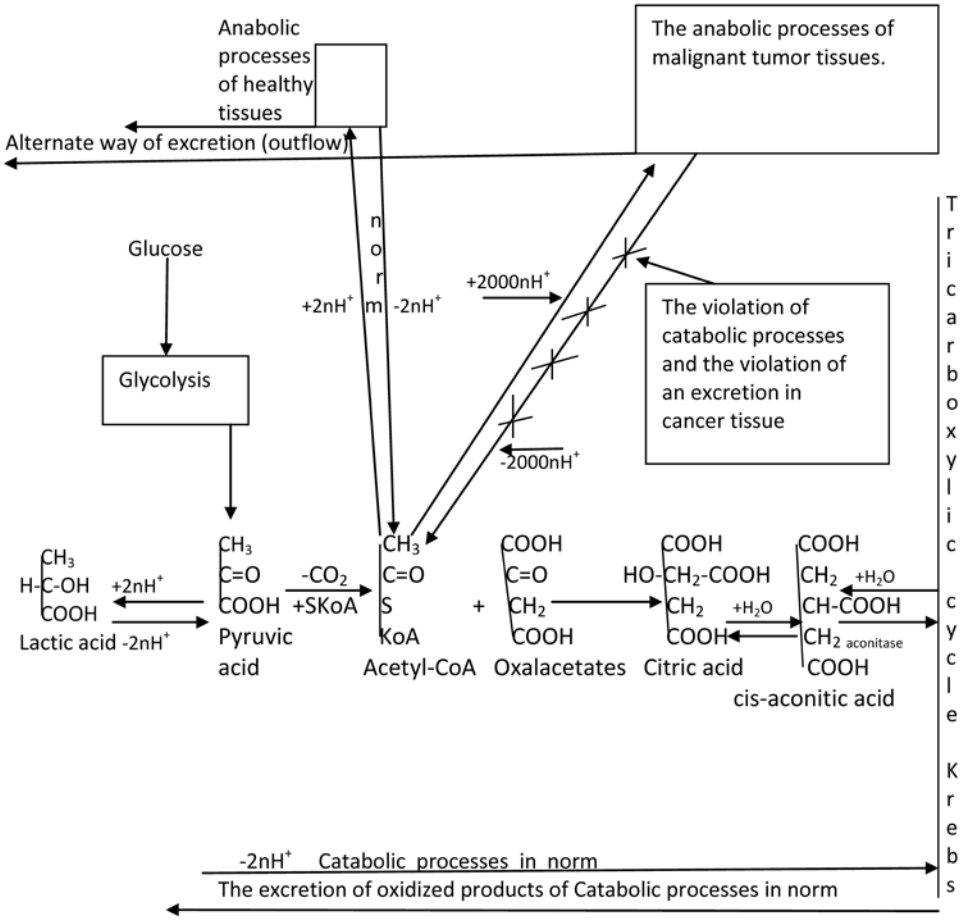

FIGURE 1. The nodal point of bifurcation in anabolic and catabolic processes. These comprise many anabolic processes with high consumption of energy and acetyl-CoA for anabolic processes in cancer tissue. The large alternative excretion of substances within the structure of rejected cells and the damage of excretion of substances via oxidative processes in cancer tissue unlike normal tissue are depicted. The accumulation of energy into lactic acid for anabolic processes is shown.

acids in the mechanism of energy accumulation for anabolic processes. This distinction of cardiac smooth muscle from the cell metabolism of transversal striated muscle fibers in skeletal muscle is characterized as follows: The pulmonary oxidative system takes over the majority of oxidative catabolic function of mitochondria action of cardiac smooth muscle in normal tissue, which prevents hypoxia and accumulation of lactic acids for anabolic processes via glycolysis. On the contrary, the transversal striated muscle fibers in skeletal muscle accumulate lactic acids because of the intensive work ($W_{str.m.}$) involved for hypertrophy of muscles via anabolic processes. These situations also prove the presence of bifurcation in the nodal point of metabolism, which divides such resisted pathways of metabolism into catabolic processes and anabolic processes (Fig. 1).

III. ONCOGENESIS FROM THE POINT OF VIEW OF THE PROPOSED CONCEPT

As the result of their operation, etiological factors (oncogenes, v-oncogenes, cancerogenes, etc.)[11–14] and anabolic (biosynthetic) processes in tumor tissue increase considerably (+2000 nH^+) as compared

with normal tissue (+2 nH^+) (Fig. 1). According to Berdinskih,[15] high biosynthetic activation of intracellular proteins on free polyribosomes unconnected with membranes in cancer tissue results in inhibition of natural gene replication processes, promoting activation of oncogenes and kinetic activity and contributing to the development of cellular cycles. In addition, Nghiem et al.[16] proposed that the action of Her2 kinase corresponded to studying the influence of genes on mutation processes. In addition, Thompson et al.[17] studied gene operations and the influence of genes on mutation processes of tumor suppressors.

However, all of these gene operations require delivery of substances across cellular membranes during the G1 phase of the cellular cycle. These substances include nucleic acids for transcription and reverse transcription RNA processes, as well as for t-RNA delivery of amino acids for translation processes and other substances for DNA replication and further cell division. Thus, it is impossible to have both DNA and RNA functions without delivering amino acids, nucleic acids, and other substances into cells for filling the G1 phase cellular cycle. This depends on the diffusion of these substances through the cellular membranes connected with the permeability of cell membranes, which is forced by the balance of the cellular electrochemical potentials (μ) from both extracellular and intracellular species in normal cells and in cancer cells. The data concerning the cellular electrochemical potentials (μ) are considered below in Section III.A.

According to Kim and Dang, the shift in metabolism in cancer tissue from catabolic processes to anabolic biosynthetic processes takes place.[18–20] In studying the mechanism of the Warburg effect, Garber noted that it is the third anabolic pathway of normal tissue metabolism supplementary to the two main catabolic pathways to generate energy in the form of adenosine triphosphate (ATP): oxidative phosphorylation in mitochondria and glycolysis in the cytoplasm.[21,22]

Although all of these metabolic pathways begin from the NPBac at the introduction level of tricarboxylic acids in the Krebs cycle in acetyl-CoA, the anabolic pathway is the resistant pathway to both catabolic pathways (Fig. 1). Preservation of the stability of these resistance processes as anabolic pathways of metabolism, and as two catabolic pathways of metabolism, supports the stability of cancer tissue, which promotes its survival (apoptosis resistance). Therefore, Elstrom et al. noted that Akt holds cells dependent to glucose consumption and glucose catabolism to maintain bioenergetics.[23] Indeed, glucose consumption and glucose catabolism maintain the stability of these resistance processes of metabolism as well as bioenergetics. As a result of the many anabolic processes in cancer tissue and the high consumption of acetyl-CoA for anabolic (biosynthetic) processes, an overload of NPBac occurs because of the remaining lack of acetyl-CoA for catabolic oxidative processes in cancer tissue. The lack of acetyl-CoA causes suppression of the development of catabolic processes. In healthy tissue, moderate processes maintain the balance between anabolic and catabolic processes. Some quantity of acetyl-CoA is formed because of pyruvate dehydrogenase (PDH) action in mitochondria of cancer cells, which maintains the oxidative catabolic function of respiration for cancer cell survival. Oxidative aerobic processes of respiration produce the greatest quantity of calories in comparison with anaerobic processes of glycolysis, maintaining temperatures from 36°C to 37.5°C (at which all enzymes in tissue operate). This is because glycolysis produces only two ATP molecules per glucose molecule, compared with 38 ATP molecules for complete oxidation via the Krebs cycle. Cancer cells alone would die without respiration (Fig. 1). Therefore, Gottlob et al. showed the influence of Akt/PKB on both glycolysis and mitochondrial hexokinase for the inhibition of apoptosis.[24] The conversion of pyruvate to acetyl-CoA by PDH in mitochondria maintains and saves respiration in cancer cells, despite the presence of many anabolic processes. The action of Her2 kinase and the other stimulators promoting aerobic processes contribute to cancer cell survival via the maintenance of oxidative catabolic respiration that also contributes to apoptosis resistance. The vast increase in lactic acid production is the necessary endergonic mechanism for the energy accumulation of an immeasurable number of anabolic processes in conditions of gly-

colysis in cancer tissue (Fig. 1). The changes in mitochondria of cancer cells must be considered not only as constant mitochondrial lesions that suppress oxidative phosphorylation, accelerate glycolysis, and suppress apoptosis,[25–27] but also as mitochondria mutations that result in maintaining the aerobic oxidative exergonic function of respiration for the survival and growth of malignant cells. According to Bonnet et al.,[25] the high potential of mitochondrial membranes ($\Delta\Psi m$) is explained by the proposed concept as the significant increase of anabolic endergonic processes in the malignant tumor versus normal tissue (e.g., Figure 1 shows that the normal tissue is 2 nH^+ and that the malignant tumor is 2000 nH^+). Changes in the potential of mitochondrial membranes and in Kv channels,[25,26,28–30] as well as the actions of cancer tissue ferments,[18–20,25,31] correspond to the state of cancer tissue metabolism in which anabolic endergonic reductive processes prevail. Inhibition of PDH with phosphorylation of PDH by kinase PDH (PDK) in cancer tissue[25] is explained by the proposed concept as the result of a shift into many anabolic processes. However, any quantity of acetyl-CoA is formed because of PDH action in mitochondria, which saves the oxidative catabolic function of respiration for cancer cell survival. In addition, much of acetyl-CoA is formed as a result of β-oxidation of fatty acids. It is known that the malignant tumor avidly consumes fats from fatty depots; thus, the patient becomes thinner, leading to cachexia. However, increased glucose uptake for glycolysis in cancer cells promotes increased acetyl-CoA with alterations in energy metabolism for excessive anabolic processes in cancer tissue. Indeed, energy accumulation in formed lactate is necessary to maintain excessive anabolic endergonic processes in conditions of intensive glycolysis in cancer tissue. In addition, a mitotic phase occurs after phase G1 in the cell cycle (i.e., cell fission that is a consequence of DNA replication processes). Cell fission (a mitotic phase) and a chemical phase of DNA replication are self-replicated processes that take place depending on participation of free radicals as the driving mechanisms of these processes.[32] Emanuel provided research regarding the presence of free radicals in the kinetics of oncologic processes.[32] However, free radicals are formed as a result of oxidizing reactions. Thus, the further development of a cellular cycle takes place as a result of oxygen radicals (O^*) use, which is generated by excessive levels of reactive oxygen species (ROS). The ROS promote superoxide radical oxygen (O^*) via hydrogen peroxide (H_2O_2). Thus, oxygen is necessary not only for cell survival but also for cell reproduction in the mitotic phase of a cellular cycle. However, the presence of oxygen (O_2) causes inhibition of glycolysis in normal tissue, which corresponds to the Pasteur effect.[33] Indeed, the identical exergonic catabolic pathways of oxidative aerobic processes and glycolytic anaerobic process suppress one another using NPBac and acetyl-CoA. Oxidative exergonic catabolic processes are equilibrated with anaerobic exergonic processes of glycolysis in cytoplasm and in extracellular matrix according to chemical potentials (μ), suppressing one another and forming the Pasteur effect in normal tissue. On the contrary, there are different metabolic pathways in cancer tissue that are resistant to one another. For example, high anabolic, endergonic, biosynthetic metabolism resists catabolic exergonic processes of aerobic oxidative metabolism and anaerobic glycolysis metabolism, violating the pathways of both of these catabolic processes. Indeed, the anabolic, endergonic, biosynthetic pathway of metabolism prevails over exergonic, oxidative catabolic processes considerably and inhibits development of catabolic metabolisms of both the aerobic oxidative pathway and the anaerobic glycolysis pathway in cancer tissue. Therefore, aerobic oxidative metabolism and anaerobic glycolysis metabolism develop independently from one another, fulfilling separate functions. These interactions of metabolic pathway resistance cause the Warburg effect in cancer tissue because anabolic processes force the separate development of aerobic oxidative catabolic processes and anaerobic catabolic processes of glycolysis. Thus, Lopez-Lazaro[33] explained the following: The formation of the ROS transforms energy of the free radicals oxygen (O^*) of a mitochondrion, as disoxic metabolism, which causes the development of the cellular cycle into mitotic phase, promoting DNA replication processes. In addition, the use of oxygen promotes cancer cell survival (apoptosis resistance)

