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Cellular and molecular characterization of the infiltrating polarized macrophages during the onset of heterotopic ossification in a mouse model of Fibrodysplasia Ossificans Progressiva (FOP)

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Genetic and Acquired Heterotopic Ossification: A Translational Tale of Mice and Men

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Abstract:

Heterotopic ossification is defined as an aberrant formation of bone in extraskeletal soft tissue, for which both genetic and acquired conditions are known. This pathologic process may occur in many different sites such as the skin, subcutaneous tissue, skeletal muscle and fibrous tissue adjacent to joints, ligaments, walls of blood vessels, mesentery and other. The clinical spectrum of this disorder is wide: lesions may range from small foci of ossification to massive deposits of bone throughout the body, typical of the progressive genetically determined conditions such as fibrodysplasia ossificans progressiva, to mention one of the most severe and disabling forms. The ectopic bone formation may be regarded as a failed tissue repair process in response to a variety of triggers and evolving towards bone formation through a multistage differentiation program, with several steps common to different clinical presentations and distinctive features. In this review, we aim at providing a comprehensive view of the genetic and acquired heterotopic ossification disorders by detailing the clinical and molecular features underlying the different human conditions in comparison with the corresponding, currently available mouse models.

Keywords:

heterotopic ossification, mouse models, fibrodysplasia ossificans progressiva, FOP, ACVR1/Alk2, BMP signalling, Activin A, GNAS1, POH, SARS-CoV-2

1. Introduction

Heterotopic ossification (HO) is a pathological process leading to the neoformation of mature bone in extraskeletal, soft tissues.

In physiological conditions, bone formation occurs during development through two main pathways: endochondral ossification, in which a cartilage intermediate is progressively replaced by osteogenic cells; and intramembranous ossification, where osteogenic cells differentiate from condensed mesenchymal cells [1,2].

Development of heterotopic bone shares striking molecular and histological features with normal bone development and can be distinguished from ectopic tissue calcification, a pathological event in which different processes lead to the deposition of insoluble calcium salts of variable composition outside bone and teeth, with the involvement of different soft tissues and organs (skin, kidney, lungs, vessels, etc.). Ectopic calcification may be observed in aging, or secondary to different pathological conditions in the presence of normal calcium and phosphorous values in the plasma (dystrophic calcification). Alternatively, it may be the result of conditions with altered plasma ion levels with precipitation of the exceeding salts (metastatic calcification) [3,4].

Usually, HO is not associated with any metabolic conditions and occurs in different, broad clinical settings.

The most common forms of HO are acquired and can occur as a complication of surgery (e.g., arthroplasty), fracture repair, in response to muscle and soft tissue trauma, severe burns, traumatic injury of brain and spinal cord. Moreover, post-traumatic HO is a common complication in combat injuries which severely prevents patients from recovering or adapting to prosthesis [5]. Genetic forms of HO are rare and include the fibrodysplasia ossificans progressiva (FOP), progressive osseous heteroplasia (POH) and other GNAS1-related conditions.

The clinical spectrum of this condition is wide, lesions range from small foci of ossification to massive deposits of bone throughout the body leading to cumulative ankylosis and severe and progressive impairment of movement [6]. This latter course is more typical of the genetic forms of HO, although also acquired lesions may be clinically relevant and relapses at the site of the lesion may occur in response to interventions aimed at removing the bone neoformation.

Regardless of the etiology, it is hypothesized that in the pathogenesis of HO the main factors that play a role are: (a) a genetic susceptibility (mendelian condition or predisposing genetic and multifactorial background); (b) an inciting event, such as different types of trauma able to function as a trigger, although not always recognizable; (c) the generation of a conductive environment at the site of injury through a complex crosstalk between the cells of the damaged tissue, inflammation and progenitor cells, that may be of multiple origin, able to differentiate into bone after receiving the appropriate signal at an available receptor.

This work provides an overview of the main forms of HO, from acquired forms to severe genetic conditions, among which fibrodysplasia ossificans progressiva, with a focus on the available mouse models mirroring the human counterparts (Table 1).

We have subdivided the human conditions into acquired and genetic forms with a description of the corresponding available mouse models. Nevertheless, this classification may be considered subjective to a certain extent, since it has become evident that injury and inflammation can accelerate and trigger HO in FOP patients as well as in animal models of genetic HO, and the high variability in predisposition of different individuals to acquired HO suggests a genetic basis for individual susceptibility.

2. Acquired Heterotopic Ossification

2.1. Post-Traumatic Heterotopic Ossification

The formation of new bone through an endochondral process is an uncommon event in the postnatal life initiated by different triggers such as fractures, traumatic events, acute trauma, combat related injuries and severe burns [7,8,78,79]. Moreover, HO is a frequent complication of orthopedic surgeries, involving hip and elbow [5,9,10,11].

The frequency of this secondary event is variable and seems to be strongly correlated with the site of trauma, the severity of the insult and also with the patient's age [8,80].

An effective therapeutic approach to prevent and treat ectopic bone formation is crucial for a positive resolution of interventions and quality of patients' life but is still not available. Up to now, the three possible therapeutic options consist of surgical excision (although relapses maybe common), treatment with anti-inflammatories and radiation therapy [7,12] with frequent limitations related to the accessibility of the involved areas.

