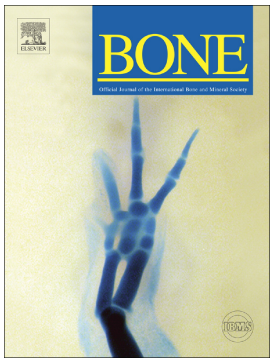

Retinoid roles and action in skeletal development and growth provide the rationale for an ongoing heterotopic ossification prevention trial

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Retinoid roles and action in skeletal development and growth provide the rationale for an ongoing heterotopic ossification prevention trial

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Abstract

The majority of skeletal elements develop via endochondral ossification. This process starts with formation of mesenchymal cell condensations at prescribed sites and times in the early embryo and is followed by chondrogenesis, growth plate cartilage maturation and hypertrophy, and replacement of cartilage with bone and marrow. This complex stepwise process is reactivated and recapitulated in physiologic conditions such as fracture repair, but can occur extraskeletally in pathologies including heterotopic ossification (HO), Ossification of the Posterior Longitudinal Ligament (OPLL) and Hereditary Multiple Exostoses (HME). One form of HO is common and is triggered by trauma, invasive surgeries or burns and is thus particularly common amongst severely wounded soldiers. There is also a congenital and very severe form of HO that occurs in children with Fibrodysplasia Ossificans Progressiva (FOP) and is driven by activating mutations in *ACVR1* encoding the type I bone morphogenetic protein (BMP) receptor ALK2. Current treatments for acquired HO, including NSAIDs and local irradiation, are not always effective and can have side effects, and there is no effective treatment for HO in FOP. This review article describes the research path we took several years ago to develop a new and effective treatment for both congenital and acquired forms of HO and specifically, the testing of synthetic retinoid agonists to block the initial and critical chondrogenic step leading to HO onset and progression. We summarize studies with mouse models of injury-induced and congenital HO demonstrating the effectiveness and mode of action of the retinoid agonists, including Palovarotene. Our studies have provided the rationale for, directly led to, an ongoing phase 2 FDA clinical trial to test efficacy and safety of Palovarotene in FOP. Top-line results released a few months ago by the pharmaceutical sponsor Clementia are very encouraging. Given shared developmental pathways amongst pathologies of extraskeletal tissue formation, Palovarotene may also be effective in HME as preliminary in vitro data suggest.

1. Introduction

The skeleton is composed of endochondral and intramembranous elements. The endochondral elements are more numerous and include the long bones, ribs, vertebrae, pelvis, cranial base and mandibular condyle, while the intramembranous elements comprise the calvaria, certain facial bones, mandible and clavicles [1]. It is not fully known why and how these two skeletogenic pathways actually evolved and persisted [2, 3], but much has been learned over the years about their cellular and molecular regulatory mechanisms [4-6]. As their name implies, the endochondral elements have a cartilaginous origin and derive from mesenchyme condensations that appear at specified locations and times in the developing embryo, undergo chondrogenesis and incipient morphogenesis, and preconfigure –and provide a blueprint for- the future mature ossified skeleton [2]. The neoformed chondrocytes within each incipient skeletal element become organized in growth plates with typical zones of proliferation, maturation and hypertrophy that sustain growth, expansion and elongation appropriate and distinct for each element, and permit the eventual replacement of hypertrophic mineralized cartilage with bone and marrow [7]. Such endochondral process continues for a long period of time spanning late embryogenesis through end of puberty at which point all the growth plates close and the skeleton reaches its maturity and final size. In comparison, the intramembranous elements derive from craniofacial ectomesenchymal and mesenchymal condensations also forming at specified locations and times in the early embryo that however, undergo ossification without an intermediate cartilaginous step [1, 4]. Their growth and expansion are largely sustained by cell proliferation and also apposition by which local progenitors are recruited from the immediate surroundings into the skeletal growth fronts, undergo osteogenesis and allow for appropriate tissue expansion, elongation and morphogenesis as prescribed for each element [8, 9].

The endochondral and intramembranous processes can be re-initiated in adults for physiologic reasons such as bone fracture repair. It is widely assumed that relatively small fractures in which the fracture lines are physically close to each other mostly repair via intramembranous ossification by which the fracture is eventually filled with new bone tissue and may undergo some remodeling to restore skeletal element outline and contour [10-12]. Large, extensive and more serious factures are instead repaired via endochondral ossification that involves formation of a large mesenchymal and vascularized callus followed by cell condensation, chondrogenesis, cartilage maturation and hypertrophy, endochondral ossification and extensive bone remodeling. There are also conditions in which intramembranous- and endochondral-like processes are re-initiated for non-physiologic reasons and responses and are associated with, or can directly cause, pathologies. On example is Ossification of the Posterior Longitudinal Ligament (OPLL), a potentially severe condition in which bone-like tissue forms and accumulates along the back and progressively alters tissue function and biomechanics [13]. Another example is Hereditary Multiple Exostoses (HME), a pediatric condition caused by mutations in the Golgi-resident enzymes EXT1 or EXT2 in which ectopic masses of cartilage form next to the growth plates of multiple skeletal elements, including long bones, ribs and vertebrae [14, 15]. The tissue masses –known as exostoses and osteochondromas- enlarge and protrude away from the growth plates, undergo endochondral ossification in their proximal region and remain continuous with bone and marrow of affected skeletal elements [16]. Because of their large number and sizes, the osteochondromas cause a number of health problems, including skeletal deformations, chronic pain and even malignancy. Most relevant here is heterotopic ossification (HO) in which extraskeletal endochondral bone forms and accumulates at diverse locations in the body at the expense of muscle and connective tissues [17, 18]. One form of HO is common and non-genetic, is triggered by trauma, invasive surgeries,