because aerobic oxidative processes generate enough energy to maintain temperatures from 36.0°C to 37.5°C for the operation of cellular enzymes. Hypoxia-inducible factor 1 (HIF-1) resists the destructive action of excessive quantities of oxygen free radicals (O*) and hydrogen peroxide (H_2O_2), maintaining processes that transform glycolytic energy into ATP molecules (oxphos) to form and consume acetyl-CoA for many anabolic processes in cancer tissue. Thus, the mechanism of transformation from healthy cell metabolism into cancer cell metabolism explains the distinction between the Pasteur effect in healthy cell metabolism and the Warburg effect in cancer cell metabolism. The interactions of metabolic pathways that resist each other, with one that prevails, cause the Warburg effect in cancer tissue. On the contrary, the Pasteur effect in normal tissue becomes apparent considering the interactions among the identical catabolic oxidative exergonic processes: aerobic oxidative metabolism and anaerobic glycolytic metabolism.[33] Examinations of mitochondrial frataxin confirm the proposed concept of a Warburg effect mechanism. In addition, this concept develops and also explains mechanisms of interaction among frataxin and ROS, reducing the accumulation of excess ROS and promoting the balance of anabolic and catabolic processes as well as the inhibition of tumor growth.[33] After all, there are the two cancer tissue conditions: 1) The development of catabolic oxidative processes resistant to anabolic processes; the augmentation of catabolic oxidative processes under the influence of frataxin inhibits anabolic processes and as well as tumor growth. 2) On the contrary, increased anabolic processes promote tumor growth, contributing to a decrease in catabolic oxidative processes and lack of mitochondrial frataxin protein at metastasis[34] (Fig. 1).

III.A. Irrepressible Tumor Growth From the Physical Chemistry Point of View

By using the Theorell formula and the concept proposed in this article, it is possible to explain the mechanism of contact inhibition of proliferating cells of healthy tissue and the absence of this phenomenon in malignant tumor tissue. The Theorell formula is as follows:

$$dn/dt = - UcA\, d\mu/dx$$

where dn/dt is the quantity of diffusing substance molecules in the unit time; U is the substance mobility; c is the substance concentration; A is the membrane area; μ is the chemical potential; and x is the molecule distance from membrane.

Chemical potential (μ) is the driving mechanism for both active and passive transport of substances across cellular membranes. It is necessary to take into account that cells of the same layer of any tissue comprise approximately identical substance concentrations (c), having identical mobility (U), identical area of cellular membranes (A), and identical molecule distance from the cell membrane (x). In normal tissue, the absence of substance diffusion (dn/dt) through the cellular membrane of tissue due to circumferential cell contact with other cells is explained by the availability of the identical chemical potentials ($\mu_1 = \mu_2 = \mu_3$, etc.) in all of those cells, which influences decreased permeability of the cellular membranes and decreased substance diffusion (dn/dt) through the cellular membrane. Therefore, the G1 phase of the cellular cycle is not filled with substances, and a contact cellular inhibition of propagating cells occurs in normal tissue. The part of the cellular membrane that is free from the cellular contact separates the cellular chemical potential from another environment chemical potential ($\mu_{cell} \neq \mu_{environment}$). Therefore, contact inhibition of cell propagation is absent here due to the increased permeability of cellular membranes and increased substance diffusion (dn/dt) through the cellular membrane filling G1 phase of the cellular cycle. This occurs in wound healing and in the growth of epidermal epithelium, nails, and hair. Cells with similar chemical potential cannot also be formed in cancer tissue because of the overload of NPBac and high consumption of acetyl-CoA for anabolic processes. Therefore, cancer cells develop in various levels of the cellular cycle as the result of different filling intensities of the G1 phase of the cellular cycle. Cancer cells thus contact other cancer cells having another chemical potential ($\mu_1 \neq \mu_2 \neq \mu_3$, etc.). This contributes to damage of cellular membrane permeability and causes the diffusion (dn/

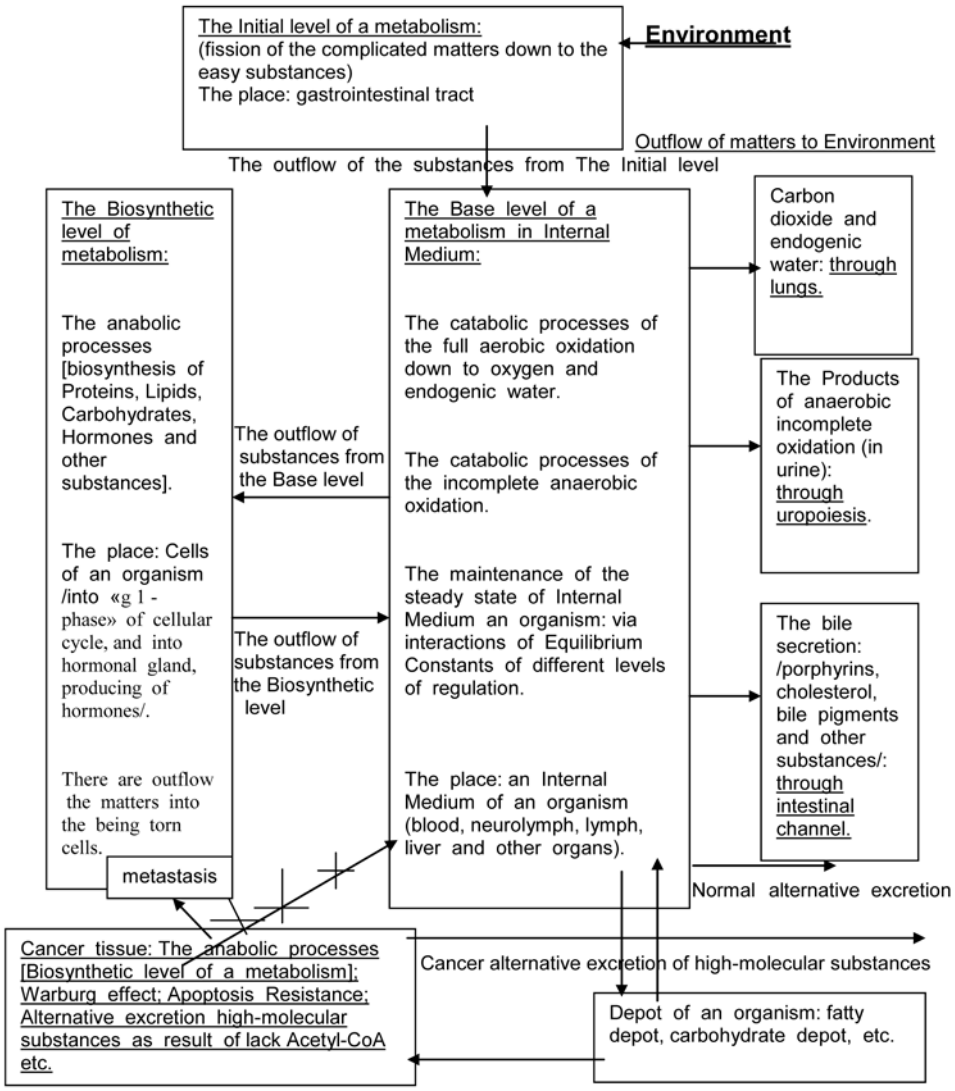

FIGURE 2. Metabolism in an organism and dysmetabolism in cancer tissue. Damage of an excretion of substances via oxidative processes in cancer tissue unlike in normal tissue is shown. Metastasis formation is also depicted.

dt) of substances into cells permanently by additionally overfilling the G1 phase of the cellular cycle. Thus, the absence of contact cellular inhibition of propagating cells arises in cancer tissue. It promotes the intensive propagation of cells and unrestrained growth of malignant tumors. According to Altenberg and Greulich[11] as well as D'Agostino et al.,[12] the cellular cycle of cancer tissue depends on the life cycle of the propagating viruses with anabolic capacity, which creates the condition that cancer cells cannot be formed with similar chemical potential. Therefore, cancer cells also have different chemical potentials ($\mu_1 \neq \mu_2 \neq \mu_3$, etc.).

III.B. Formation of Metastasis and Nonhealing Cancer Ulcers

A cancer tumor is located inside the organism; in this example, the human organism is used as the

environment.[35–37] Therefore, cancer uses substances from the organism depot (fat depot, carbohydrate depot, etc.) for cancer tissue metabolism, and the patient thus grows thin (Fig. 2). Anabolic processes (in Fig. 1, +2000 nH^+ and the long arrow upward) cause the blockage of excretion (outflow) of synthesized high-molecular substances from cancer tissue by the oxidative metabolic pathway (in Fig. 1, –2000 nH^+ and crossed long arrow directed downward) because of the overload of NPBac and the lack of acetyl-CoA for oxidative processes in cancer tissues. Therefore, the alternative pathway of high-molecular substance excretion takes place within separate cells. The nonhealing cancer ulcer is thus formed because of the rejection of these cells from the malignant tumor into the environment. However, viable cells can be rejected from the malignant tumor tissue into the internal medium of the organism (blood or lymph) and, upon being diffused by lymph or blood current, reach healthy locations of the extracellular matrix, forming metastases (Fig. 2). Initially, there is no overload of NPBac and lack of acetyl-CoA for oxidative processes in the new location. In the extracellular matrix, there remains a temporary capability of the normal pathway of substance excretion via oxidative processes. Thus, metastasis of the malignant tumor is not formed here temporarily. However, many high-molecular substances are being formed again in these places. The situation of NPBac overload is repeated and new metastases are formed. Thus, there is the condition in which the cancer tumor is prepared to form metastasis: The biosynthetic anabolic endergonic processes prevail in this condition (+2000 nH^+), which promotes the blockade of the excretion of substances via oxidative processes (outflow), derived as the result of biosynthetic anabolic endergonic processes, and there is increase in the tumor cell mitochondrion potential ($\Delta\Psi m$). The other condition, when the cancer situation is not yet prepared to form metastases, is characterized by the fact that there is still no blockade of the excretion of substances via oxidative processes (outflow), derived as the result of biosynthetic anabolic endergonic processes: Oxidative catabolic processes of excretory substances (–2000 nH^+) counterbalance biosynthetic anabolic processes (+2000 nH^+), synthesized in the malignant tumor, and there is no increase in the tumor cell mitochondrion potential ($\Delta\Psi m$). Therefore, the overload of NPBac and lack of acetyl-CoA, which is the carrier of K^+ ions in the Kv channel, contribute to the low expression of K^+ in the Kv channel of malignant tumors.[25,28–30] This also happens together with two conditions: (1) cancer cells produce a lack of the endogenous frataxin protein, and (2) an excess of frataxin increases oxidative metabolism, whereas ROS accumulation remains unaffected by frataxin.[34] This observation will be compounded by representation of the dual conditions of a tumor tissue in which the state promoting tumor growth and metastasis formation is either formed or not formed. Thus, mitochondrial frataxin takes part in mechanisms to restore the balance of ROS operation in a cell such that it does not decrease excessive oxidative action (i.e., frataxin promotes functions of ROS, restraining redundant oxidative processes).[34]

IV. EXPLANATIONS FROM THE POINT OF VIEW OF THE PROPOSED CONCEPT AND PREVIOUSLY UNEXPLAINED RESULTS

Elstrom et al. reported that they were surprised that the proliferation of malignant cells did not increase in cultures with the activated serine/threonine Akt kinase, although there was stimulation of glucose consumption in the transformed cells without affecting the rate of oxidative phosphorylation.[23] Bellacosa et al. also found it interesting that tumor cells rarely display increased size in comparison to their normal counterparts, in spite of the mTOR/cIF4E pathway that is often activated in human tumors.[38] However, additional increases in acetyl-CoA are not formed in these mechanisms; therefore, the advancement of the strengthening of anabolic endergonic processes and the increase in proliferation is failed.