The study of different types of nonhereditary ectopic bone specimens has revealed that osteogenic differentiation in damaged tissues is the result of lymphocytic recruitment and migration, followed by fibroproliferation and vascularization that leads to mature bone formation through a cartilage intermediate [13]. The involvement of the innate immune system is certainly crucial although the exact role and balancing of the different components still need to be better clarified.

The understanding of the HO core process including the strong relationship between the inflammation, pro-osteogenic stimuli and precursor cells requires the elements and pathways that contribute to the activation of the endochondral differentiation leading to ectopic bone formation to be defined [25].

Mouse Models of Post-Traumatic Heterotopic Ossification HO

The etiology of acquired HO still remains unclear as there are many factors contributing to its development, including inflammation, hypercalcemia, hypoxia and immobilization [81]. Since little is known about the underlying causes and the pathophysiological mechanisms of acquired HO, it has become difficult to develop new mechanism-based animal models. Some uncertainties also remain on their accuracy in reproducing human features of HO [14]. Anyway, nowadays there are many animal models able to reproduce at least some of the features of typical HO.

Bone morphogenetic protein (BMP) injection/implantation models. BMPs are signalling molecules belonging to the family of the transforming growth factor β (TGF-β) involved in the homeostasis and differentiation of a wide range of tissues, including cartilage and bone [15,82]. In the context of HO, several studies have been performed to better understand the role of BMPs in the induction of osteogenesis.

Some BMPs, such as BMP2 and BMP4, are able to induce potent inflammatory reactions and their injection, with or without additional injury, is followed by a robust endochondral ossification process [16,83]. In particular, a BMP2 injection, together with mild cardiotoxin-mediated muscle injury, elicits infiltration of CD11b macrophages and endochondral ossification in less than 10 days [17,84].

In contrast, other BMPs, such as BMP9, are able to induce a weak inflammatory response and require other inflammatory stimuli to trigger heterotopic ossification [85]. To ameliorate the efficacy of the induction protocol, new strategies were developed for the delivery of BMPs in the muscles, including the implantation of BMP-loaded biomaterials, such as matrigels and sponges [86]. These strategies allow a slow, but constant, release of BMPs avoiding an impairment of muscular function at the same time.

Another interesting approach comes from microporous calcium phosphate ceramic particles. These molecules do not release BMPs, but their implantation is still able to induce HO since these biomaterials improve the adhesion, proliferation and differentiation of cells, showing an improved osteoinductive ability [87]. However, the biological mechanisms underlying this process are not entirely understood, even if some theories assume that their physicochemical and structural characteristics may play a key role in this context.

Still, there are some debates concerning the nature of such models, since large amounts of BMPs are suddenly introduced into the muscle, giving rise to a nonphysiological HO development [14]. However, implantation models are frequently used since they can reproduce in a reasonable way the features of human HO, both from a molecular and a histological point of view. Furthermore, these models allow a local activation of the BMP signalling in the tissue, thus avoiding systemic effects. BMP4 overexpression has been observed in lesions of FOP patients suggesting that implantation models could also be appealing for the study of other forms of HO including the genetic forms [16].

These implantation models have been therefore particularly useful in investigating the process of ectopic ossification, which cells are involved in the onset and progression of HO [18,61,84] and are also amongst the most straightforward in vivo models to test new pharmacological approaches to inhibit HO [17,88].

Achilles tenotomy model. The ossification of the Achilles tendon is a rare event that can occur as a consequence of trauma or surgery. [89,90,91]. This process was investigated in animal models in which it was possible to induce HO following a trauma, such as tendon squeezing or dissection [92,93]. Some works have demonstrated that one of the key events underlying ectopic bone formation in the Achilles tenotomy models is hypoxia. A low oxygen tension environment is translated into an increase of hypoxia inducible factor (HIF)-1α expression and in turn, HIF-1α enhances angiogenesis, chondrogenesis and finally osteogenesis [94]. Nowadays, Achilles tenotomy models are sometimes used due to their straightforwardness and reproducibility [81] even though they are still of doubtful relevance for humans, since ectopic bone formation in the Achilles tendon is a rare event, often associated with other pathologic conditions such as rheumatoid arthritis and ankylosing spondylitis [93].

Burn/tenotomy model. A further evolution of the tenotomy model is represented by the burn/tenotomy model. In humans, burn injuries are traumatic events commonly causing HO, even though it is hard to predict where the ossification will occur [95]. The burn/tenotomy model involves the combination of tendon dissection and burn injury on the dorsal skin, which is required in order to elicit a systemic inflammatory state [19]. This mouse model develops HO in the areas that received the dissection with high frequency, such as the calcaneus, ankle joint, and tibia/fibula of the limb. As observed for the Achilles tenotomy model, mice receiving the burn/tenotomy treatment show an increase in HIF-1α expression, which plays a pivotal role in the development of traumatic HO [96]. Here as well HIF-1α supports angiogenesis, by upregulating VEGFA in the injured area, creating a proper microenvironment for subsequent endochondral ossification [97]. This model presents some advantages: in fact, by combining tenotomy and burn injury, the ossification sites are more predictable and HO develops in an accelerated way. In addition, HO induction does not require the administration of exogenous molecules [19,96,97,98]. Nevertheless, both BMP and TGF-β signalling pathways are upregulated after the burn/trauma, but with a difference to the genetic FOP model (see below), activin A does not appear to play a role in the ectopic ossification [99].

The burn/tenotomy model has been widely used to investigate which cells are involved in the onset and progression of HO after trauma, highlighting the critical role of several types of circulating mesenchymal cells and cells of the innate immune system [20,21,100,101].