burns and ensuing inflammation and is thus particularly common amongst severely wounded soldiers [19]. There is also a congenital, severe and often fatal form of HO that occurs in children with Fibrodysplasia Ossificans Progressiva (FOP), is usually initiated by local inflammation and flare-ups and is driven by activating mutations in *ACVR1* encoding the type I bone morphogenetic protein (BMP) receptor ALK2 [20]. Current treatments for injury- or surgery-induced HO include systemic administration of non-steroidal anti-inflammatory drugs (NSAIDs), local prophylactic low-dose irradiation directed to affected site, or combination of both [21, 22]. These common treatments are associated with reduced HO incidence, but are not always effective and cannot be given after combat-related injuries [23]. Symptomatic HO masses can be removed by surgery, but this procedure requires further hospitalization, may have complications including infections and blood loss and could even instigate another round of HO [24]. There are even fewer therapeutic possibilities for patients with FOP [25]. This disease is exceedingly reactive and aggressive, and surgery nearly always triggers a far more serious round of HO and bone accumulation and is thus strickly avoided. Because of the connection between flare-ups and HO formation, the current standard of care for FOP patients includes a brief 4-day course of high-dose corticosteroids starting within 24 hrs of a flare-up [25]. Though this treatment can reduce inflammation and pain, it does not consistently prevent the progression to HO [26]. Given the limitations in current therapeutic options, potency and applicability, what could be alternative, safer and more effective treatments for HO? In this review, I will describe the rationale and path we followed over the last few years to address that critical question. We considered principles of developmental biology and molecular mechanisms of skeletogenesis to predict plausible therapeutic targets for HO prevention. This led us to select the retinoid signaling pathway and its nuclear receptors, and we have indeed demonstrated that synthetic retinoid agonists –and in particular Palovarotene- are potent inhibitors of both

acquired and congenital forms of HO in mouse models [27-29]. Happily, these studies have elicited a current phase 2 clinical trial to test the effectiveness of Palovarotene against HO in FOP patients (NCT02190747). Given its ability to inhibit ectopic endochondral ossification regardless of instigating mechanisms, Palovarotene may actually have broader therapeutic applicability, including prevention of osteochondroma formation in HME as preliminary data below suggest.

2. Retinoid signaling roles in skeletal patterning and development

Vitamin A is an essential molecule with a multifaceted biology that plays roles in numerous processes, including skeletogenesis [30, 31]. It is normally stored in the liver and adipose tissue as retinyl esters that upon need, are esterified to retinol which is released into the circulation bound to carrier proteins (retinol-binding proteins, RBPs) given its hydrophobicity [32]. The largely inactive retinol is taken up by cells in many tissues via the transporter STRAT6 [33] and converted by the sequential action of cytoplasmic retinol dehydrogenases (RDHs) and retinaldehyde dehydrogenases (RALDHs) into biologically-active *all-trans* retinoic acid (*at*RA) [34]. Other active retinoids, including *9-cis* and *13-cis* RA, are produced in certain organs, but usually at lower concentrations [35]. The retinoids are ferried to the nucleus by cellular retinoid binding proteins (CRABPs) [36] where they interact with, and regulate the transcriptional activity of, retinoic acid receptors (RARs) and retinoid X receptors (RXRs) [37-39]. The RARs and RXRs are present as heterodimeric RAR-RXR complexes and are bound to retinoic acid response elements (RAREs) located in enhancer regions of target genes. The RAREs are composed of two hexameric direct repeats (DRs) of the sequence (A/G)G(T/G)TCA separated by 5 or 2 base pairs (DR5 and DR2, respectively). The DR5 and DR2 configurations provide RAR-RXR binding specificity since similar hexameric repeats separated by 3 or 4 nucleotides (DR3 and DR4) serve as the response elements for vitamin D

receptor/RXR complexes and thyroid hormone receptor/RXR complexes, respectively [40, 41]. This striking regulatory feature is referred to as the 3-4-5 rule [39]. The RAR-RXR heterodimers can bind to the RAREs in the absence of active retinoids, and such unliganded complexes exert transcriptional repression function in association with co-repressors such as nuclear receptor co-repressors 1 and 2 (NCOR1 and 2) recruiting histone deacetylase and Polycomb complexes [42]. Retinoid binding to the RAR-RXR heterodimers allows for protein conformational changes and replacement of co-repressors with co-activators (NCOA1, 2 and 3) along with histone acetylases and other factors, leading to chromatin relaxation and activation of target gene expression (reviewed in [43, 44]). Given that transcriptional repression and activation work in close concert to establish and regulate basic but essential tissue-specific gene expression patterns, the ability of RAR-RXR complexes to exert both roles depending on absence or presence of ligands endows them with broad biological functionality and relevance [39, 44]. It should be noted that the cellular levels of *at*RA and other retinoids markedly differ in different tissues and cell types or at different stages of tissue development and maturation, and those levels are strictly controlled and maintained by differential expression and activity of retinoid-producing enzymes (see above) versus retinoid-catabolic CYP26 enzymes [44-46]. Indeed, global ablation of *RALDH2* is embryonic lethal [47]. As part of fine-tuned regulatory loops, *at*RA regulates the expression of its own signaling pathway and metabolic genes, including the *RARs*, *CYP26A1* and carrier proteins [48, 49], and acts both as an autocrine and paracrine factor upon release and diffusion from producing cells [50]. Thus, the repressor and activation functions of the RAR-RXR complexes are tissue specific, can be modulated depending on local ligand levels and gradients, and can be altered within the same tissue or organ over developmental stages.

The vertebrate genome contains three RAR genes (*RARα, RARβ* and *RARγ*) and three RXR genes (*RXRα, RXRβ* and *RXRγ*). Several of these genes are expressed in specific and dynamic spatio-temporal patterns during embryogenesis, though *RARα, RXRα* and *RXRβ* are uniformly expressed in mouse embryos [51, 52]. Significant insights into RAR and RXR roles in skeletal development and patterning have come from the analysis of mouse embryo mutants lacking one or more of these genes. With one exception (*RXRα*), ablation of single *RAR* or *RXR* genes is largely well tolerated indicating that there is significant functional redundancy amongst these receptors [31, 45]. However, compound mutant embryos do have severe and pervasive defects in multiple organs and structures, including the developing skeleton, that were documented and analyzed in seminal studies and have been summarized in comprehensive reviews [31, 37, 45, 53]. As an example, compound *RARα/RARγ*-null mouse embryos were found to exhibit profound defects in the craniofacial, axial and appendicular skeleton [54]. The most severely affected elements in the skull were those in the midfacial region and cranial base deriving from mesectoderm and paraxial mesoderm [55], and included frontal and nasal bones, otic capsule, cranial vault and ethmoid and sphenoid bones. Upper jaw tooth germs were missing, but mandible and temporomandibular joint and condyle appeared normal. The axial skeleton of the compound mutant embryos exhibited homeotic transformation of several vertebrae, and in particular anteriorization of C2 and C5 that often displayed ectopic ribs. Significant malformations were noted that included vertebral body misshape, fusion of neural arches, rib misalignment and fusion, and sternum defects. The homeotic transformations are particularly interesting since the identify of each vertebra is under strict control by *Hox* genes [56, 57] several of which are found to be direct targets of RA signaling and action [58]. In addition, endogenous *at*RA is present in the form of a two-tailed gradient along the dorsal embryonic trunk including the somites, with higher levels in mid-region and tapering down toward

the cephalic and caudal ends that express RA catabolizing CYP26A1 [59, 60]. Thus, the vertebral homeotic transformation in compound *RARα/RARγ*-null mouse embryos may have resulted from altered patterns of *Hox* expression, subpar responses to local ligands and gradients, and/or modulation of RAR activation vs repressor function.