In describing the interrelationship between anti-apoptotic factors (Bcl-2, Bcl-X_L, Mcl-1, etc.) and pro-apoptotic factors (Bax, Bak, etc.) in permeability processes of the mitochondrial membrane, Gogvadze et al.[39] did not investigate causes promoting prevalence of one factor over other factors in different conditions. Therefore, the authors could

not explain the interaction mechanisms of these facts and expressed plenty of doubts. However, these doubts can be explained by using the Theorell formula and the proposed concept: The mechanism of permeability of a mitochondrial membrane depends on interactions of chemical potentials (μ) inside a mitochondrion and in cellular cytoplasm. The participation in these mechanisms of pro-apoptotic and anti-apoptotic factors, as well as other factors, depends on states of mitochondria in hypoxia (under HIF-factor) with the prevalence of glycolysis or in oxidative phosphorylation. These conditions occur as a result of tumor development in an organism, such as tumor growth, metastases, and so forth. Therefore, primary mechanisms of these conditions are: (1) the interaction of a cell with an extracellular matrix, and (2) the permeability of a cellular membrane, depending on chemical potentials (μ). Unlike the work by Gogvadze et al.,[39] our explanation of the mechanism of tumor growth is based on the studied mechanisms of the cellular cycle in the different conditions that are necessary for cell reproduction and for tissue growth.

Hsu and Sabatini noted that the increase in lactic acid production cannot be explained, originally described by Warburg, and suggested that lactate must result from the metabolism of nonglucose substrates.[40] However, the proposed concept explains that increased lactic acid production is the necessary endergonic mechanism for energy accumulation in anabolic processes in hypoxia promoted by factor HIF-1 and the lack of energy because of glycolytic metabolism (Fig. 1).

Christofk et al. concluded that tumor cells preferentially use glucose for purposes other than oxidative phosphorylation, and that this metabolic switch may be required to support cell growth.[41] They expressed the following doubt: "How tumour cells establish metabolic phenotype Warburg effect and whether it is essential for tumorigenesis as is yet unknown."[41(p. 230)] However, it is possible to suppose that the switch establishing the metabolic phenotype Warburg effect of PDK in tumor cells connects with the metabolic shift to anabolic processes in cancer tissue. This is because the PDK in cancer tissue contributes to energy accumulation in lactic acid. The many anabolic processes in cancer tissue are needed for considerable increases in PDK activity for huge quantity of lactate formation, unlike normal tissue.

According to the Pasteur effect, the presence of O_2 (aerobic processes) is known to cause glycolytic inhibition. However, the main phenomenon of cancer is the activation of glycolysis in aerobic processes, according to the Warburg effect. Therefore, Lopez-Lazaro has inquired about why and how this phenomenon occurs.[33] The proposed concept indicates that there are two resistance pathways of metabolism: catabolic oxidative exergonic processes (aerobic oxidative metabolism and anaerobic glycolytic metabolism) and anabolic reductive endergonic biosynthetic processes. The anabolic endergonic biosynthetic pathway of metabolism prevails over exergonic oxidative processes considerably, and inhibits both aerobic oxidative metabolism and anaerobic glycolytic metabolism in cancer tissue. Therefore, aerobic oxidative metabolism and anaerobic glycolytic metabolism develop independently from one another and do not inhibit one another from fulfilling separate functions. Thus, these resistance interactions to metabolic pathways cause the Warburg effect in cancer tissue, separating the independent processes of aerobic oxidation and glycolysis. On the contrary, the Pasteur effect in normal tissue becomes apparent considering the interactions between the identical catabolic oxidative exergonic processes: aerobic oxidative metabolism and anaerobic glycolytic metabolism. Therefore, these identical pathways of metabolism suppress one another, which corresponds to the mechanism of the Pasteur effect[33] because of the use the same nodal point of bifurcation of anabolic and catabolic processes.

IV.A. Explanation the Mechanisms of the Other Authors' Experiments Using the Proposed Concept

Other authors who investigated Warburg effect could not explain the results of some of their experiments since the significance of acetyl-CoA in anabolic processes was unknown at that time. Now the significance of acetyl-CoA in anabolic processes of

metabolism has been proven as the biosynthesis of porphyrins, cholesterol, fatty acids, lipids, proteins (via glycine), nucleic acids (via glycine → thymin synthetase system → thymine), and other substances. Therefore, results of earlier research can be explained using the proposed concept.[3–6,8–10] Warburg found that aerobic glycolysis is characteristic for the metabolism of tumor tissue, unlike healthy tissue. This feature of the metabolism of tumor tissue has been termed the "Warburg effect." In studying the Warburg effect, results from many separate experiments did not make it possible to explain the blanket mechanism of the effect. Potter, Le Page, and Busch made an important attempt to explain the Warburg effect mechanism. Because Potter and Le Page did not observe increases of subsequent products of tricarboxylic acids in the Krebs cycle in tumor tissue in spite of a large increase of glycolytic products, unlike healthy tissue, it was impossible to stimulate the formation increase of these products.[8] Also, the blockade of some links of tricarboxylic acids in the Krebs cycle did not result in the accumulation of citric acid and hydroxy-butanedioic acid accordingly in cancer tissue as compared with normal tissue.[7,9,10] Potter, Le Page, and Busch suggested an assumption to explain the results of their experiments.[8–10] With particular focus on acetyl-CoA, they proposed the concept of a bottleneck (non-blockade) in the point of cancer metabolism at the acetyl-CoA level.[8–10] Thus, in explaining their research, Potter, Le Page, and Busch suspected that there was a bottleneck in the metabolism of cancer tissue at the introduction level of tricarboxylic acids in the Krebs cycle (acetyl-CoA).[7–10] This assumption could not fully explain the mechanism of the Warburg effect. Developing this assumption from the point of view of the current knowledge, we have explained the Warburg effect mechanism using the proposed concept (see above). As such, the mechanisms of the other authors' experiments were explained from the point of view of modern knowledge (Fig. 1).

The respiratory coefficient is lower in cancer tissue than with glycolysis in normal tissue, due to the absence of increased CO_2 with aerobic oxidation. However, the respiratory coefficient is higher in cancer tissue than in normal tissue with aerobic oxidation of respiration.[3,4] This occurs because the necessary oxygen consumption for energy generation with aerobic oxidation of respiration remains the same for cell survival in cancer tissue (apoptosis resistance) as well as in normal tissue. However, the absence of increased CO_2 in cancer tissue is explained by the transfer in cancer tissue metabolism at the NPBac–acetyl-CoA level from oxidative exergonic catabolic processes into reductive endergonic anabolic processes.[2–10]

Compared with normal tissue, the absence of increased oxygen consumption occurs in cancer tissue with the attachment of excess intermediate products of tricarboxylic acid in the Krebs cycle, including lactate, pyruvate, succinate, fumarate, malate, and so forth.[5,6] Unsuccessful attempts to observe the increase of oxidative processes and products of these processes, as well as the absence in the increase of oxygen consumption, are explained by the transfer in cancer tissue metabolism at the NPBac–acetyl-CoA level from oxidative exergonic catabolic processes into reductive endergonic anabolic processes.[2–10] These results could not be explained by earlier authors[3–6] because at that time it was not known that acetyl-CoA participates in anabolic biosynthetic processes.

Similar results have been shown with calculation of the Meyerhof index for both malignant tumor tissue and for normal tissue[7] (i.e., oxygen consumption is approximately identical in malignant tumor tissue and normal tissue despite the high level of glycolysis in malignant tumor tissue). Earlier authors were unable to explain these results at that time.[7] Increased oxygen consumption does not occur in cancer tissue as compared with normal tissue, in spite of high glycolysis in cancer tissue. This is because plenty of anaerobic catabolic processes (glycolysis) of metabolism are replaced in NPBac into anabolic processes in cancer tissue, but are necessary for energy accumulation in aerobic catabolic processes. Thus, oxygen consumption for cancer cell survival remains the same in cancer tissue as in healthy tissue. The necessary energy generation with aerobic oxidation of respiration into mitochondria for survival cells are identical both in malignant tumor tissue and in normal tissue. Therefore, catabolic aerobic processes do not change themselves, and the Meyerhof

index remains the same in both cancer tissue and in healthy tissue.[7]

An absence in the increase of catabolic products of metabolism in tricarboxylic acids in the Krebs cycle occurs in spite of the high level of glycolysis, and it is impossible to observe the increase of these products experimentally.[7–10] Accordingly, there is an absence in an increase of citric acid and hydroxy-butanedioic acid that is caused by a blockage of some links of tricarboxylic acid in the Krebs cycle, as compared with normal tissues.[9,10]

Indeed, it is impossible to explain the action suppressors considering the results of experiments Potter and Busch, who studied blocking the tricarboxylic acids in the Krebs cycle (TCA cycle), and their experiments have resulted in an absence in increased quantities of products that preceded the blockage in cancer tissue, unlike normal tissue.[9,10]

Accordingly, the absent increase in citric acid and hydroxy-butanedioic acid by the blockage of some links of tricarboxylic acid in the Krebs cycle, as compared with normal tissue,[9,10] is also explained by the shift in metabolism of cancer tissue at the NPBac–acetyl-CoA level from oxidative exergonic catabolic processes into reductive endergonic anabolic processes. Indeed, it would be impossible to explain where the surplus of substances and energy goes (which arises at the blockage of the TCA cycle) that has not been spent in exergonic catabolic oxidizing processes of the TCA cycle. Increased concentrations of lactic acid in cancer tissue do not correspond to the quantity of the surplus of substances, which arise at the blockage of the TCA cycle. The surplus of substances occurs because of the shift in metabolism of cancer tissue at the NPBac–acetyl-CoA level from oxidative exergonic catabolic processes into reductive endergonic anabolic processes, causing an increased quantity of synthesized substances.