Michelsson's model. This model is also known as the "immobilization−manipulation model" and was first ideated by Michelsson who was able to induce HO in rabbit quadriceps by repeated and intense immobilization of the knee joint, which could be similarly reproduced in other joints [22]. This model turns out to be particularly useful to understand the role of inflammation in HO, in particular an increase in the level of prostaglandins has been observed before proper bone formation [23]. Moreover, it has been shown that the formation of HO can be prevented by separating the femur and the quadriceps with the insertion of a plastic membrane. The interaction between bone and muscle appears to be pivotal for the onset of HO.

However, this model has received some criticism for what concerns the development of HO, since it is unclear whether the newly formed bone could be considered as ectopic bone or as a dystrophic calcification [24]. Furthermore, although Michelsson's model paved the way for the study of HO in rabbits, its relevance in the context of other mammals, like mice, remains to be investigated.

2.2. Neurogenic Heterotopic Ossification

Neurogenic heterotopic ossification (NHO), affecting soft/extraskeletal tissue surrounding hip, shoulder and elbow joints, is a spontaneous consequence of injuries involving the central nervous system.

The primary damage leading to HO can directly involve traumatic brain injury (TBI) and spinal cord injury (SCI) but can also be a consequence of isolated nontraumatic neurological events such as stroke and cerebral anoxia [26,27,28].

The etiology and the severity of the primary neurological damage, the patient's post trauma management, coma, ventilation support, autonomic dysregulation, spasticity and the gap between trauma and rehabilitation are all factors that can strongly contribute to the risk of ectopic bone formation and influence the HO locations and volumes [26,27].

The pathogenic mechanism of NHO is not yet well understood. The study of human lesions reveals some important points to be further investigated, such as the contribution of neuro-inflammation signals (e.g., substance P, calcitonin gene-related protein, CGRP, etc.) deriving from the damaged peripheral or central nervous system and the responsiveness of different resident precursor cells that activate the wrong repair process [25,29,30,31].

Very recently, Meyer and colleagues described four patients with a severe form of SARS-CoV-2 infection (COVID-19) developing HO of the hips and shoulders. All the patients required intensive care, with mechanical ventilation and a prolonged immobilization period [32]. The relationship between HO and COVID-19 is not clear. However, SARS-CoV-2 infection induces a potent systemic inflammation state, triggers macrophage activity and the production of inflammatory cytokines at tissue level, defined as a "cytokine storm" [102]. Mechanical ventilation may affect acid-base homeostasis thus inducing hypoxia. Moreover, severe infection spreads to the central and peripheral nervous system with high risk of encephalitis, stroke, and severe neuro-muscular illness [33].

These events, together with the prolonged immobilization of the patients, are all critical factors able to drive HO formation in COVID-19 patients. As commented by Meyer and coll., occurrence of this complication may be currently underestimated in severely affected patients and might further impact their rehabilitation.

Mouse Models of NHO

Spinal cord injury (SCI) mouse model. NHO is a frequent event and occurs in about 20 to 30% of patients following spinal cord injury [34]. From here, the necessity arose of developing a mouse model aimed at better understanding the features of ossification after SCI. In these models, this form of trauma is usually simulated by either a laminectomy of the dorsal spine, followed by a transection of the spinal cord and muscle injury by cardiotoxin injection [35] or by injury induction with a weight drop followed by the injection of a small dose of BMP2 [34]. Both models reproduce what is observed in patients with SCI that develop NHO, since ossification forms rapidly. Moreover, mice with SCI-induced NHO mirror the development of ectopic bone from the histological

point of view, presenting a formation of lamellar bone with large amounts of osteoblasts, osteoclasts and osteocytes. However, since the procedure induces paraplegia of the mice, special care is needed to ensure the survival of the experimental animals, therefore this model may have a more limited use for large scale study.

The role of inflammation has been investigated also in NHO development. In particular, resident macrophages have been shown to produce several factors critical to the maturation and maintenance of newly formed bone, such as BMPs and Oncostatin M [31,35]. Therefore, macrophages may be another therapeutic target for the treatment of NHO.

Traumatic brain injury (TBI) model. NHO has been investigated in rats, with an attempt to create a model that could allow heterotopic ossification to be studied in association with TBI, coupled with other forms of peripheral injuries commonly observed in patients. In particular, these injuries consist of femoral fracture and muscle injury. It has been observed that after 6 weeks, 70% of the rats that received both forms of injuries, together with TBI induction, showed ectopic bone in the injured hindlimb [36]. Interestingly, only 20% of rats receiving both femoral fracture and muscle injury without TBI induction presented ectopic bone. For what concerned the ossification, joints showed the presence of ectopic bone as observed in human NHO patients; furthermore, it appeared to be more severe in rats in which TBI was combined with the other two forms of injuries compared to rats in which TBI was not induced [36]. Curiously, TBI has been proven to have a negative effect on bone healing in a rat model, while callus formation was exacerbated, probably as a consequence of the activation of different metabolic and inflammatory pathways [37].

Anyway, there are still some limitations concerning this model of NHO because of the lack of an assessment of the contribution of each individual injury to the development of ectopic bone formation, and of the clues concerning the histological analysis of the newly formed ectopic bone in tissues. Further studies may allow better clarification of these points.