The appendicular skeleton was affected as well in dual *RARα/RARγ*-null mouse embryos, and the defects appeared to be more severe in forelimbs than hindlimbs by E18.5 [31, 54]. These defects included: fusion of several digits at the level of flanking soft tissues; loss of digit 1 in some mutants and polydactyly with six digits in others; reduction in overall limb length with proportional decreases in skeletal elements lengths; delayed ossification; and sporadic absence of certain elements and in particular radius and fibula. While the exact causes of these multiple and complex changes remain to be clarified, it is possible that the digit fusion was caused by lack of regression and death of interdigit mesenchyme. This mesenchyme was shown to strongly express the retinoid synthesizing enzyme *RALDH2* [61] and also β-galactosidase in *RARE-LacZ* reporter mice [59], signifying that it is *at*RA rich and responsive. Thus, absence of the two RAR genes in mutant embryos may have altered its developmental fate, reduced its regression, and elicited digit fusion.

This brief synopsis makes it abundantly clear that retinoids and RARs play very important, complex and multiple roles in skeletal patterning and development, likely as a result of their ability to influence the expression of a broad array of target genes and to modulate diverse and essential processes ranging from cell proliferation, differenation and survival as well as morphogenesis.

3. Antithetic relationship between retinoid signaling and chondrogenesis

As reiterated above, chondrogenic differentiation of mesenchymal and ectomesenchymal cell condensations emerging at multiple and prescribed sites throughout the early embryo allows the formation of the initial cartilaginous skeleton serving as the blueprint of the future mature endochondral skeleton. Given their fundamental nature, chondrogenesis and cartilage formation have been studied extensively and for decades, and much has been learned about their complex molecular regulation as well as the numerous local and systemic factors influencing mesenchymal cell differentiation, growth plate function and endochondral ossification (schematic in Fig. 1), as comprehensive and illuminating reviews make clear [4, 5, 62-64]. An intriguing and long-standing aspect of these processes is that the mesenchymal condensations need to proceed to chondrogenesis rather than alternative developmental paths such as osteogenesis, fibrogenesis or adipogenesis, and there has been unabated interest in identifying the mechanisms underlying this key developmental fate decision [65, 66]. Several pathways serve to steer and facilitate the decision to chondrogenesis including *SOX* genes and TGFβ/BMP signaling [4], but concurrently and as importantly, other counteracting pathways need to be shut down. One such mechanism is the Wnt/β-catenin signaling pathway which would otherwise promote osteogenesis [67]. A second mechanism is the retinoid signaling pathway [68] as elaborated next.

Early interest in retinoid signaling in chondrogenesis and skeletogenesis was boosted by the finding that *at*RA appeared to be present in the form of an anterior-to-posterior grandient across the early developing limb buds, leading to the suggestion that it represented the first vertebrate morphogen ever identified [69]. Though this thesis turns out to be not fully solid [70], it generated great interest at the time, including delineating whether and how *at*RA would act at the cellular developmental level to guide cell differentiation and morphogenetic decisions within limb progenitor populations committing to different lineages. We were the first to show several years ago

that *at*RA potently inhibited chondrogenic cell differentiation while having no obvious effect on myogenic cells, the second major limb progenitor population [71]. Since the limb chondrogenic cells derive from the lateral plate mesoderm while the myogenic cells are somitic in origin, the data showed also that limb progenitor populations with distinct origins and fates responded differentially to *at*RA, reinforcing the idea that *at*RA and retinoid signaling could operate within a developing field to influence fate decisions within local progenitor populations. In good correlation, data from the *RARE-LacZ* reporter mouse study cited above clearly showed that retinoid signaling was undetectable within limb mesenchymal condensations undergoing chondrogenesis and cartilage formation [59], though it was very strong in neighboring non-chondrogenic cells. The data consolidated the idea that chondrogenesis normally requires absence of –or a steep drop in- retinoid signaling to initiate and proceed.

Subsequent studies by Underhill and coworkers greatly expanded knowledge and insights in this area. In a series of illuminating and well crafted cellular and molecular studies, this group showed that transcriptional repression by unliganded RAR receptors is actually required for chondrogenesis and expression of master chondrogenic genes and signaling factors, including *Sox* genes and bone morphogenetic poteins (BMPs) and their receptors [61, 72, 73]. The authors monitored the expression patterns of *RARs* and also genes involved in *at*RA synthesis and catabolism during chondrogenesis in vivo and in vitro, and found that very early preskeletal progenitors expressed *RAR*α but the cells switched to *RAR*γ expression during their chondrogenic commitment and differentiation. This switch was accompanied by marked decreases in *RALDH2* and *CRABP* expression and concurrent increases in *CYP26* expression, underlying the notion that chondrogenesis requires depletion of endogenous retinoids and carrier proteins and concurrent transcriptional repression function by unliganded RARs, mostly mediated by RARγ. Interestingly,

the authors also showed that treatment of limb mesenchymal progenitors with a synthetic retinoid inverse agonist (that increases RAR repressor function) greatly stimulated chondrogenesis and cartilage formation, reaffirming the important positive role of RAR-mediated transcriptional repression in chondrogenesis.

In concurrent studies, we focused on further cartilage development and growth plate function and found that while *RARα* and *RARβ* were expressed at low levels and in rather diffuse manners, *RARγ* was prominently expressed in the resting, proliferative and pre-hypertrophic zones of growth plate and was down-regulated in the hypertrophic zone [74-76]. We created conditional mouse mutants deficient in *RAR* expression in cartilage by mating floxed *RAR* mice with *Col2-Cre* mice [77]. Compound *RARα/RARγ*- or *RARβ/RARγ*-deficient mice exhibited significant growth retardation by 3 weeks postnatally, and their growth plates were defective and contained very low amounts of aggrecan, a normally abundant cartilage matrix component. Direct quantification of endogenous retinoids using tissue microdissection and LC-MS/MS analytical procedures [78] showed that the growth plates were largely devoid of endogenous retinoids, reaffirming the idea that the RARs were unliganded and were exerting repression function. We interrogated multiple plausible co-repressors and found that *ZAC1* was prominently expressed in growth plate and its expression pattern fully overlapped that of *RARγ*. Interestingly, *RARγ* and *ZAC1* were able to physically interact with each other, and *ZAC1* over-expression in cultured chondrocytes under retinoid-free conditions boosted chondrocyte phenotypic expression and functioning, likely mediated by increased *SOX* gene expression. Similar responses were obtained when the chondrocytes were treated with a synthetic reverse agonist that as pointed out above, increased *RAR* repressor function.