V. EXPLANATION OF THE MECHANISMS OF MODERN RESEARCH FROM THE POINT OF VIEW OF THE PROPOSED CONCEPT

As a result of great increases in anabolic endergonic processes, the derived molecular changes in cancer cells influence activation of Akt, Bcl-2, and PKB.[13,14,24,26,27] The increased permeability of cellular membranes of cancer cells according to the Theorell formula results from their structure changes, which determine mutation of tyrosine-kinase receptors of growth factors such as epidermal growth factors, platelet-derived growth factors, and receptor protein tyrosine kinases.[36,42] Also, the high biosynthetic activation of nonsecreting intracellular proteins on free polyribosomes unconnected with membranes[15] is the result of gene replication processes, as well as activation of natural oncogenes and kinetic activity, and contributes further to the development of cellular cycles. It is also necessary to consider that the chemical cell potential (μ) of cancer cells depends on the disturbed interaction between anabolic and catabolic processes in cancer tissue metabolism. Indeed, cancer metabolism takes part in correspondence with the mechanism of the phenomenon known as absence of contact inhibition of proliferating cells in malignant tumors. Therefore, metabolic converged pathways in the mitochondria are not independent from each other, and it seems that glycolytic phenotype is also associated with a state of apoptosis resistance.[25,27]

Akt encodes a protein serine-threonine kinase, and takes part in the metabolism of amino acids participating in anabolic processes of synthesis oncoproteins. In addition, the Akt oncogene, which encodes a protein serine-threonine kinase, is associated with enhanced glucose uptake and aerobic glycolysis even independent of HIF-1.[22] Akt, which stimulates glycolysis and induces resistance to apoptosis, activates hexokinase 2 (HK-2), an enzyme catalyzing the first and irreversible step in glycolysis.[23] Mitochondria interact with activated HK-2 in cancer, resulting in suppression of cell death while supporting cell growth via enhanced glycolysis that maintains cell life, even in the presence of oxygen (Warburg effect).[43] Kim and Dang studied the mechanism of PDH and PDK interaction and concluded that PDK1 is identified as a direct HIF-1 target gene in hypoxic cells.[19] PDK1 phosphorylates and inactivates the mitochondrial PDH complex. The suppression of PDH by PDK1 inhibits the conversion of pyruvate to acetyl-CoA, thereby attenuating mitochondrial respiratory function. Because nonhypoxic stabiliza-

tion of HIF through oncogenic events has been observed, Kim and Dang hypothesized that PDK1 levels may be upregulated by HIF in nonhypoxic tumor cells, which would divert pyruvate from PDH and result in increased lactate production.[19] Indeed, the anabolic metabolism shift to lactic acid is the glycolytic stage of energy accumulation for anabolic processes, which contributes to suppression of PDH by PDK1 for inhibition of the conversion of pyruvate to acetyl-CoA. This provides the endergonic conversion of pyruvate to lactic acids and also attenuates mitochondrial aerobic function (Fig. 1). Therefore, many glycolytic enzymes have been recognized to also regulate apoptosis, and several oncoproteins induce the expression of glycolytic enzymes.[18,19]

Explanations of the results of experiments using the phenomena of cell death and survival of cells via the mutation in cancer tissue[41] possibly correspond to the mechanism of apoptosis resistance. Akt, via its downstream mediator glycogen synthesis kinase 3 (GSK3), induces the translocation of hexokinase to the mitochondrial membrane, where it binds to the voltage-dependent anion channel (VDAC), suppressing apoptosis.[19,31] Inhibition of GSK3 in cancer cells causes unbinding of hexokinase from the VDAC channel, which induces apoptosis.[31] This suggests that perhaps the metabolic phenotype in cancer is due to a potentially plastic mitochondrial remodeling that results in suppressed oxidative phosphorylation, enhanced glycolysis, and suppressed apoptosis.[25] All of these data correspond to the overload of NPBac and lack of acetyl-CoA, which is the carrier of some anions that also influence on the VDAC via the translocation of HK-2 to the mitochondrial membrane, and may also be connected to the action of Akt. In addition, some suppressors of tumor metabolism, including Akt, participate in anabolic processes in which acetyl-CoA is also consumed for amino acid synthesis. It is on this basis that we can suppose a possible mechanism of suppression of catabolic processes by some suppressors via the consumption of acetyl-CoA for anabolic processes, because suppression does not block oxidizing glycolytic processes. Therefore, in studying the mechanism of action of suppressors of glycolysis, Kim and Dang did not note blocking by suppressors of the TCA cycle.[18,19] Considering the shift to anabolic processes in cancer tissue, the results of research by Ferguson and Rathmell[44] suggest that the shift toward the anabolic reductive reactions promotes tumor growth. Such a shift also promotes changes in chemical and electric potential of anion mitochondria channels (VDACs)[23,43,44] in tumor tissue. The synthesis of FITO-ATP uses the stored $\Delta\Psi m$ energy of the Kv channel of the malignant tumors to synthesize substances by anabolic biosynthetic systems. Therefore, PDH is inhibited by phosphorylation in the malignant tumor as the result of actions of reductive anabolic processes.[12] According to Thompson's model, tumors create energy by starting with upstream gene mutations that activate Akt and ending with cancer cells continuously consuming glucose, both aerobically and anaerobically.[22] Indeed, cancer requires consumed glucose for energy generation via catabolic oxidative processes, and the generated energy is used for anabolic processes in cancer growth or in lactic acid production for the energy accumulation needed in anabolic processes. In an investigation of interactions between glycolytic and respiration processes, Garber raised the question of how cancer cells benefit from the Warburg effect.[22] The author suggested that cancer cells could benefit from glycolysis in many pathways.[22] Thus, Gottlieb and Thompson contend that a boost in glycolysis in addition to respiration, which continues unabated, generates energy more quickly than in normal cells that overwhelmingly rely on respiration.[21] Werma's work suggests that glycolysis leads directly to anaerobic hypoxic conditions via HIF-1 activation, which further boosts metabolism.[22] Developing these suggestions, Dang supposes that shutting down respiration functions protects cancer cells from mitochondria damage that occurs when cellular respiration functions are abnormal under hypoxic conditions.[20,22] However, the proposed concept explains that the cancer cells benefit from the Warburg effect. Thus, upstream gene mutation under the v-oncogene action creates the malignant transformation that leads to cancer metabolism and the Warburg effect. Cancer metabolism is characterized by many anabolic biosynthetic processes with the consumption of a lot of energy and acetyl-CoA. In addition, cancer metabolism

requires the anaerobic hypoxic condition via HIF-1 activation for glycolysis, in which an acetyl-CoA is constituted. The suppression of cancer metabolism because of acetyl-CoA deficiency is not a blockade for further respiration processes because respiration produces far more energy than glycolysis. Therefore, some acetyl-CoA is consumed for respiration, despite the huge consumption of acetyl-CoA for anabolic processes (Fig. 1).[37] Only the blockage of excretion (outflow) via oxidative processes the high-molecular molecule, which are formed as the result of huge biosynthesis, occurs in cancer tissue because of a lack of acetyl-CoA (Fig. 1). The benefit from the shutting down of respiration processes in cancer cells is that this protects cancer cells from mitochondria damage when cellular respiration functions are abnormal under hypoxic conditions. Mitochondria can operate only under normal respiratory function. Damage of the mitochondria leads to damage of respiratory function and to cell death because of a lack of energy generation for exergonic processes, which produce plenty of calories for the maintenance of the normal temperatures (36°C–37.5°C) at which all of the fermentation functions in cancer tissue take place. The metabolism in a mitochondrion (on the first pathway of development) promotes acetyl-CoA formation because the phosphorylation of PDH by PDK is suppressed, which contributes to a metabolism shift from mitochondria to cytoplasm to increase development of metabolism on the second pathway of glycolysis with plentiful formation of additional quantities of acetyl-CoA.[21,22] However, the mechanism of the Warburg effect also promotes energy accumulation via lactic acid concentration for the many anabolic processes in cancer tissue. It causes the benefits of the Warburg effect in the condition of cancer metabolism. One of the consequences of NPBac overload in cancer tissue is that it also leads to disintegration of cadherin-catenin complexes of cancer tissue membranes connected with "α-catenin" loss and disturbance of integrin receptors on those membranes. It reduces adhesive and associative membrane properties in extracellular matrix of cancer tissue.[16,35,36] The separated cancer cells can be combined with cadherins and integrins to the matrix of the healthy tissues, which have no NPBac overload in extracellular matrix. Thus, the formation of metastasis corresponds to the described mechanism, which is based on the proposed concept (Figs. 1 and 2). Other discoveries also do not contradict the proposed mechanism of the Warburg effect; rather, they supplement each other. These discoveries include: oncogene detection (mos, src, ras, TP53, BUB1, MYC, CAC25B, MCM4, Brca2, Brca2, Blm, v-raf, v-ras, v-scr, BcL-x, etc.); oncogene suppressors (p-53, Rb, etc.)[11,16,35,36,41]; cyclin-dependent kinases ensuring mutagenesis; detection of the mechanism of dysfunction of tyrosine-kinase of transmembranous receptors reacting with oncogenes, change of growth factor, and so forth; integrins, cadherins, and selectins; mutagenesis polyribosomes; and Akt, Bcl-xL, PKB, RI3K.[15,16,36,42]

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Functional Roles of Osteoactivin in Normal and Disease Processes

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ABSTRACT: Osteoactivin (OA) protein was discovered in bone cells a decade ago. Recent literature suggests that osteoactivin is crucial for the differentiation and functioning of different cell types, including bone-forming osteoblasts and bone-resorbing osteoclast cells. Here, we review the literature to date on various regulatory functions of osteoactivin, as well as its discovery, structure, expression, and function in different tissues and cells. The transcriptional regulation of osteoactivin and its mechanism of action in normal and diseased conditions with special emphasis on bone are also covered in this review. In addition, we touch on the therapeutic potential of osteoactivin in cancer and bone diseases.

KEY WORDS: osteoactivin, glycoprotein nmb, DC-HIL, HGFIN, PMEL17, type I transmembrane glycoprotein, adhesion, osteoblasts, osteoclasts, cancer

I. DISCOVERY OF OSTEOACTIVIN AND RELATED FAMILY MEMBERS

The initial identification of osteoactivin (OA) emerged from studies using an animal model of osteopetrosis.[1] Other groups have also identified the same protein in different species and have designated different names, such as glycoprotein nonmelanosoma protein B (gpnmb) in melanoma cell lines[2–6] and melanocytes,[7,8] dendritic cell heparan sulfate proteoglycan integrin dependent ligand (DC-HIL) in dendritic and T cells,[9–11] and human hematopoietic growth factor inducible neurokinin (HGFIN) in tumor cells.[12] To avoid confusion, here we use the term OA and review the literature on its structure, its role in physiology, and its pathophysiology. We will also briefly discuss the mechanism of action of OA in cellular functions and the potential use of OA as therapeutic target for certain disease conditions.

II. HOMOLOGY AND STRUCTURE/ FUNCTION OF OSTEOACTIVIN

OA was first isolated and cloned from the long bones of a rat model with osteopetrosis.[1] The protein coding region of the OA cDNA is composed of 1716 base pairs and it codes for a protein of 572 amino acid residues. Sequence comparison of OA revealed 77% homology to gpnmb,[5] HGFIN protein,[12] and mouse DC-HIL.[10] In addition, OA is homologous to quail neuroretina protein-71 (QNR-71) (65% homology),[13] and 60% homology to human Pmel-17/gp100 melanocyte-specific protein.[14] Subsequently, mouse gpnmb protein[15] was identified to be homologous to human and mouse OA sequences. A comparison of rat and mouse OA protein sequences revealed 88% identity.[1] The OA gene was mapped on different chromosomes in different species: chromosome 4 for rat, chromosome 6 for mouse, and chromosome 7 for human OA,[16] respectively.