3. Genetic forms of Heterotopic Ossification

Heterotopic ossification may also represent the most relevant clinical feature of three genetic diseases, fibrodysplasia ossificans progressiva (FOP), progressive osseous heteroplasia (POH) and Albright hereditary osteodystrophy (AHO). These are all rare conditions, inherited as autosomal dominant traits and characterized by the occurrence of bone neoformation in extraskeletal tissues. Nevertheless, these diseases significantly differ in the underlying genetic causes and pathways involved, clinical presentation and course, and in the differentiation process leading to the ectopic bone formation.

3.1. Fibrodysplasia Ossificans Progressiva (FOP)

Fibrodysplasia ossificans progressiva (FOP, OMIM135100) is a rare genetic disorder with an estimated average prevalence of 1–1.5/2,000,000 and one of the most severe conditions of HO.

The typical clinical presentation of FOP is characterized by the presence of a peculiar congenital malformation of the great toes that could be considered the first clinical sign of the disease, although other congenital anomalies (malformation of the thumbs, fusion of cervical vertebrae, digit reduction defects, etc.) and clinical signs (presence of tibial osteochondromas) may be present with variable expression and frequency [38]. HO of soft tissues, such as skeletal muscles, tendons, ligaments and joints starts in childhood and progresses throughout the life evolving to entrap patients in a second skeleton.

HO usually occurs with an episodic course consisting of acute phases called flare-ups alternating with quiescent phases of the disease activity. The study of the natural history of FOP reveals that flare-ups are preceded in more than 80% of the analyzed patients by symptoms like swelling, pain, or decreased mobility [38,39]. However, FOP progression can be extremely variable and unpredictable, not all the flare-ups may result in ectopic bone formation. On the other hand, HO may progress also with a creeping course, in the absence of a clinically relevant acute phase [38,39]. HO may be initiated or exacerbated by several factors such as trauma, vaccinations, surgical or medical interventions, infections, or may initiate without a recognizable trigger [38,39]. As such, early diagnosis of FOP is mandatory to prevent behaviors or procedures that might be harmful for the patient.

All these observations have suggested the importance of inflammation and immune response in the etiology of the disease. This is further supported by the histological studies performed on human specimens of biopsies obtained from patients before the diagnosis of FOP. In early lesions, the degeneration of the damaged tissue is evident and elicits a strong inflammatory response with tissue infiltration by different types of immune cells (monocytes, macrophages, lymphocytes, mast cells) [40,41]. Then, after a fibroproliferative phase, ectopic bone forms through a classical endochondral ossification process. This latter is further sustained by the markedly hypoxic microenvironment, generated by inflammation in the early FOP lesions, which enhances the BMP signalling and promotes HO formation [42]. The heterotopic bone has the features of a mature trabecular bone with marrow elements, with the same mechanical, physical and metabolic properties of the orthotopic bone.

The genetic cause of FOP is a gain-of-function mutation of the ACVR1/Alk2 gene. The gene encodes a type I receptor for bone morphogenetic proteins (BMPs) [43], a wide group of secreted factors belonging to the TGF-β family of proteins. ACVR1/Alk-2 forms functional complexes at the cell membrane with type II receptors able to bind BMP ligands, thus activating both Smad-dependent and independent intracellular signalling pathways involved in osteogenesis and bone homeostasis [103].

The R206H is the most commonly recurrent mutation in FOP, affecting a highly conserved residue within the GS domain of the protein [38,43], whereas rare cases may be associated with different variants affecting the same functional region of the receptor or the kinase domain [71,72,73,74,75].

The mutation causes constitutive activation of the receptor which becomes hypersensitive to BMPs and, most importantly acquires a new, disease-specific feature by perceiving Activin A (ActA) as an agonist [49,50]. Activin A belongs to the same family of BMP ligands. However, usually it does not show osteogenic properties and although able to bind wild-type ACVR1/Alk2, in normal conditions this represents a non-transducing/inhibitory complex [49,50]. In contrast, binding of ActA to the mutated receptor carrying FOP-associated variants triggers the downstream Smad1/5/9 signalling [49,50,51], enhances the endochondral ossification of primary connective tissue progenitor cells of FOP patients [52], thus promoting HO formation.

Noteworthy, different types of immune cells (macrophages, dendritic cells, T and B lymphocytes, natural killer cells) are able to secrete and to respond to ActA, with a broad range of different modulatory actions on the inflammation process [53].

The different mouse models of FOP currently available are providing crucial insight into the role of ActA and the signalling pathways involved, the origin and nature of the different progenitor cells that contribute to the ossifying lesions, the role of inflammation and the importance of the microenvironment (hypoxia, etc.), and provide the basis to preclinical studies to develop targeted therapies.

Mouse Models of FOP

With the aim to reproduce the condition of the dysregulated BMP signalling occurring in FOP, several strategies have been adopted in mice.

BMP ligand overexpression mouse models. The first genetic strategy of mimicking FOP in vivo was to overexpress the BMP proteins involved in HO. BMP4 was highlighted as a key factor in FOP pathogenesis, therefore dysregulation of its expression was investigated in the FOP pathogenic context. The development of a model of BMP4 overexpression required the identification of a proper promoter that could drive its expression efficiently. Several promoters were investigated, but most of them were not able to induce postnatal HO or led to the onset of developmental abnormalities [104,105]. The only promoter that could induce the overexpression of BMP4, thus leading to proper HO formation was the neuron specific enolase (Nse) promoter [54].