Together, the studies above provided clear evidence that chondrogenesis and cartilage and growth plate functioning are promoted by silencing of retinoid signaling and by unliganded RAR repressor function. Indeed, data from both *RARE-LacZ* reporter mice [59] and our direct biochemical measurements demonstrate that prechondrogenic mesenchymal condensations, developing cartilage and growth plate are all largely devoid of endogenous retinoids. This is also a reflection of the avascular nature of these tissues, making it less likely that they would receive circulating retinol, but also their differential expression of *RALDH2* and *CYP26* [68, 76].

4. Retinoids as a possible treatment for HO and related conditions

It is the evidence and insights from all the above studies and related work that led us to conceive the idea over a decade ago that retinoids could be an effective preventive treatment for HO. As summarized above, both acquired and congenital forms of HO are largely endochondral processes that start with mesenchymal condensations and chondrogenesis and proceed with cartilage maturation and ossification. Thus, treatment with active retinoids should readily block HO given that chondrogenesis and cartilage development normally require lack of retinoid signaling and unliganded *RAR* repressor function. A first problem we confronted at that time was that though essential to life and diverse in structure, natural endogenous retinoids –and *at*RA in particular- do not discriminate amongst the RARs and activate all isoforms, making the potential biological consequences of a putative treatment with natural retinoids too broad and possibly unmanageable in the skeleton. Luckly, several pharmaceutical companies in the eighties and nineties had embarked in the production, characterization and testing of large arrays of synthetic retinoid agonists that were found to have preferential binding and selectivity for RARα, RARβ and RARγ [79-81]. Aiming to

target the earliest possible step in progenitor cell commitment to chondrogenesis when RARα is strongly expressed [68], we decided to first test the synthetic RARα agonist NRX195183, also in consideration of the fact that this drug had already been extensively studied, was being tested in mice for other potential therapeutic uses, and had already passed FDA phase 1 [80, 82, 83]. To make sure that the drug had effective anti-chondrogenic activity, we tested it in micromass cultures of mouse embryo limb mesenchymal cells that are a popular system for chondrogenesis analyses [84]. After cell seeding, cultures were mainatained in standard control low serum medium or medium containing: rhBMP2 (to boost chondrogenesis); 1 or 3 μM agonist; or rhBMP2 plus agonist (Fig. 2). Staining with alcian blue on day 5-6 of culture showed that while chondrogenesis was increased by rhBMP2 treatment (Figs. 2C-2D) over control (Figs. 2A-2B) as expected, it was largely prevented by agonist treatment regardless of presence or absence of rhBMP2 treatment (Figs. 2E-2H). There are several experimental animal models of acquired HO, and a convenient and highly consistent one involves the subcutaneous implantation of a scaffold containing rhBMP2 [85]. Working under my guidance, my research technician Ms. Tiffany Morrison utilized this model and optimized drug delivery by gavage. She implanted Matrigel containing 1 μg rhBMP2 at two subdermal sites along the ventral abdominal wall of 2 month-old female mice and monitored HO formation and progression over time. Starting on day 1 from implantation, half of the mice received vehicle (30% DMSO/70% peanut oil) by gavage once or twice/day, while companions received vehicle containing NRX195183 (final dose about 30 mg/kg). Fig. 3 shows an example of results she obtained. The data unequivocally showed that ectopic HO-like cartilaginous masses had formed in control vehicle-treated mice by day 12-14 and displayed strong alcian blue cartilage staining (Figs. 3A-3E). In contrast, the Matrigel scaffolds retrieved from agonist-treated mice were essentially negative for alcian blue staining and devoid of cartilage (Figs. 3F-3J). Heartened by these exciting

and novel observations, we carried out additional and more extensive experiments sponsored by a grant from the Department of Defense. We found that the HO-like masses in control mice contained not only ectopic cartilage but also endochondral bone, marrow, TRAP-postive osteoclasts and numerous blood vessels by day 12 or 14, but all these parameters had been markedly reduced in agonist-treated mice. These effects were accompanied by sharp decreases in gene expression of *SOX9, collagens X* and *XI* and *RUNX2*. The study provided the first evidence ever that a retinoid agonist-based strategy could effectively counter and even prevent the formation of HO-like lesions [27].

Though impressive, the pharmacological strategy above required fairly high doses of RARα agonist to be fully effective [27]. In addition, while RARα is expressed during the early phase of mesenchymal cell commitment, the resulting chondrogenic cells switch to expressing RARγ which is also expressed in fully differentiated chondrocytes and growth plate [68, 74, 76]. Thus, a therapy against HO based on RARγ agonists could potentially be more effective because it would act on both committed chondrogenic progenitors as well as chondrocytes. We set out to test this tantalizing hypothesis using several synthetic RARγ agonists that if all effective, would also allow us to establish a drug class effect. The first adult mouse models we tested were the subdermal one described above and a muscle-injury model that mimics trauma-induced HO which is common after invasive surgeries such as hip replacement [86] or severe trauma such as that occurring in combat theaters [19]. We monitored and evaluated the HO process over time in the whole animals or after surgical resection of the lesions, using soft X-ray, μCT to estimate bone volume/total volume (BV/TV), histology and histochemistry, and protein and gene expression analyses. We found that several RARγ agonists reduced HO formation in both models and did so to varying degrees. One of the most effective agonists was NRX204647 that markedly reduced HO at any dose tested (0.12 to

1.2 mg/kg by daily gavage), likely as a reflection of its high binding affinity for RARγ [87]. Two other agonists –CD1530 [88] and R667 [89]- displayed substantial anti-HO activity at the high end of doses tested (1.2 to 12 mg/kg and 0.12 to 4 mg/kg, respectively). When some of the agonists were used in *RARγ*-null mice, HO was not inhibited, attesting to drug selectivity and specificity. In addition, if the agonists were given within 5-6 days from whem HO had been first induced, the drugs were still effective, indicating that they enjoyed a fairly good window-of-opportunity. To clarify mechanisms of action, we carried out in vitro studies with chondrogenic cells and found that RARγ agonist treatment markedly reduced the levels of phosphorylated SMAD1/5/8 that normally transduce pro-chondrogenic BMP signaling [5]. The treatment also reduced the overall cellular steady-state SMAD1/5/8 levels via shunting the proteins for proteasome-mediated degradation, indicating that the drugs were globally interfering with the BMP signaling pathway and the chondrogenic differentiation program.