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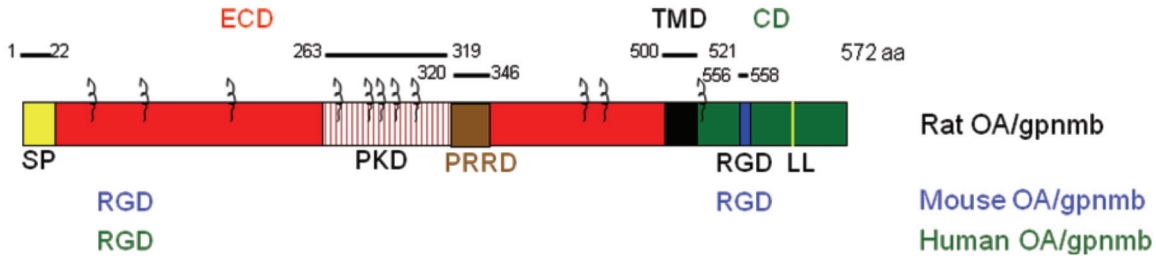

FIGURE 1. Primary structure of osteoactivin (OA) protein and its homology to other family members. (A) Schematic diagram of the primary structure of OA drawn to scale. The protein consists of three main parts: the extracellular domain (ECD), the transmembrane domain (TMD), and the cytoplasmic domain (CD). Numbers correspond to amino acid position. SP, signal peptide; PKD, polycystic kidney disease domain; PRRD, proline rich repeat domain; TMD, transmembrane domain; LL, dileucine sorting sequence; RGD, integrin binding domain. Note the presence of an RGD domain in the C-terminus of rat OA, whereas mouse OA has two RGD domains, one in the N-terminus and the other in the C-terminus, and human OA has only one RGD domain in the N-terminus.

Due to its high homology to Gpnmb and Pmel17, OA was suggested to belong to the Pmel17 gene family.[16] Based on the predicted protein sequence, OA is classified as a type I transmembrane protein consisting of three main domains: an N-terminal extracellular domain (ECD) or luminal domain (amino acids 23–500), a middle short transmembrane domain rich in hydrophobic residues (amino acids 501–521), and a C-terminal cytoplasmic domain (amino acids 522–572) (Fig. 1). The first 22 amino acids at the N-terminal domain constitute a signal peptide that aids the entry of OA into its secretory pathway. The N-terminal extracellular domain can be further divided into three subdomains, including an Arg-Gly-Asp (RGD) domain, a polycystic kidney disease–like domain (PKD), and a proline-rich repeat domain (PRRD). Each of these domains has specific functions. Interestingly, the RGD domain is present in mouse and human OA in its N-terminal domain, but is absent in the N-terminal domain of rat OA. Interestingly, the RGD domain is suggested to function as an attachment site for integrins and contributes to integrin-mediated cell attachment and spreading.[1,10,17] The PKD domain has an immunoglobulin-like folding structure that plays a role in protein-protein and protein-carbohydrate interactions.[18] The PRRD domain function is not clear in OA but has been linked to O-linked glycans of Pmel-17.[19] The transmembrane domain has an alpha helical structure and is suggested to play a role in anchoring OA protein to the cell membrane. OA also contains a di-leucine amino acid sorting signal sequence in close proximity to the C-terminal domain, with a potential role in sorting the protein through the rough endoplasmic reticulum (RER).[20–23] The roles of the different domains of OA have not yet been determined experimentally. However, one study showed that the PKD and, to a lesser extent, the PRRD domains negatively regulate T-cell proliferation.[9] The physiologic role of different domains of Pmel-17 has been well characterized in melanocytes.[19,20,24–30]

III. PROCESSING AND LOCALIZATION OF OSTEOACTIVIN

OA mRNA and protein are localized in different tissues and cells, including Kupffer cells in the liver[31–33]; myocytes in the muscle[34]; lymphatic tissues, where it is expressed by antigen-presenting cells (APCs)[10,11,35]; melanocytes[36]; bone marrow macrophages[37,38]; dendritic cells[10,39]; endothelial cells[10]; and bone, where it is expressed in osteoblasts,[1,17,40–44] osteoclasts,[45] and osteocytes.[44] OA expression is also reported in primary cells and immortalized cell lines, including NIH-3T3 fibroblast cell lines,[33] C2C12 myoblast cell lines,[34] RAW 264.7 macrophage cell lines,[38] retinal epithelial pigment cells,[15] melanocyte cell lines,[8,36] melanocytes in human skin,[46] primary bone marrow

macrophages,[38] primary osteoblasts,[1,17,40,41,43] osteoclast-like cell lines,[45] and chrondrocytes.[44,47] Altered OA expression has also been reported in pathological conditions such as osteoarthritis,[47] breast cancer,[48–52] melanoma,[2,36,46,53,54] and glioblastoma.[55]

Processing of OA protein has been examined in different cell types. In melanocytes, OA is reported to be localized in the RER.[46] Another study on dendritic cells demonstrated subcellular localization of OA and identified that OA was predominantly present in intracellular compartments and lower levels of OA protein were detected in the plasma membrane. Similar results were reported in COS-1 cells that were transfected with OA.[10] Immunocytochemistry staining of OA in dendritic cell line suggests intracellular localization of DC-HIL (OA). Most of the DC-HIL (OA) staining was observed in large vesicles located in the perinuclear region, as well as in small vesicles located towards the periphery.[10] Taken together, these results demonstrate that DC-HIL (OA) mostly localizes in the cytoplasm, and is also present at lower levels on the cell surface.[10]

In macrophages, the perinuclear localization of OA suggests its presence in the Golgi apparatus. This localization was studied using several approaches. OA has been reported to co-localize with beta-COP, a protein that associates with membranes of the Golgi complex and is important for trafficking.[56] In addition, staining of OA was visualized in the RER and endosomal compartments following treatment with brefeldin A (BFA), a drug that disrupts the Golgi network.[17] Since the Golgi apparatus is the site of protein sorting within the cell, it is most likely that OA accumulates in the Golgi and is transported to the plasma membrane or is secreted upon specific stimuli. Ripoll et al. reported that OA is translocated into small vesicles located toward the cell periphery following activation with interferon-gamma and lipopolysaccharide (LPS).[56] These studies suggest that the association of OA with macrophage secretory pathways could provide an explanation of its effects on cytokine production/secretion.[45] Subcellular localization studies on OA showed that OA was specifically localized in stage III and VI melanosomes. This observation was confirmed by studying subcellular fractions techniques of pigmented (MNT-1) and non-pigmented (WM266-4) melanocyte cell lines. In these cells, OA is localized in stage III and VI melanosomes, suggesting that OA may play a role in late melanogenesis.[36]

Data from independent studies on osteoblasts and osteoclasts suggest that the localization of OA into different cellular compartments and its functions are dependent on processing of OA.[17,45] We have extensively studied the localization and processing of OA in osteoblasts. Using Western blot analysis, we showed that OA protein has two isoforms: one is transmembranous and the other is secreted into the conditioned medium of primary osteoblast cultures. Fractionation of osteoblast cell compartments showed that the mature, glycosylated OA isoform of 115 kDa is found in the membranous fraction. Both OA isoforms (secreted and transmembranous) are found in the cytoplasmic fraction of osteoblasts. These observations were further confirmed using immunofluorescent co-localization of OA with markers for the RER and for the plasma membrane. We examined the processing of OA by overexpressing EGFP-tagged OA in osteoblasts.[17] During the first 24 h of expression, OA was found to be localized in vesicular and endosomal-like structures. After 40 h of transfection, OA was found either anchored to the plasma membrane or was secreted into the culture medium. In addition to these studies, we also transfected HA-tagged OA into MC-3T3-E1 osteoblasts and observed that HA-tagged OA was localized with GM130, a marker for the Golgi apparatus (Fig. 2). Further studies on OA trafficking and processing may lead to new information on the roles of OA in mediating trafficking of other molecules within osteoblasts.

Evidence that supports OA protein shedding by ectodomains in vitro and in vivo is described below. In muscle myocytes, Furochi et al. showed that shedding of OA in skeletal muscles of mice required unloading stress, such as denervation, whereas overexpression of OA in C2C12 cells (myoblast cell line) was sufficient for ectodomain shedding of OA.[34] ADAM12 is one the sheddases that causes shedding of OA in muscle cells.[34] A study in melanocytes demonstrated that PMA and CaMI mediated by disintegrins and metalloproteinases, specifically

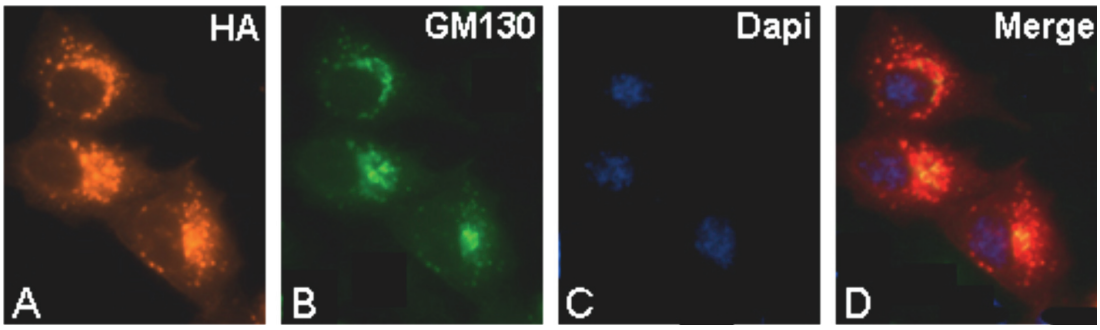

FIGURE 2. Localization of exogenously transfected osteoactivin (OA) protein in osteoblasts. MC3T3-E1 osteoblasts were transfected with HA-OA for 24 h. Cells were then labeled with anti-HA antibody (**A**), anti-GM-130, Golgi marker (**B**), followed by secondary antibodies conjugated with Cy3 (orange signal in **A**) and FITC (green signal in **B**). Cells were also labeled with Dapi for nucleus (blue signal in **C**). (**D**) Merged image (red signal) of A, B, and C. Note that exogenously expressed OA labeled with anti-HA is co-localized with Golgi marker, suggesting that tagged OA protein can be expressed, trafficked, and processed in osteoblasts.

ADAM10, induce ectodomain shedding of OA.[34] However, the mechanism responsible for the shedding of OA in these cells has not been clearly elucidated. A recent study on breast cancer cells identified that ADAM10 sheddase resulted in ectodomain shedding of Gpnmb (OA) ectodomain from the surface of breast cancer cells. This study hypothesized that the ectodomain shedding of Gpnmb (OA) may be responsible for endothelial cell migration, which promotes angiogenesis in breast cancer and other oncogenic cells.[48]

III.A. Processing and Modification of Osteoactivin Protein

Sequence analysis of OA showed that the protein is heavily O- and N-linked glycosylated.[17,58] Processing of OA protein has been documented in a variety of cell types, including muscle and bone cells. A study reported that OA protein exists as two different isoforms of 116 and 97 kDa. Treatment with endoglycosidases H and F, enzymes responsible for preventing sugar modifications, reduced the intact forms to bands with a molecular weight (MW) of 67 kDa, corresponding to the MW of the nonglycosylated OA protein.[17] Thus, due to high mannose-type glycosylation, the intact forms of OA have apparent MWs of 97 and 115 kDa.[17] Two secreted isoforms of OA have also been reported in muscle with MWs of 90 and 100 kDa.[34] These secreted forms of OA accumulated in the conditioned media of OA-transfected C2C12 myoblasts are reported.[33]

In the XS52 dendritic cell line, Northern blot analysis showed that DC-HIL (OA) mRNA (2.9 kb) was expressed at high levels; however, this expression was less in J774 and RAW macrophages cell lines, while it was undetectable in other cell lines examined.[10] A study on COS-1 cells transfected with DC-HIL (OA) identified two isoforms of OA of 90 and 110 kDa using anti-DC-HIL antibody raised against the N-terminus domain of OA. Another study reported that OA exists in two isoforms of 95 and 125 kDa in XS52 DC lysates using the N-terminus OA antibody described above. Bioinformatics studies on DCHIL suggested that it is heavily glycosylated and has 11 putative N-glycosylation sites. The estimated MW of the DC-HIL (OA) protein was much larger than that predicted from the full length amino acid sequence (67 kDa), which could be due to N or O-glycosylations on DC-HIL (OA). In order to test for N-glycosylation in DC-HIL (OA) protein, XS52DC cells were treated with N-glycosidase. N-glycosidases reduced the MW of the intact isoform of DC-HIL (OA) to two bands of 76 and 66 kDa. The discrepancy in the MW of non-glycosylated DC-HIL (OA) may be due to the differential N- and/or O-glycosylation, which may also explain the variation in MW between native

(non-glycosylated) DC-HIL (OA) in XS52 DC and its recombinant form in COS-1 cells.[10]

It is the late Golgi-modified glycosylated isoform (115 kDa) of OA that undergoes ectodomain shedding to release secreted OA (100 kDa) into the medium, leaving a 20-kDa C-terminal fragment at the plasma membrane.[34,36] Pmel17, a homolog of gpnmb (OA), undergoes a similar pattern of processing.[59] Multiple cells types—including human keratinocytes,[60] macrophages,[56] and HEK293, COS-7,[15] and NIH-3T3 cells[34]—show differential cellular localization of OA during its processing, which might explain, at least in part, the biological functions of OA in different physiological systems.