Before the development of the ACVR1/Alk2 mutated transgenic mice (see below), the Nse-BMP4 transgenic mouse has been the most used model for studying BMP overexpression in FOP. Nse-BMP4 mice mirror in a fair way the progressive formation of heterotopic bone seen in FOP patients and, as in humans, some sites like the diaphragm, tongue and extraocular muscles are spared from HO development. However, no malformations in the great toe and in the joints were observed, which are typical of FOP [54]. This model has been used for understanding which cell types can differentiate in the osteogenic lineage and for studying the events that trigger HO [54].

Interestingly the progeny deriving from the mating of Nse-BMP4 mice with mice overexpressing Noggin, an inhibitor of BMP4, do not develop FOP. Moreover, local

injection of Noggin in a mouse model of BMP4-induced HO rescues the animals from developing heterotopic ossification in the site of injection, showing that the use of BMP inhibitors may be effective for the treatment of HO-related diseases [106].

Hyperactive ACVR1/Alk2 models. One of the first mouse models used to study FOP was actually generated to investigate the role of ACVR1/Alk2 during development. This model was obtained by the expression of a Cre-inducible transgene consisting of the human ACVR1/Alk2 cDNA carrying the engineered Q207D substitution (also known as constitutively active Alk2, caAlk2) [107].

This mutation causes the substitution of a glutamine with a negatively charged residue, namely aspartic acid, in the GS domain of the receptor, leading to constitutive activation of the downstream Smad-dependent cascade [107].

After the discovery of ACVR1/Alk2 as the causative gene in FOP, this mouse was considered useful to model the disease phenotype, since intramuscular expression of the caAlk2 transgene was able to induce ectopic endochondral bone formation with joint fusion and functional impairment [55].

In this model, global postnatal expression of ACVR1/Alk2Q207D obtained by mating the mice with ubiquitously expressed inducible Cre (CAGGCreERT) did not develop HO. HO was observed when ACVR1Q207D mice were injected at specific sites with adenoviral vectors containing the Cre recombinase. Curiously, when mice with the global activation of the mutation were injected with control adenovirus, HO was developed as well [55].

These results led to the hypothesis that HO formation was dependent on the presence of both the ACVR1Q207D and an inflammatory trigger/environment [55,108].

The engineered ACVR1Q207D mutation has never been described in humans in association with FOP. However, the substitution of the same residue by a glutamic acid, Q207E, has been reported in rare cases of FOP [38]). Although Q207D and Q207E may look similar since they both introduce a negatively charged residue, these mutations have different impacts on the receptor function. In fact, ACVR1Q207D was shown to be constitutively activated by an irreversible loss of inhibitory GS domain conformation occurring upon the first phosphorylation event, which is not observed in ACVR1Q207E and ACVR1R206H [56]. Although ACVR1Q207D shows some functional features

different from the naturally occurring ACVR1Q207E and ACVR1R206H mutants, the ACVR1Q207D mouse model presents a robust, BMP-signalling dependent HO formation and is extensively used in the preclinical development of inhibitory compounds and drugs [55,57].

Acvr1R206H mouse models. As soon as the FOP mutations were identified, great effort was put in place to produce a more disease-relevant animal model. As mentioned above, the great majority of FOP patients carry the same R206H mutation. The first models were obtained by introducing the Acvr1R206H mutated gene in the murine endogenous locus [58]. Even though the endogenous mutation led to the development of classic FOP features, like digit malformation, joint fusion and other skeletal anomalies, most of the progeny encountered problems of perinatal lethality. This allowed only the chimeric mice with estimated 70% to 90% mutated cells to be studied, but still the problem concerning perinatal lethality limited severely the applicability of this model [58]. For this reason, new strategies had to be explored.

In order to overcome the perinatal lethality, a model of conditional knock-in mutation was developed Acvr1[R206H]/FlEx [50]. When Acvr1R206H expression is induced postnatally upon tamoxifen-inducible Cre-mediated recombinase, HO is triggered and develops between 2 and 4 weeks, apparently without the need of additional injury. This model provided some important insights concerning the molecular mechanisms of FOP and made possible the investigation of the aberrant role of ActA in FOP. As previously stated, ActA has an inhibiting activity towards ACVR1/Alk2 in a wild-type background, but in patients and mice presenting the R206H mutation, ActA ends up being a powerful activator of the receptor, thus inducing HO [50]. The same floxed transgenic line has been used to develop another Acvr1R206H mouse model [59]. In this work upon doxycycline-induced Cre recombination and cardiotoxin-mediated muscle injury, complete HO developed in 2 weeks. Using this model, the immune system has been shown to play a pivotal role in FOP heterotopic bone formation. Cell types such as macrophages, mast cells and neutrophils have been observed in FOP lesions of Acvr1R206H mice after injury [59]. Moreover, these cells persisted at high levels during bone formation, instead of returning to preinjury levels, and increased production and persistence of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1b, further strengthen the

hypothesis of a sustained proinflammatory environment in FOP lesions of Acvr1R206H mice [59]. Depletion of mast cells and/or macrophages has been proven to reduce HO in Acvr1R206H mice, indicating that these cells may be candidate targets for pharmacological treatments in FOP [59]. However, a better clarification concerning the cell composition of the inflammatory infiltrate in FOP lesions is still needed.