But would the retinoid agonists be able to inhibit the far more severe congenital forms of HO? To tackle this critical question, we used the only transgenic mouse line available at the time and originally created by Dr. Y. Mishina (University of Michigan) that encodes a Cre-inducible constitutively-active ALK2^{Q207D} mutant protein and EGFP [90]. This particular mutation is not amongst the 13 *ACVR1* mutations described so far in FOP patients, including the most common ALK2^{R206H}, that are not constitutive-active, have a slight increase in basal activity and are ligand dependent [91]. Thus, HO formation in the ALK2^{Q207D} line was expected to be even more aggressive than that driven by naturally occurring FOP mutations. To induce HO, we injected the left anterior tibial muscles in P7 ALK2^{Q207D} mice with adeno-Cre along with cardiotoxin to induce local muscle damage and inflammation. Extensive and aggressive HO developed by 4 to 5 weeks from induction in mice that had received daily vehicle treatment by gavage, but HO had nearly been

prevented in companion mice receiving RARγ agonists as revealed by μCT analysis. To confirm data, vehicle and agonist-treated HO mice were injected intraperitoneally with alizarin complexon at P29, P31 and P33, sacrificed at P35 and processed for stereomicroscopic fluorescence analysis of affected leg site. Strong alizarin signal was observed in vehicle-treated mice but not in agonist-treated mice, confirming that HO formation had been suppressed by agonist treatment.

Given that the the $ALK2^{Q207D}$ mice were expected to have a stronger HO phenotype than mice carrying one of the human *ACVR1* mutations, the above data were quite encouraging by implying that the RARγ agonists could be even more effective against human mutations. However, this still needed to be proven directly. Thus, we joined forces with colleagues at the University of Pennsylvania who are leaders in FOP research [26] and with researchers at Regeneron Pharmaceuticals who had developed an inducible knock-in transgenic mouse line encoding the most common human FOP mutation, $ALK2^{R206H}$ [92]. For these experiments, we purposely tested only R667 –renamed Palovarotene in the meantime- for several reasons. Chiefly, Palovarotene had been extensively tested in patients with emphysema in a large phase 2 FDA-approved clinical trial [93]. The idea behind those efforts was the well known beneficial effects that retinoids have on epipthelial cell differentiation and re-epithelization of lung lining [94]. The drug was found to be effective, safe and well tolerated with minimal side effects, even when given daily over a 2 year period. Because of this auspicious clinical profile as well as its effectiveness against HO in mice [28], this drug had actually long been our lead compound. We induced HO in 1 month-old mice by turning on transgene expression and production of $ALK2^{R206H}$ by doxycycline while inducing leg muscle inflammation by cardiotoxin injection as above. While extensive HO formed in vehicle-treated mice after a month or so that led to severe reduction of leg motion, there was a marked decrease in HO in the Palovarotene-treated mice as well as improved leg motion and a local

decrease in mast cells [95]. The latter cells were originally invoked in congenital HO pathogenesis for their roles in inflammation [96], and we have also observed a sharp decrease in the number of mast cells as well as macrophages following Palovarotene treatment in a mouse HO injury model [29].

Because HO in FOP patients starts early in postnatal life, we mimicked this situation by turning on the expression and production of $ALK2^{R206H}$ throughout the developing embryonic limbs by mating the knock-in $ALK2^{R206H}$ mice with *Prx1-Cre* mice [97]. Interestingly, neonatal mutant mice displayed the characteristic large toe malformation which is typical of FOP infants, and developed extensive HO by 1 month. In addition, there was a significant reduction in average long bone length and alterations in chondrocyte proliferation and maturation in their growth plates, accompanied by excess pSMAD1/5/8 distribution and abnormal *PTHrP* expression. To administer Palovarotene as early as possible, the drug was given to the lactating mothers until day 14 postnatally, and drug treatment was then continued by direct oral administration to the pups until 1 month of age. The treated mice displayed a sharp reduction in limb HO as well as clear improvements in both long bone length and growth plate organization and function. These experiments produced an additional and most remarkable finding. It has long been known that retinoid treatment could affect the normal functioning of the growth plate particularly at young age, given that the growth plates are normally devoid of endogenous retinoids as described above [75]. Indeed, companion WT mice receiving Palovarotene from their lactating mothers and then directly until 1 month of age did display some appreciable changes in their growth plates, mainly consisting of a reduction in aggrecan accumulation and a slight decrease in growh plate height. Remarkably, the growth plates in Palovarotene-treated mutant mice at 1 month of age were instead wholly normal and closely resembled those in control vehicle-treated WT mice. The data clearly indicate

that WT and mutant growth plates and mice respond quite differently to drug treatment. Retrospectively, this is actually not surprising because a congenital mutation could have pleiotropic effects and alter multiple processes and mechanisms, including cell and tissue responses to a given drug. We interpreted our data to indicate that by being able to reduce BMP signaling [28], Palovatotene treatment had "normalized" pSMAD1/5/8 signaling in mutant ALK2^{R206H}-expressing growth plates, allowing these structures to maintain a normal organization and phenotype and in turn sustaining normal long bone lengthening.

At present, we are carrying out studies to further unravel the mechanisms of action of Palovarotene in the control of chondrogenesis and thus, HO initiation. We find that the drug directly and effectively blocks chondrogenesis in limb bud mesenchymal micromass cultures under basic conditions (Figs. 4E-4F) compared to control (Figs 4A-4B). Exogenous rhBMP2 treatment greatly stimulated chondrogenesis (not shown) and pSMAD1/5/8 levels (Fig. 4I, second lane), but both such stimulations were inhibited by Palovarotene treatment (Fig. 4I, fourth lane). We previosuly showed that retinoid agonists enhance the anti-chondrogenic pathway Wnt/β-catening signaling [98] and additional data suggest that they may modulate ERK1/2 signaling, another anti-chondrogenic mechanism [99]. Together, the potency of Palovarotene in HO may reflect its ability to differentially modulate key regulatory mechanisms and alter the balance between pro- and anti-chondrogenic pathways. As pointed out in the Introduction, there are other rare diseases that involve ectopic chondrogenesis, excess BMP signaling and extraskeletal endochondral ossification, including HME [16, 100]. As indicated above, this pediatric disease is caused by loss-of-function mutations in *EXT1* or *EXT2* and a consequent deficiency in heparan sulfate (HS), and is characterized by ectopic chondrogenesis along the growth plate leading to formation of osteochondromas [16]. As we showed previously, HS deficiency (elicited by genetic, enzymatic or