Interestingly, OA is also reported to have distinct subcellular localization in macrophages.[45] During early (1–3 days) osteoclast differentiation induced by RANKL (receptor activator of nuclear factor kappa-B ligand), OA was localized in the perinuclear region. Further treatment with RANKL for 5–7 days induced the trafficking of OA protein to late endosomes and lysosomes. This endocytic pathway could target OA into the plasma membrane. The plasma membrane-bound OA is suggested to be secreted to induce osteoclast differentiation.[45]

In addition to native protein (65 kDa), OA also presents as a secreted glycoprotein (115 kDa) that undergoes posttranslational modifications, including both O- and N-linked glycosylation, that are crucial for its regulatory functions in osteoblasts[17] and other cell types including osteoclasts. Recent studies in osteoclasts have identified three different glycosylated isoforms (80 kDa, 100 kDa, and 139 kDa) of OA, in addition to the native isoform (65 kDa).[45] The differences in MWs of OA isoforms in osteoclasts could be due to different glycosylation patterns (O- versus N-), as previously documented in osteoblasts.[17] Abdelmagid et al. reported that glycosylation of OA is required for osteoblast differentiation and function. These observations were demonstrated by treatment of osteoblast cultures with anti-OA antibody that neutralized the secreted effects of glycosylated isoforms of OA.[17]

Abdelmagid et al. also used fractionated osteoblasts to distinguish the presence of native and glycosylated proteins in different cellular compartments.[17] Glycosylated OA was present both in cytoplasmic and membranous fractions, whereas the native-unprocessed form of OA was found only in the cytoplasmic fraction. These observations suggested that glycosylated OA functions either as a membrane-bound protein (it is secreted being part of the matrix) or a secreted protein that plays a role in osteoblast differentiation.[17] The differences in the processing of truncated OA as compared with full-length OA were performed using DBA2J mice.[17] DBA2J mice have a naturally occurring point mutation in the *Osteoactivin* gene that results in the expression of truncated OA protein with only N-terminal 150 amino acid residues.[8,61] Immunofluorescent analysis revealed a perinuclear localization of truncated OA. In contrast, full-length OA was found in punctate vesicle-like structures localized toward the peripheral cytoplasmic compartments.[17] C-terminus–deficient OA in osteoblasts obtained from DBA2J mice was found to be retained in ER/Golgi, and hence resulting in defective processing, glycosylation, trafficking, and secretion of OA in comparison with their normal littermate DBA2 mice osteoblasts. There is also a possibility of increased trafficking of truncated OA into the lysosomal and proteosomal degradation pathways in DBA2J osteoblasts, resulting in altered differentiation and function in osteoblasts. Similar reports of altered trafficking were observed in the silver Pmel17 mouse with truncated Pmel17 protein,[62] a homolog of OA. Another study to characterize function of truncated OA used bone marrow-derived stromal cells that are osteoblast progenitor cells from DBA2J mice, and the results showed that truncated OA had reduced osteoblast differentiation in comparison with their normal littermates. These results implicated that glycosylated full-length OA is crucial for osteoblast development and function.[17]

IV. OSTEOACTIVIN FUNCTIONS IN TISSUE PHYSIOLOGY AND PATHOLOGY

The cellular functions of OA have only recently emerged, and have shown that OA has the ability to regulate cell proliferation, adhesion, differentia-

tion, and synthesis of extracellular matrix proteins in various cell types in both normal and pathological processes.[1–4,7–10,16,31–33,41,43,55,57,63–66] Below is a description on the functional role of OA in tissue physiology and pathology (summarized in Table 1).

IV.A. Osteoactivin in the Liver

Using in situ hybridization, OA expression was detected in sinusoid-lining cells. Whereas the total liver contained only traces of OA mRNA, isolated Kupffer cells expressed abundant amounts of OA, which further increased with time in culture.[57] In an acute liver injury model in the rat, induced by carbon tetrachloride (CCl4), OA expression was greatly increased and reached a maximum of expression 48 h after injection.[57] Using immunohistochemistry techniques, OA protein expression was localized in pericentral inflammatory cells and CD68-positive sinusoid-lining cells. In the human liver, OA expression was increased in fulminant hepatitis and paracetamol intoxication. This enhanced expression of OA in the acutely inflamed liver protected the liver from hepatic fibrosis.[32] Taken together, OA is expressed at high levels in normal and inflammatory liver macrophages, suggesting a significant role for OA in acute liver injury.

Subtractive hybridization analysis showed that OA is highly expressed in liver samples of rats with hepatocellular carcinoma in comparison with the normal liver. The same study also linked high expression of OA with metastatic potential of rat hepatoma cells both in vitro and in vivo.[31,55] In summary, during acute stages of injury inflammation of the liver, OA expression is enhanced in normal and inflammatory liver macrophages and protects the liver from hepatic fibrosis. However, as the inflammation progresses to chronic conditions, enhanced OA promotes invasion of injured hepatic cells to cause hepatocellular carcinoma. Further studies are warranted to dissect the functional role of OA in liver physiology and pathology.

IV.B. Osteoactivin in Muscle

A microarray analysis of rat gastrocnemius muscle undergoing atrophy following denervation showed an eight-fold upregulation of OA expression.[33] This led to studies that examined the role of OA in muscle regeneration. Using immunofluorescent staining, OA expression has been reported in muscle fibers and especially in the sarcolemma of myofibers.[33] The potential role of OA in muscle regeneration has been studied in mice in a model of muscle denervation, which resembles the loss of nerve supply caused by diseases such as polio or by chemical or physical injury to the nerve.[67] Skeletal muscle denervation causes muscle atrophy, and studies have shown an increase in OA expression associated with increased expression of matrix metalloproteinase (MMP)-3, MMP-9, and vimentin following denervation.[67] The expression of MMP-3 and MMP-9 was mainly present in fibroblast-like cells infiltrated into denervated muscle. However, OA was expressed in the sarcolemma of myofibers adjacent to these fibroblast-like cells. The role of OA in myocytes may involve the activation of infiltrated fibroblasts. Further studies to confirm the functional role of OA in muscle were performed using transgenic mice ubiquitously overexpressing OA under the control of a cytomegalovirus (CMV) promoter. Muscle denervation in these mice further enhanced the expression of MMP-3 and MMP-9 in fibroblasts infiltrated into gastrocnemius muscle when compared with the muscles of wild-type mice. Additional studies have shown that overexpression of OA in the NIH3T3 fibroblast cell line, but not in the C2C12 myoblast cell line, induced expression of MMP-3, suggesting that OA might functionally target fibroblasts.[67] These studies suggest that OA may function as an activator for fibroblasts infiltrated into denervated skeletal muscles and may play an important role in regulating degeneration/regeneration of extracellular matrix.

To address whether this OA-mediated increase in MMPs in skeletal muscle affects the regeneration of denervated skeletal muscle, OA-transgenic mice were subjected to long-term denervation for 70 or 90 days. Long-term denervation caused severe degeneration of myofibers and fibrosis in the skeletal

TABLE 1. Summary of Reported Functions of Osteoactivin (OA) and Related Family Members

	System	Organ/Cell	Reference
GPNMB Biologic Event/Association	Low-metastatic melanoma cell lines		Weterman et al. (1995)[5]
	Gene linked to developing retinal pigment and epithelium and iris	Mouse osteoblasts	Bächner et al. (2002)[15]
	Expressed in endothelium during human malignancy	Human ovarian carcinoma cells	Ghilardi et al. (2008)[73]
	Genome-wide expression in osteoarthritis, new candidate genes	Human cartilage	Karlsson et al. (2010)[47]
	Essential for tissue repair	Mouse kidney and macrophages	Li et al. (2009)[72]
OA Biologic Event/Association	Cloning and characterization	Mouse osteoblasts	Safadi et al. (2001)[1]
	Genes encoding human and mouse OA		Owen et al. (2003)[16]
	OA in normal and disease human and rat liver		Haralanova-Ilieva et al. (2005)[57]
	Downstream effector of BMP-2	Mouse osteoblasts	Abdelmagid et al. (2007)[41]
	Osteoblast differentiation and function	Mouse	Abdelmagid et al. (2008)[17]
	Inducer of angiogenesis	Mouse and human breast cancer cells	Rose et al. (2010)[48]
HGFIN Biologic Event/Association	Pigmentary glaucoma in DBA/2J mice		Anderson et al. (2001)[79]
	Interacts with substance P	Human bone marrow fibroblasts	Bandari et al. (2003)[12]
	Cell cycle regulation	Human	Metz et al. (2005)[42]
	Upregulation in monocytes/macrophages during end-stage renal disease	Mouse and human	Pahl et al. (2010)[37]
DC-HIL Biologic Event/Association	Cloning	Dendritic cells, Langerhans cells	Shikano et al. (2001)[10]
	Inhibits human allogenic T cell responses		Chung et al. (2009)[35]
	Promotes growth of melanoma in mice		Tomihari et al. (2009)[46] Tomihari et al. (2010)[69]
	Treatment for activated T-cell–driven disease		Akiyoshi et al. (2010)[68]

muscle of wild-type mice.[33] However, overexpression of OA protected skeletal muscle from such changes. Infiltration of fibroblast-like cells and collagen deposition in the muscles were sustained at lower levels after long-term denervation in the skeletal muscle of these OA-transgenic mice as compared to wild-type mice. This cytoprotective effect of OA was supported by the expression of regeneration/degeneration-associated genes in the gastrocnemius muscle during denervation. Denervation significantly upregulated the expression of antifibrotic genes, such as glypican-1 and decorin-1, in the gastrocnemius

muscle of OA transgenic mice, compared with wild-type mice. In contrast, overexpression of OA caused a significant reduction in the denervation-induced expression of elongation factor 1A-1, an indicator for the persistence of degenerated cells. These results suggest that the OA-mediated increase in MMPs in skeletal muscle might be useful for protecting injured muscle from fibrosis, leading to full regeneration after denervation.