This model, as well as a different Acvr1R206H-knock-in floxed strain, in which expression of Acvr1R206H is Cre-dependent and under the control of the endogenous Acvr1 locus (Acvr1tnR206H), that has been generated independently [60], have been used to characterize the cells that can contribute to the endochondral ossification, in particular fibroadipogenic precursors (FAP) [60,109].

3.2. Progressive Osseous Heteroplasia (POH) and GNAS1 Related Conditions

Progressive osseous heteroplasia (POH, OMIM 166350) is an ultrarare genetic disease that begins in early childhood with widespread heterotopic ossifications at dermal and subcutaneous fat level, and progresses with the involvement of subcutaneous and deep connective tissues [61,62,63]. The disease is mainly sporadic but recurrence with an autosomal dominant inheritance has been also reported [64]. The genetic causes of the disease are loss of function mutations of the Gs-α isoform of the of the GNAS1 gene, in the inherited paternal allele [63,65,66,67].

The early manifestation of the disease is a maculopapular rash caused by patchy areas of bone within the dermis, present at birth or appearing some weeks later. Then, HO progresses from the skin and subcutaneous fat to deep connective tissues (subcutaneous fat, muscles, tendons, ligaments, fascia) by severely impairing joint mobility and limb growth. HO associated with POH is not triggered by trauma, infections, nor associated with metabolic abnormalities, and develops through an intramembranous differentiation process [61,63,65,68].

The GNAS1 locus is characterized by a complex epigenetic regulation with the synthesis of different transcripts with mono and biallelic expression. As such, besides POH both constitutive and somatic mutations in the GNAS1 gene with the differential involvement of the maternal or paternal alleles, result in a broad spectrum of phenotypes that may include HO, and a variety of clinical signs such ad skeletal malformations, hormone

alterations and obesity. This group of diseases are Albright hereditary osteodystrophy, (AHO) pseudopseudohypoparathyroidism (PPHP) and different types of pseudohypoparathyroidism (PHP) [70,71].

In the context of heterotopic ossification, patients affected by AHO share a constellation of clinical manifestations including short stature, brachydactyly, obesity and ossifications limited to the subcutaneous layer (subcutaneous ossification, SCO) that could be considered the peculiar characteristics of this disorder. SCO occurs spontaneously or secondary to trauma, can cause pain and affect daily life quality and surgical removal does not guarantee a definitive resolution [63,72,73].

GNAS1 encodes the stimulatory alpha subunit (Gαs) of the G protein complex. This latter transduces extracellular signals received by transmembrane receptors called G protein-coupled receptors (GPCRs) to cellular mediator by stimulating the activity of the hormone-sensitive adenylyl cyclase. Each G protein is a heterotrimer composed of an α, β, and γ subunit. Gαs-mediated signalling interacts with the Wnt and Hedgehog pathways, both crucial regulators of skeletal development, remodeling and injury repair [110].

Moreover, GNAS1 has a crucial role in skeletal development and homeostasis by regulating different processes of skeletal cell maturation. In 2011, Pignolo et al. observed that the altered GNAS1 expression promoted the osteoblast differentiation by unbalancing the differentiation of the multipotent connective tissue progenitor cells towards osteogenesis at the expense of adipogenesis [111]. Furthermore, the central role of Gαs has been demonstrated in the correct formation of skeleton bone by inhibiting/limiting Hedgehog (Hh) signalling in mesenchymal progenitor cells. Loss of function mutations in the GNAS1 gene leads to the upregulation of Hh that is considered sufficient to induce HO in GNAS1-related conditions [110,112,113].

Mouse Models of POH and AHO

Mouse models of POH. The rCre-Gsα mouse model is a transgenic murine model expressing the Cre recombinase under the control of human renin (hRen) promoter, which can excise the GNAS1 gene when flanked by loxP sites [69]. Unexpectedly, this kind of mutation had no major effects on the renin-angiotensin system and the urinary concentrating ability of rCre-Gsα mice was preserved [69]. Interestingly, mutated mice

show marked abnormalities in the spleen due to fibrous connective tissue deposition, which are not found in human POH patients.

On the other hand, this model reproduces some of the common features of human POH, in particular soft tissue mineralization and ossification, which may also extend to subdermal connective tissues [69]. Furthermore, ossification has been found also in the skeletal muscles adjacent to the long bones of the forelimb, which is another common site of ossification observed in human patients [62]. Surprisingly, the rCre-Gsα mouse model reproduces well most of the common features observed in human POH patients, showing that Gsα has a fundamental role in mineralization and bone development. Still, deeper studies concerning spleen fibrosis observed in this model may be needed, in order to prevent undesired effects in mice.

Mouse models of AHO. Targeting the GNAS1 gene has been the most direct strategy to mimic AHO in mice models. The first genetic approach was performed by targeting the exon 2 of the GNAS1 gene, whose homozygous deletion is associated with postnatal lethality [74]. Different phenotypes were observed in these mice depending on the maternal or paternal origin of the allele. The animals with the maternal inherited mutation presented resistance to PTH, were obese and hypometabolic, whereas the paternal origin of the mutation was translated into lean and hypermetabolic mice [75]. The deletion of the exon 2 in the chondrocyte lineage, gave rise to ectopic cartilage formation in the growth plate area of the tibia, showing that GNAS1 is a negative regulator of chondrocyte differentiation [114]. Still, no traces of ectopic bone were observed in these models. In this regard, targeting the exon 1 of the GNAS1 gene turned out to be a more successful strategy [76]. In this murine model was observed the presence of subcutaneous ossification by 12 months of life, a typical feature of AHO affected patients. Furthermore, no differences concerning the maternal or paternal origin of the allele were observed, both in terms of ossification frequency and histological appearance [76]. On the other hand, male mice had more severe and widespread ossification in the subcutaneous tissues, indicating that probably androgens may accelerate the ossification process. Other studies also showed that the deletion of the exon 1 of the GNAS1 gene was related to a decrease in sensitivity to PTH and TSH, with increased circulating levels of these hormones, with more severe phenotypes associated to the maternal origin of the mutation [77].