pharmacological means) greatly stimulated chondrogenesis in limb mesenchymal cell micromass cultures and concurrently stimulated pSMAD1/5/8 levels [100]. As a pharmacological tool in those studies, we used the small chemical compound Surfen which is a HS antagonist [101]. To determine whether Palovarotene is able to counter the stimulation of chondrogenesis caused by functional HS deficiency, we treated the micromass cultures with Palovarotene in absence or presence of Surfen (Fig. 4). Clearly, while Surfen treatment did increase chondrogenesis (Figs. 4C-4D) over control (Figs. 4A-4B) as expected [100], Palovarotene was able to fully block such increase (Figs. 4G-4H). The anti-chondrogenic potency of Palovarotene was reiterated by the finding that it markedly decreases expression of typical cartilage gene markers, including *Sox9*, aggrecan (*Acan*), collagen 2 (*Col2a1*) and collagen X (*Col10*) (Fig. 4J). Thus, Palovarotene could have therapeutic value and relevance for HME also since it can block BMP signaling and chondrogensis needed for osteochondroma formation.

5. An ongoing phase 2 clinical trial for FOP

Our 2011 study [28] showing that retinoid agonists including Palovarotene were effective against both acquired and congenital forms of HO received significant attention. Amongst those drugs, Palovarotene was particularly attractive as a possible HO therapeutic because it had already undergone close clinical scrutiny and pharmacodynamics analyses and had been tested in the phase 2 emphysema trial, appearing both effective and safe [93]. Luckily, our study caught the attention of experts and interested parties, and the pharmaceutical company Clementia was created to explore Palovarotene's therapeutic potentials in FOP. Indeed, in 2014 Clementia was able to initiate a phase 2 randomized, double-blind, placebo-controllled efficacy and safety study of Palovarotene against

HO initiation and progression in FOP patients (NCT02190747). As specified at *ClinicalTrials.gov*, the trial aimed to enroll 40 patients, and outcome measures included plain radiographs and low dose CT scans to assess amount or area of new heterotopic bone formation over time. Additional analyses included assessment of active range of motion, pain and swelling, duration of active symptomatic flare-ups, and overall physical function. Clementia announced top-line results in October 2016 indicating that happily and promisingly, several positive trends were observed in patients receiving Palovarotene treatment, including decreases in: the fraction of patients developing HO; time of resolution of a flare-up; and patient-reported pain linked to the flare-up area. The trial is ongoing and is in an open-label extension at the moment.

6. Conclusions and implications

Natural retinoids are fundamental components of life as elaborated above. This was conspicuously demonstrated a while back by the finding that global ablation of *RALDH2* in mice was incompatible with survival, and the mouse embryo mutants died at mid-gestation, failed to undergo axial rotation, lacked limb buds and had gross craniofacial abnormalities [47]. These aberrations were wholly caused by lack of endogenous retinoid production since they were rescued by systemic maternal administration of *at*RA. In addition to *at*RA or *9-cis*-RA, retinol can be metabolized to *11-cis*-retinaldehyde that is essential for light absorbance and vision [102]. Natural retinoids are also exploited as therapeutics for certain conditions such as: acute promyelocytic leukemia in which the *at*RA treatment is likely to act by inducing terminal differentiation of leukemic cells [103]; and acne in which *13-cis*-RA (isotretinoin) treatment likely acts to change sebaceous gland activity and phenotype [104]. The creation of synthetic retinoids with RAR

isoform selectivity promised to open new therapeutic avenues and opportunities initially [87], but the successes have been limited so far. Should Palovarotene prove itself to be an effective and safe treatment for congenital HO in FOP patients as the recently announced top-line results indicate, this would not only represent a landmark contribution for treating this otherwise often fatal and currently uncurable disease, but would also reiterate the potentials of synthetic retinoid-based therapies and inspire further research. Because acquired HO develops via a similar endochondral ossification as congenital HO does -at least in most cases-, Palovarotene should likely be efficacious as a preventive measure against this common condition [27-29] that affects patients undergoing extensive surgeries as well as those suffering from extensive burns, neural tissue damage or severe combat wounds [18]. Notably, we have recently shown in collaboration with Drs. T. Davis, J. Forsberg and coworkers at the Naval Medical Research Center in Bethesda, MD that Palovarotene is in fact able to effectively inhibit HO in a blast-trauma rat model that closely mimics HO pathogenesis in soldiers sustaining combat injuries or other forms of blast trauma [105]. Lastly, given the involvement of ectopic chondrogenesis, excess BMP signaling and endochondral bone formation in osteochondroma formation in HME, Palovarotene may turn out to be an effective treatment for this condition as well, indicating that shared pathogenic pathways in HO and HME – and most notably excessive BMP signaling- offer common targets of therapeutic intervention.

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support and commitment to our work over the years. Our mandate was to provide proof-of-principle evidence using laboratory animals that a synthetic retinoid agonist-based therapy was effective against both acquired and congenital forms of HO. Should Palovarotene prove to be as effective against congenital HO as clearly suggested by the top-line clinical trial data and should it be eventually approved for FOP treatment, I do very much hope this achievement will lead to an additional clinical trial to test Palovarotene against acquired forms of HO caused by invasive surgery, burns, brain damage or combat wounds. I would like to express my sincerest gratitude to Clementia whose visionary and resourceful officers have made the FOP trial possible. I would also like to thank: the many colleagues, postdocs and research technicians who participated in the HO studies over the years; Dr. P. Chambon for generously donating the floxed *RAR* mouse lines; and Dr. C. Mundy for providing the data on micromass cultures and insights into data analysis and interpretation. Due to the concise nature of this review chapter, not all relevant and deserving literature and authors could be cited.

Figure legends

Fig. 1. Schematic depicting the major developmental steps underlying endochondral bone formation. This process becomes overtly appreciable in the early embryo with formation of mesenchymal cell condensations in trank and limbs; ectomesenchymal condensations also form in the craniofacial region. Cell condensation is followed by chondrogenic cell differentiation, chondrocyte proliferation, maturation and hypertrophy in the growth plate, and is then brought to conclusion with invasion of hypertrophic mineralized cartilage by osteoprogenitors and blood vessels and replacement of cartilage with endochondral bone and marrow. Studies by many laboratories have delineated and characterized distinct genes and pathways that promote and regulate each of these developmental steps [4-6]. The two stop signs point to the developmental steps that are inhibited by natural and synthetic retinoid agonists, including *at*RA and Palovarotene, offering a strategy to block ectopic cartilage and bone formation at extraskeletal sites in pathologies such as HO and HME.