IV.C. Osteoactivin and Inflammatory Cells

T-cell activation is regulated by a number of different mediators expressed by APCs. DC-HIL (OA) is highly expressed in these APCs.[10] Shikano et al. also generated a soluble recombinant DC-HIL (OA) and observed that DC-HIL (OA) bound to activated, but not resting, T cells.[11] T-cell activation (via and anti-CD-3 antibody) was attenuated in the presence of DC-HIL (OA).[11] DC-HIL (OA) also inhibited reactivation of T cells previously activated by APC stimulation.[11] Taken together, these findings show that DC-HIL (OA) is a negative regulator of T-lymphocyte activation. Further studies showed that DC-HIL (OA) suppresses T-cell activation via binding to syndecan-4 ligand (SD-4), a type 1 transmembrane heparin sulfate proteoglycan expressed on T cells.[11] DC-HIL (OA)/SD-4 binding phosphorylates SD-4's intracellular tyrosine and serine residues.[35,68] DC-HIL (OA) also has the ability to inhibit tumor repressive T-cell activation and promotes growth of melanoma in mice.[69] It has been proposed that the exploitation of the DC-HIL (OA)/SD-4 pathway may lead to treatment of human T-cell diseases including melanoma.[69]

Macrophages have the ability to differentiate into osteoclast cells upon stimulation with RANKL via the nuclear factor kappa-B (NF-κB) pathway. OA expression is upregulated in murine RAW 264.7-derived osteoclast-like cells upon NF-κB stimulation.[45] Using a macrophage cell line, RAW 264.7 cells, OA gene expression was measured via microarray analysis[45] and OA was found to be highly expressed in mature osteoclasts. In addition, this expression was further upregulated upon RANKL-induced differentiation.[45] These results were confirmed using real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis.[45] The authors further reported that OA was present in vivo as demonstrated by immunocytochemical staining of OA in mouse femoral sections. In addition, in vitro inhibition of OA protein using anti-OA antibodies reduces cell size, number of nuclei, fusion, and bone resorption activity of osteoclasts, suggesting that OA plays an important functional role in osteoclastogenesis.[45]

OA expression is also reported in liver macrophages.[57] Harlanova-Ilieva et al. examined OA expression in liver macrophages in normal and acutely injured rat livers.[57] In a carbon tetrachloride-induced model of acute liver injury, analyses such as RT-PCR, Northern blot analysis, in situ hybridization, and immunohistochemistry show that OA expression was highest in rats with acute liver injury.[57] These results suggest that OA plays a role in acute liver injury.[57]

HGFIN (OA) expression has recently been studied in monocytes and monocyte-derived macrophages in hemodialysis patients and healthy subjects.[37] Blood samples were taken from both patients and control subjects, and monocytes were isolated via density gradient centrifugation and by using real-time RT-PCR and Western blot analyses. The investigators concluded that HGFIN (OA) expression was markedly increased in monocyte-derived macrophages isolated from patients within the hemodialysis group.[37] These findings suggest that HGFIN (OA) may play a role in different renal pathologies.

IV.D. Osteoactivin in Bone Cells

Recently, OA has emerged as a vital glycoprotein for the differentiation and function of both types of bone cells: osteoblasts and osteoclasts. The high level of OA expression in bone was initially described in a model of osteopetrosis in the rat and its expression was shown to increase during osteoblast differentiation and function.[1] In primary rat osteoblast cultures, OA expression increases during their development, with maximal expression during the final stages of

differentiation, that is, the matrix mineralization stage (days 17–21). To identify the specific role of OA during osteoblast development, osteoblast cell cultures at different stages of development were treated with either anti-OA functional blocking antibody or OA antisense oligonucleotides to neutralize secreted OA protein or to downregulate OA expression, respectively. Both anti-OA antibody and OA antisense treatment blocked osteoblast differentiation and function associated with inhibition of matrix maturation and calcium deposition. This effect was independent of cell proliferation and viability.[16,17] Subsequent studies examined the effects of overexpression of OA using a CMV promoter to drive OA cDNA expression in osteoblasts. These experiments showed that overexpression of OA induces osteoblast differentiation and function with no effect on cell viability or proliferation, confirming an important role for OA in osteoblast differentiation and function.[17]

Targeted studies have been performed to further elucidate the roles of the functional domains of OA during osteoblast differentiation. A study to delineate whether the N- or C-terminus of the OA protein is involved in osteoblast differentiation was performed using functional blocking antibodies directed against either the N- or C-terminus region of the protein. The study by Selim et al. revealed a crucial role for the C-terminus of OA in osteoblast differentiation.[40] These results are in agreement with the observation of a significant decrease in differentiation of osteoblast progenitor cells obtained from DBA2J mice that lacked the C-terminus domain of OA.[17] Interestingly, the C-terminus of OA contains the integrin-binding RGD motif. Generally, integrin receptors interact via the RGD motif on the adhesion ligand[70]; however RGD-independent interactions also exist.[71] To characterize the role of the RGD motif, Selim et al. used short synthetic peptides with a C-terminal OA sequence wherein conserved aspartic acid (D) in a RGD motif was mutated to glutamic acid (E), RGE. OA-derived peptides were then used to examine whether these peptides stimulate osteoblast differentiation in an RGD-dependent manner.[40] Both peptides showed similar results on osteoblast differentiation and function, suggesting that the effect of OA-derived peptides is RGD independent. The information gained from the above-mentioned study on OA-derived peptides is important; however, these findings cannot be completely correlated with the role of the RGD motif in the full-length OA protein. Further studies using mutations in the RGD motif and/or their flanking sequences in full-length OA will help to elucidate the role of the RGD motif of OA in bone cell differentiation and function.

OA expression was also investigated during active bone regeneration using a model of fracture repair in the rat. Our laboratory recently reported the temporal and spatial expression patterns of OA mRNA and protein in a femur fracture model in the rat.[44] Both OA mRNA and protein expression in intact long bones and growth plates were observed; in addition, OA mRNA and protein levels were also evaluated in fracture calluses collected at several time points up to 21 days postfracture (PF). OA mRNA and OA protein were found to be highly expressed in osteoblasts localized in the metaphysis of the intact long bone (tibia), and in hypertrophic chondrocytes localized in the growth plate, as determined by in situ hybridization and immunohistochemistry, respectively. Using a rat femur fracture model, Northern blot analysis showed that the expression of OA mRNA was higher in day-3 and day-10 PF calluses when compared with intact rat femurs. Using the in situ hybridization technique, we examined OA mRNA expression during fracture healing and found that OA expression was temporally regulated. Positive mRNA signals were seen as early as day 3 PF and reached a maximal intensity of expression at day 5 PF, followed by decreasing levels of OA mRNA at day 21 PF. The highest level of OA mRNA levels (day 5 PF) correlates with the peak of chondrogenesis during fracture repair. We also observed a higher level of OA mRNA expression in the soft callus compared to unfractured intact femurs. Similarly, we detected high levels of OA protein by immunohistochemistry throughout the reparative phase of the hard callus compared to unfractured intact femurs. Interestingly, the secreted OA protein was also detected within the newly made cartilage matrix and unmineralized osteoid tissue. Taken together, these data suggest the possibility that OA plays an important role in

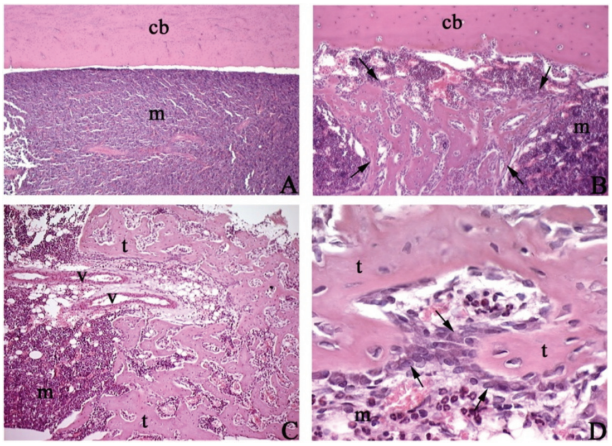

FIGURE 3. Effect of recombinant osteoactivin (rOA) protein on bone formation in vivo. Photomicrographs of hematoxylin and eosin–stained sections of the diaphysis from control (**A**) and rOA (**B–D**) injected femurs. (**A**) Low-power photomicrograph showing normal cortical bone (cb) and marrow (m) in a saline/BSA-injected control femur. There was no evidence of de novo bone formation anywhere in the marrow cavity. (**B**) Low-power photomicrograph of similar region shown in **A**, but in rOA-injected femur showing a large island of newly formed woven bone (outlined by arrows). (**C** and **D**) Higher-power photomicrographs of bone formed in response to rOA showing bony trabeculae (t) lined with active osteoblasts. In some areas, especially at the periphery, there are rows of osteoblasts and/or osteoprogenitors piled two or more layers deep (arrows). Note that the marrow (m) immediately adjacent to newly formed bone has an abundance of vascular channels (v). Magnifications: A and B = ×60, C = ×130, and D = ×200.

bone formation and serves as a positive regulator of fracture healing.[44]

To examine the effect of OA on bone regeneration, we tested whether recombinant OA (rOA) protein could elicit an osteogenic response in vivo. We chose to use a local delivery system in which rOA was administered via a single injection into the marrow cavity of the femur. This model has been used to test the anabolic response of other known osteoinductive stimuli, including BMP-2 and PGE_2. Adult male rats (12–16 wks of age) were used in this study. Briefly, a small area on the dorsal surface of the femur (just distal to midshaft) was exposed surgically through a small skin incision. A small hole was made through the cortical bone using a 27-gauge bit on a dental drill, and a Hamilton syringe was used to inject a small volume (20 mL) of saline containing rOA (1 mg) into the marrow cavity. The hole was immediately plugged using Vetbond Tissue Adhesive (a veterinary form of superglue; 3M, St. Paul, MN), the incision was sutured, and the animals recovered quickly and uneventfully. Control rats were injected with the same volume of saline or 1% bovine serum albumin (BSA) in saline. After 1 wk, the animals were euthanized and femurs were removed for radiographic and histological analyses. In every femur (total of 6 rats) injected with rOA, the radiographs showed areas of increased radiodensity within the marrow cavity compared with saline- or BSA-injected controls (data not shown). Histological evaluation revealed that rOA-injected femurs had islands of newly formed woven bone within the marrow cavity (compare Fig. 3B with A). The newly formed bony trabeculae were lined with rows of active, cuboidal osteoblasts (Fig. 3C and D). There were many blood vessels in the marrow immediately adjacent to these islands of bone, and multiple rows of osteoblasts and/or osteoprogenitors were often observed near the periphery. There was no evidence of an osteogenic response in any of the control-injected rats (Fig. 3A). These results clearly show that the rOA is an osteoinductive agent in vivo.

V. OSTEOACTIVIN AND DISEASE PROCESSES

V.A. Osteoactivin and Inflammation

The glycosylated isoform of OA, also referred to as DC-HIL, is highly expressed in APCs including epidermal Langheran cells (immature dendritic cells), leukocytes including CD14+ monocytes (precursor macrophages),[35] and myelomonocytic cell lines. Its expression is also highly upregulated during macrophage differentiation[56] and in patients with asymptomatic stages of HIV infection.[72] Binding of OA expressed on APCs to syndecan-4 (heparin sulfate bearing glycoprotein)[11] on activated T cells inhibited T-cell activation.[35] OA has been reported to inhibit the anti-CD3–induced T-cell response,

decrease secretion of proinflammatory cytokines, and block their entry into the S-phase of the cell cycle.[35] Collectively, these studies suggest that OA functions as a negative regulator for allogenic/T-cell interactions.[9] Overexpression of OA in the macrophage-like RAW264.7 cell line resulted in decreased production of the inflammatory cytokines IL-6 and IL-12 and the inflammatory mediator nitric oxide in response to lipopolysaccharide.[56] However, LPS treatment of the DBA2J mice that had truncated OA caused enhanced production of proinflammatory cytokines, suggesting that OA acts as a negative regulator of macrophage inflammatory responses.