4. Conclusions

Heterotopic ossification represents a pathological process that may occur in a broad spectrum of clinical presentation, as an isolated/acquired sign or as a feature of a genetic condition, from small and self-limiting lesions to progressive forms that cause severe disability.

In this work, we have summarized the different presentations of HO in humans, with attention to both acquired and genetic forms such as FOP. Most importantly, we have provided a systematic comparison between the human condition and the corresponding animal model (Table 1), highlighting the adherence and differences with the human counterpart thus underlining the strengths and the critical points of each.

The availability of a condition-relevant animal model is of critical importance: to clarify in detail the molecular and cellular mechanisms featuring the progression of the disease and to provide preclinical evaluation of promising therapeutic agents.

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S.C. and R.G. wrote and revised the manuscript, the two authors contributed equally to this work; R.B. and S.B. conceived and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare that there is no conflict of interest.

HO Classification	Human Condition		Mouse Models of HO	
Acquired HO	**Inciting Event/Condition**	**Features**	**Inciting Event**	**Features**
Post-traumatic HO	• Fracture • Trauma • Combat related injuries • Severe burns • Arthroplasty/Surgery [5,7,8,9,10,11,12,13]	• Correlation with the site of trauma and the severity of the injury • Strong inflammatory response	BMPs injection /implantation models [14,15,16,17,18]	• Robust HO • No BMP-related systemic effects • HO may vary depending on the type of BMP applied • Applicable for genetic forms of HO • Nonphysiological HO development
			Achilles tenotomy model Burn/tenotomy model [19,20,21]	• HO in the injured hindlimbs • High inflammatory response • HIF-1α increased expression
			Michelsson's model [22,23,24]	• HO as a consequence of bone–muscle interaction • Inflammatory infiltrate increases prostaglandin levels • Unclear HO entity • Mostly used in rabbits
Neurogenic HO	• Spinal cord injury • Traumatic brain injury • Encephalitis • Stroke • Severe myopathy and neuropathy • Prolonged immobilization [25,26,27,28,29,30,31,32,33]	• Correlation with the severity of the injury • Post trauma management complications • COVID-19 related complications	Spinal cord injury (SCI) mouse model [31,34,35]	• Rapid neurogenic heterotopic ossification (NHO) • Well-reproduced NHO histology • NHO requires additional inflammatory stimuli • Paraplegic mice • Animal survival may be affected
			Traumatic brain injury (TBI) mouse model [36,37]	• NHO in joints and in the injured femurs • NHO severity increases with multiple injuries • No exogenous molecules
Genetic forms of HO	**Genetic cause**	**Features**	**Genetic background**	**Features**
FOP (OMIM 135100)	Gain of function mutations of *ACVR1/Alk2*: alteration of the BMP signalling and acquired responsivity to Activin A [38,39,40,41,42,43,44,45,46,47,48,49,50,51,52,53]	• Congenital malformation of the great toes • Variable association with other skeletal anomalies (thumb and digit malformation, fusion of cervical vertebrae, etc.) • Development of tibial osteochondromas • Endochondral HO • Spontaneous and trauma-induced HO • Flare-ups with preosseous swelling and inflammation • Involvement of skeletal muscles, tendons, aponeuroses, fascia • Progressive and severely disabling	BMP ligand overexpression mouse models (Nse-BMP4) [54]	• FOP-like phenotype • Progressive endochondral ossification • No great toes malformation • Inflammatory infiltrate in lesions
			$Alk2^{Q207D\text{-}floxed}$ ($caAlk2^{fl}$) mouse model [55,56,57]	• Postnatal and progressive HO after Cre induction • Different response to BMP inhibitors • Different ACVR1 conformation
			$Acvr1^{R206H/+}$ chimeric mouse model [58]	• Malformed first digits and hindlimbs • Postnatal and progressive HO • Limited and impaired mobility • Postnatal lethality • Only progeny with 70–90% mutated cells available for analysis
			$Acvr1^{[R206H]FlEx}$ knock-in mouse model [50,59] $Acvr1^{tmR206H}$ knock-in mouse model [60]	• Postnatal and progressive HO after Cre induction • Altered Activin A signalling • Inflammatory infiltrate • No great toes malformation • No postnatal lethality
POH (OMIM166350)	Loss of function mutation of *GNAS1* (paternal allele) [61,62,63,64,65,66,67,68]	• Maculopapular rash • HO of the skin and dermis mainly by intramembranous differentiation process • Progressive involvement of subcutaneous and deep connective tissues • Progressive may be severely disabling	rCre-Gsα mouse model [69]	• HO in soft dermal tissues • HO is invasive • Ossification in skeletal muscles adjacent to the long bones • Fibrotic spleen
AHO (OMIM103580 & 612463)	Loss of function mutation of *GNAS1* (mainly maternal transmitted) [63,70,71,72,73]	• Short stature • Brachydactyly • Obesity • Subcutaneous ossifications • Other skeletal anomalies • +/− endocrine abnormalities such as multihormone resistance *	$Gnas^{E2+/-}$ mouse model [74,75]	Maternal mutation • PTH resistance • Obesity • Hypometabolism Paternal mutation • Lean mice • Hypermetabolism • No HO formation
			$Gnas^{E1+/-}$ mouse model [75,76,77]	• Decreased sensitivity to PTH and TSH • Increased circulating PTH and TSH • Subcutaneous HO

*,"+, presence" and "− , absence".