Fig. 2. Antithetic relationship between chondrogenesis and retinoid signaling. (A-H) Bright field images show micromass cultures of mouse embryo limb bud mesenchymal cells that were maintained in control medium (A-B) or medium containining; (C-D) 100 ng/ml rhBMP2; (E-F) 1 μM retinoid agonist; and (G-H) both rhBMP2 and agonist. Note that while rhBMP2 treatment greatly stimulated chondrogenesis as indicated by a sharp increase in alcian blue staining (C-D), treatment with agonist blocked both basal and rhBMP2-stimulated chondrogenesis. Histogram to the right shows quantification of staining levels by optical density and image analysis $\pm$ S.D.

Fig. 3. Inhibition of ectopic cartilage formation by retinoid agonists. (A-E) Bright field images show ectopic cartilage masses in control vehicle-receiving mice that had been implanted

with Matrigel/rhBMP2 mixtures at two ventral subdermal sites. Ectopic masses were harvested on day 12 from implantation and stained with alcian blue. Each mouse was implanted at 2 sites and the images show 5 sets of ectopic masses from 5 distinct mice in one representative experiment. (F-J) Images show 5 sets of Matrigel/rhBMP2 implants retrieved on day 12 from 5 companion mice that had been administered RARα agonist NRX195183 (≈30 mg/kg) by daily gavage. Note the dramatic decrease in alcian blue staining compared to controls.

Fig. 4. Analyses of the anti-chondrogenenic activity of Palovarotene. (A-H) Images show mouse embryo limb bud mesenchymal micromass cultures grown until day 6 control conditions (A-B) or presence of 5 μM Surfen (C-D), 100 nM Palovarotene (E-F) or both (G-H), with the latter condition indicated as S+P. Cultures were first stained with alcian blue to reveal the extent of chondrogenesis (top row images). After documenting the data, the cultures were counterstained with hematoxylin to reveal the overall cell population (bottom row images). (I) Immunoblot analyses of pSMAD1/5/8 levels in control micromass cultures and companion cultures treated with 25 ng/ml rhBMP2, 100 nM Palovarotene or both (indicated as B + P). Equal protein loading per lane was verified by immunoblot anlaysis of total SMAD1 and GAPDH (middle and lower rows). (J) qPCR quantification of expression levels of indicated cartilage marker genes in control and Palovarotene-treated micromass cultures on day 6.

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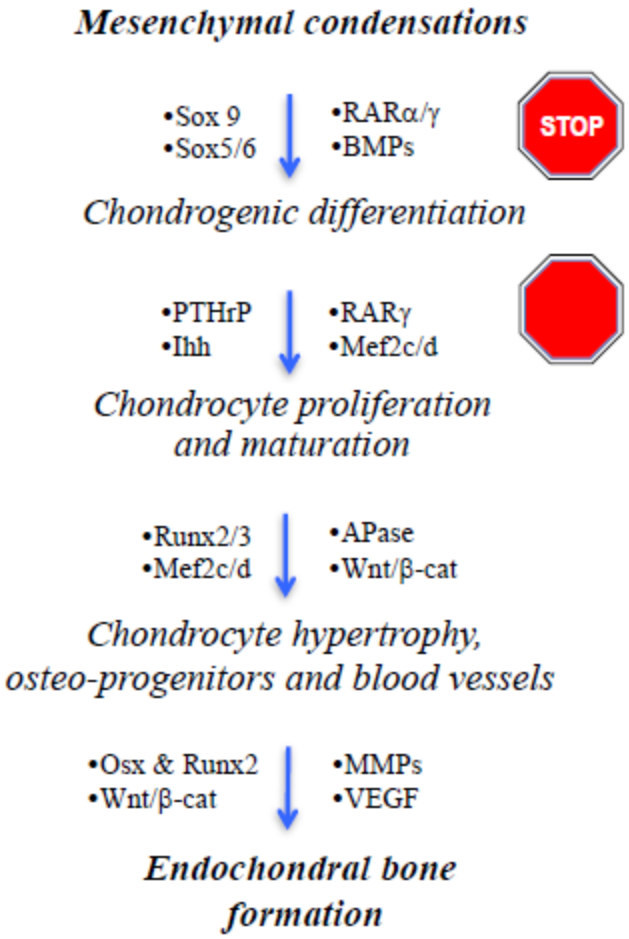