V.B. Osteoactivin in Cancer

A study comparing the expression of OA in normal and tumor-derived endothelia showed a high expression and localization of OA in the blood vessels of tumor-derived endothelia, whereas very low OA expression was observed in normal endothelia.[73] Another study of African American populations indicated that hypermethylation of the OA gene promoter was associated with a high incidence and aggressiveness of colorectal cancer.[74] There is substantial evidence to correlate the level of OA expression with the metastatic potential of tumors, including breast[50,55] and pancreatic cancers.[55] A similar pattern of OA expression was also observed in in vitro models of malignant tumors such as in glioma, hepatoma, and breast cancer cell lines, respectively. These studies implicated a high OA expression as being responsible for their enhanced migration and invasive character.[5]

MMP-3 enzymes degrade extracellular matrix and are strongly associated with epithelial mesenchymal transition, enhanced cell migration, and invasion leading to tumor metastases.[75,76] A study of 4TI-mouse mammary carcinoma cells showed that downregulation of OA decreased MMP-3 expression, whereas overexpression of OA significantly enhanced MMP-3 expression, suggesting a role for OA in tumor aggressiveness. Interestingly, forced overexpression of OA in weakly metastatic breast cancer cells enhanced osteolytic bone metastases in vivo.[50] A correlation of high levels of OA expression in breast cancer with estrogen receptor negative status and increasing tumor grade has also been reported. There also exist contradictory reports suggesting that OA acts as a tumor suppressor in breast cancer cell lines. Furthermore, these studies also show that the tumor suppressor protein p53, along with other cytokine-mediated transcription factors, interacts with the OA promoter and regulate its expression.[51,77] Thus, the role of OA in tumor progression is still not completely understood. An antibody directed to the extracellular domain of human OA and conjugated to the cytotoxic agent MMAE (CR11-vcMMAE) has been developed.[78] In vitro assays and xenograft models have shown that protease cleavage releases the cytotoxin MMAE specifically within tumor cells that express high levels of OA. These studies have led to the use of the CR11-vcMMAE antibody in phase I/II clinical trials for the treatment of patients with stage III and stage IV metastatic melanoma.[3,4] Studies on the role of OA in tumor progression and invasion are quite promising; however, due to recently published contradictory reports on the effects of OA in breast cancer cell lines, the mechanism of action of OA in cancer progression still needs to be further explored.

VI. ANIMAL MODELS OF OSTEOACTIVIN

With many promising research avenues to pursue OA functions, a few animal models have been generated to date that demonstrate its novel function in various tissues. Transgenic mice that overexpress OA under the CMV promoter showed increased muscle mass and enhanced expression of MMP-3 and MMP-9 in fibroblasts in a model of denervated skeletal muscle.[33] Another study in transgenic rats that overexpress OA by two-fold in the liver showed that OA attenuates the development of hepatic fibrosis by suppressing platelet-derived growth factor receptor-α (PDGFR-α) and tissue inhibitor of metalloproteinase-1 (TIMP-1), the key genes required for disease pathogenesis.[32]

A very exciting animal model DBA2J is the natural mutation in the OA gene in the mouse causing a premature stop codon that results in the generation of a truncated OA protein of only 150 amino acids.[7,8,79,80] Initial observations suggest that these mice develop normally. Preliminary studies using this animal model focused on the severe eye phenotype associated with the mutation. Data generated by the Simon group[7] suggest that the presence of truncated OA/Gpnmb causes mice to exhibit both iris pigmentary dispersion and iris stromal atrophy. Both phenotypes are strongly associated with the development of pigmentary glaucoma. These mice also have increased macrophage function.[56] Our group also generated transgenic mice that overexpress OA ubiquitously. These mice develop a skeletal phenotype that is associated with decreased bone mass (unpublished observations). With the development of these animal models, there has been an emerging focus on OA and its effects on different cellular and pathological processes. However, there is still a great deal of work that needs to be done to determine the functions of OA and its mechanism(s) of action.

VII. MOLECULAR REGULATION OF OSTEOACTIVIN EXPRESSION

Little is known about the molecular regulation of OA expression when compared with the vast literature related to OA and its functional role in the regulation of other molecules.

A study in the rat used carbon tetrachloride (CCl4) to induce acute injury in the liver and showed that OA expression was strongly enhanced with CCl4 treatment, whereas treatment of dexamethasone showed decreased OA expression in the acutely injured liver compared with the normal liver.[57] In addition, high levels of OA expression have been reported in humans with fulminant hepatitis and paracetamol intoxication.

Studies in patients with end-stage renal disease have also provided some insight into the regulation of OA.[37] In a study by Pahl et al., patients undergoing dialysis exhibited a marked upregulation of colony-stimulating factor (CSF) and interleukin (IL)-6 and a downregulation of IL-10, thus pointing to the potential role of antiinflammatory cytokines (IL-10) and pro-inflammatory cytokines (IL-6) in the regulation of OA as well as CSF.[37]

Some insights on the regulation of OA gene expression have emerged from therapeutic targets in malignancy. For, instance treatment with imatinib and inhibitors of the ERK pathway enhanced cell-surface expression of OA in melanoma cells,[78] thus indicating that the ERK pathway plays an important role in OA regulation. Understanding the regulation of OA is a priority for many reasons, including its potential role as an emerging therapy in breast cancer.[48] It has recently been shown in clinical trials utilizing CDX-011, an antibody-drug conjugate (OA-auristatin E) to treat breast cancer, in combination with ERK pathway inhibitors, to increase the sensitivity of breast cancer cells to CDX-011 treatment.[48]

Molecular studies on OA identified BMP-2 as a stimulating factor for OA expression via Smad1 signaling in osteoblasts.[41] This study suggested that OA acts as a downstream mediator of BMP-2 effects, as treatment of osteoblasts with OA antisense oligonucleotides inhibits BMP-2–stimulated osteoblast differentiation and functions. Furthermore, the OA promoter has multiple Smad1 binding sites that could regulate OA transcription following BMP-2 stimulation.[41] Promoter bashing studies from our laboratory suggest that Runx2 and Smad1/4 transcription factors regulate OA transcriptional activity (unpublished observations).

Like osteoblasts, osteoclasts also show a temporal increase in OA expression during their differentiation.[45] Co-localization of OA with β1 and β3 integrins is suggestive of its role in osteoclast cell adhesion. Additional studies have shown that OA expression is induced by RANKL, an osteoclast differentiation factor via MITF (microphthalmia-induced transcription factor).[38,81] Therefore, it is clear that OA is an important molecule in both osteoblast and osteoclast differentiation and is a key target for bone-specific transcription factors that integrate multiple osteogenic regulatory signals in bone.

VIII. MECHANISMS OF ACTION FOR OSTEOACTIVIN

DC-HIL (OA) is potentially involved in endothelial adhesion of dendritic cells. High levels of OA expression in dendritic cells suggest it to be involved in their migration. DC-HIL (OA) is distinguishable from other adhesion molecules on the cell surface—such as Thy-1, Ly-5, and NCAM—and is capable of interacting with heparin, whereas they do not function as ligands for integrins. However, DC-HIL (OA) serves as one of the counter-receptors for integrins. This character of DC-HIL (OA) may indicate that dendritic cells display not only common but also unique mechanisms for their transendothelial migration.

To study the localization of DC-HIL (OA) on dendritic cells, Shikano et al. performed flow cytometric analysis using soluble Fc fusion protein. This study showed that DC-HIL-Fc is localized to the cell surface of dendritic cells.[10]

Osteoblast cells cultured on OA-coated surfaces showed enhanced cell adhesion, spreading, cytoskeletal organization, and formation of focal adhesion complexes when compared with osteoblast cells grown on an untreated surface. These findings suggest that OA plays a role in cell adhesion (unpublished observation). In addition to its effects on cell adhesion, osteoblast cells adhered on OA-coated surfaces for a longer duration and showed enhanced osteoblast differentiation (unpublished observation). However, opposing effects were observed upon treatment of osteoblast cells with functional-blocking antibody to OA.[17] Similar roles of OA were observed in osteoclast cells, where treatment of osteoclast progenitor cells with an OA antibody decreased osteoclast fusion and migration and resulted in smaller osteoclast cells with decreased resorption activities.[45] Collectively, these findings suggest that OA stimulates a variety of cellular processes, ranging from initial attachment of bone cells to their migration, differentiation, and function.

Based on the current literature, there are two potential mechanisms proposed for the mediation of OA effects in bone cells. The first mechanism proposes that OA directly regulates specific signaling pathways that modulate the expression of genes involved in the differentiation of bone cells. According to this mechanism, secreted OA binds to yet-unknown receptors on the osteoblast/osteoclast cell surfaces and initiates signaling cascades to promote adhesion and their differentiation into mature osteoblast/osteoclast cells. The second mechanism suggests that secreted OA acts as an extracellular matrix protein and binds to integrins[45] and the integrins-co-receptor syndecan-4 complex.[35] It is the interaction of OA with integrins that induces a signaling mechanism and aids in cell adhesion, migration, cell survival, and differentiation. In osteoclasts, OA was demonstrated to be co-localized with β1, β3 integrins were expressed on the osteoclast surface, and treatment of osteoclast precursor cells with inhibitory RGD peptide (that inhibits integrin binding) decreased osteoclast cell fusion and resulted in smaller-sized osteoclasts, an effect similar to that observed on treatment with functional-blocking OA antibody.[45] These findings indicate that OA regulates osteoclast cell fusion, migration, and resorption through integrin receptors. Integrins have been shown to be essential for osteoblast differentiation by the use of function-blocking antibody to αvβ1 integrin.s[82,83] Unpublished work from our laboratory shows OA to be associated with αvβ1 integrins in osteoblasts. Collectively, these studies suggest that OA functions as an extracellular matrix protein, interacts with integrin receptors, and stimulates differentiation and function of osteoblasts and osteoclast cells. However, based on the evidence from these two studies, one cannot rule out the possibility of OA function through still unidentified receptors.

IX. CONCLUSIONS AND FUTURE PROSPECTS OF OSTEOACTIVIN

OA has emerged as an important anabolic factor in bone formation. Due to an increasing incidence of osteoporosis in the aging population, especially in postmenopausal women, a better understanding of the mechanisms involved in bone formation and resorption will help in the development of novel therapeutic agents that not only suppress bone loss

but also stimulate new bone formation. Because OA has effects on both osteoblasts and osteoclasts, it has potential for use in the treatment of both systemic and localized forms of bone loss. It is also associated with regeneration of extracellular matrix tissue following an injury in cases of tissue repair. OA also has immunosuppressive roles in T-cell immune response and macrophage infiltration. Finally, OA is also associated with aggressiveness and invasiveness of tumors both in vitro and in vivo. Due to its high expression in tumors, an antibody to OA conjugated to a toxic drug, CR-vcMMAE, has recently been developed and is currently in phase I/II clinical trials for the treatment of advanced melanoma and breast cancer. In conclusion, OA is a promising molecule with immense therapeutic potential.

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TABLE OF CONTENTS FOR VOLUME 20
CRITICAL REVIEWS™ IN EUKARYOTIC GENE EXPRESSION

Page Range of Issues
Issue 1, 1–86; Issue 2, 87–180; Issue 3, 181–273; Issue 4, 275–324

NUMBER 1

NUMBER 2

NUMBER 3

NUMBER 4

SUBJECT INDEX FOR VOLUME 20
CRITICAL REVIEWS™ IN EUKARYOTIC GENE EXPRESSION

Page Range of Issues

Issue 1, 1–86; Issue 2, 87–180; Issue 3, 181–273; Issue 4, 275–357

AUTHOR INDEX FOR VOLUME 20
CRITICAL REVIEWS™ IN EUKARYOTIC GENE EXPRESSION

Page Range of Issues

Issue 1, 1–86; Issue 2, 87–180; Issue 3, 181–273; Issue 4, 275–357