Figure 1

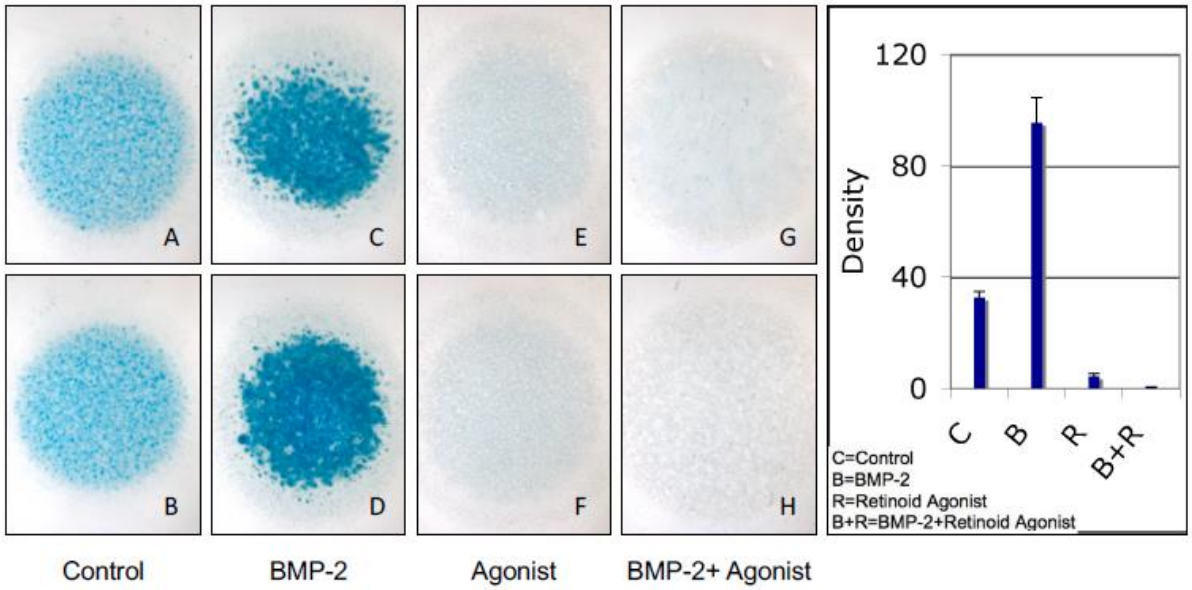

Figure 2

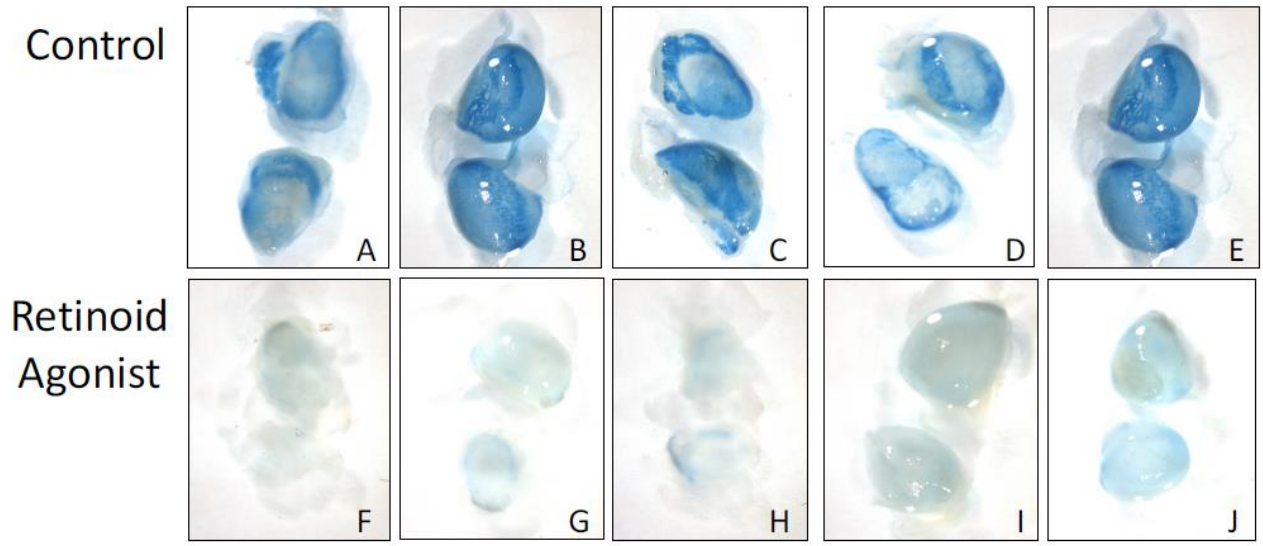

Figure 3

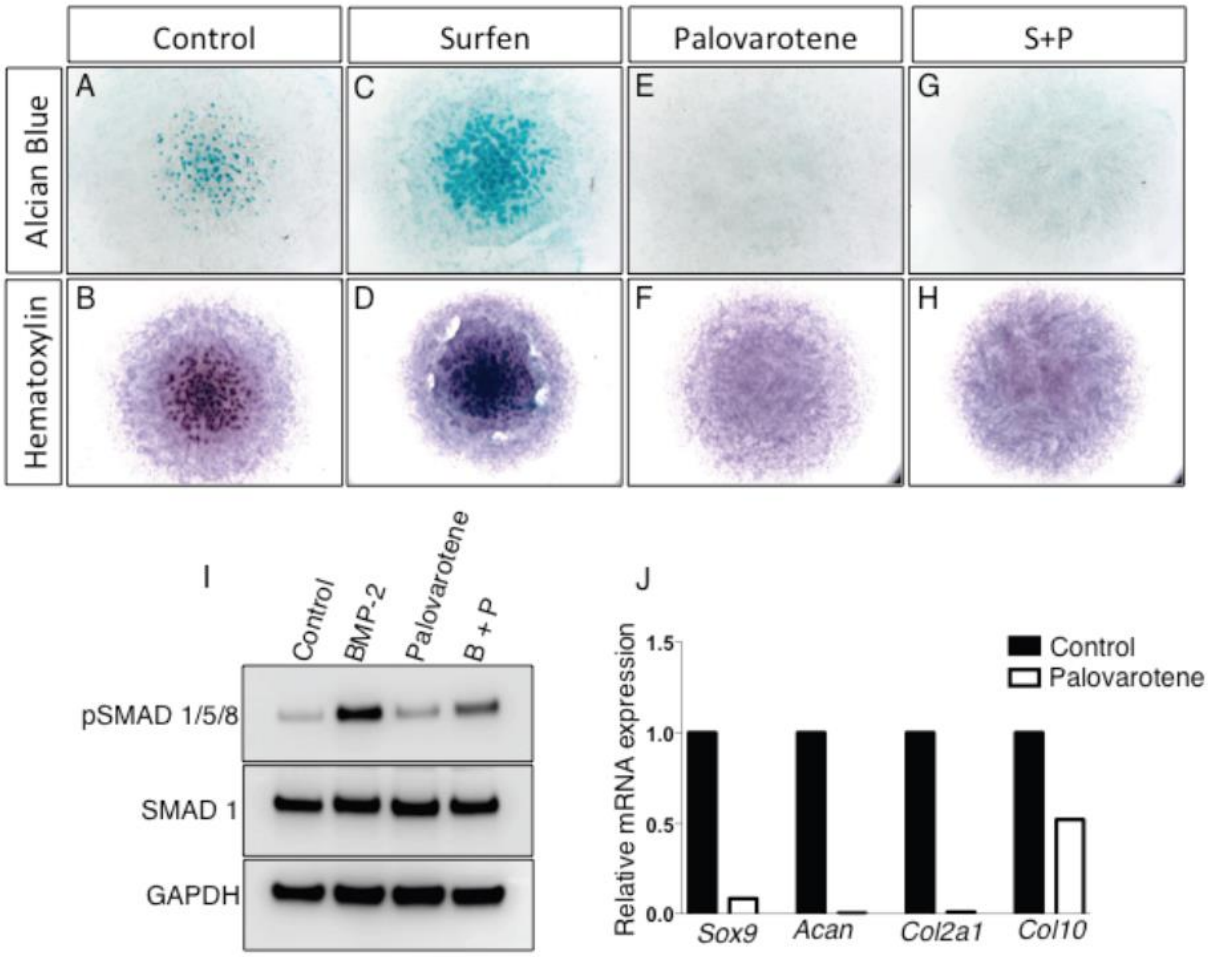

Figure 4