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Chapter 2

Cellular and molecular characterization of the infiltrating polarized macrophages during the onset of heterotopic ossification in a mouse model of Fibrodysplasia Ossificans Progressiva (FOP).

Abstract

Fibrodysplasia Ossificans Progressiva (FOP) is a rare congenital disease that results in heterotopic ossification (HO) in skeletal muscles. It arises from a gain-of-function mutation (R206H) in the Acvr1 gene encoding for the activin type I receptor, which leads to the aberrant activation of the bone morphogenetic proteins and activin A signalling pathways. Patients experience episodic inflammatory flare-ups in skeletal muscles that trigger HO. Macrophages still have an unclear role in the tissues where HO occurs and need a better characterization.

To model FOP we used the Acvr1R206H$^{lox/lox}$;Gt(ROSA26)SorCreERT2 conditional transgenic mouse strain. Computerized tomography (CT) revealed that tamoxifen induced FOP mice develop ectopic bone after receiving muscle injury at 14 and 21 days.

To investigate how the innate immune system is involved in the onset and progression of HO, we depleted circulating monocytes by performing four intravenous injections of clodronate liposomes in FOP mice.

CT scans showed that ectopic bone formation in macrophage-depleted FOP mice was significantly lower compared to controls at 14 and 21 days after injury.

To get more insights on the early signalling leading to HO, single-cell RNA sequencing was performed on muscles of FOP mice 5 and 7 days after pinch injury.

Bioinformatic analysis revealed that fibro-adipogenic progenitors (FAPs) were enriched in pathways related to chondro/osteogenesis and hypoxia in FOP mice. Furthermore, FOP macrophages expressed higher levels of osteoclast differentiation markers and displayed an upregulated pro-inflammatory profile.

Overall, these data confirm that FOP mice can reliably reproduce the features observed in patients and that macrophages are crucial for HO. Finally, single-cell transcriptomics

indicates that macrophages and FAPs are committed to form a cellular niche that promotes and sustains bone formation already at early timepoints in FOP mice.

1. Introduction

1.1 Heterotopic ossification

Heterotopic ossification is a pathological process characterized by the formation of ectopic bone in soft tissues such as muscles, tendons, ligaments, and peri-articular areas. Physiologically, mature bone tissue can be formed via two major mechanisms during development: endochondral ossification and intramembranous ossification. In both processes, the progenitor cell is a mesenchymal precursor. What distinguishes them are the mechanism by which ossification occurs and the sites affected [1]. In the process of intramembranous ossification, mesenchymal cells directly differentiate into osteogenic cells [1]. The bones formed through this process are typically flat bones such as the skull, sternum, ribs, and shoulder blades [1]. On the contrary, in the process of endochondral ossification, bone formation occurs through a cartilaginous intermediate and mainly affects the long bones [1]. The process begins with a condensation of mesenchymal stem cells, which differentiate into chondrocytes, which are progressively replaced by osteoblasts and osteoclasts [1].

During heterotopic ossification, ectopic bone tissue can be formed through the process of endochondral ossification, intramembranous ossification, or both; the newly formed bone is histologically and molecularly indistinguishable from normal bone [2].

Traditionally, heterotopic ossification is divided into acquired and genetic forms. Acquired or post-traumatic forms are among the most common and often arise as complications following surgery, fractures, trauma and burns [2]. These also include neurogenic heterotopic ossifications, which occur as a result of spinal cord injury, head trauma, encephalitis and stroke [2].

Between 2020 and 2021, several cases of heterotopic ossification following SARS-CoV-2 (COVID-19) infections have been reported. Although the etiopathogenesis of this type of ossification remains unclear, a possible explanation could be given by the systemic inflammation caused by the infection and the prolonged immobilization to which patients are subjected [3].

In contrast to acquired ones, genetic forms of heterotopic ossification are rare and generally have a more severe course. These include progressive bone heteroplasia (POH), hereditary Albright osteodystrophy (AHO), and fibrodysplasia ossificans progressiva (FOP) [4]. The first two are caused by inactivating mutations in the GNAS1 gene while FOP is caused by activating mutations in the Acvr1 [4] gene. All of these diseases are caused by mutations in a single gene; this implies that these genes play a role in important regulatory mechanisms in cell fate selection and bone formation [5].

1.2 Fibrodisplasia Ossificans Progressiva (FOP)

Fibrodysplasia ossificans progressiva (MIM 135100), one of the most severe and disabling forms of heterotopic ossification known to date, is a rare genetic disorder characterized by congenital skeletal malformations and the progressive development of endochondral ectopic bone in soft tissues such as muscles, tendons, and ligaments [6,7]. The estimated average prevalence is about one case in two million individuals, without any predisposition of gender, geographical origin or ethnicity [5].

Two main clinical features define the classic phenotype of FOP: congenital malformation of the big toes and progressive heterotopic ossification [8]. The latter follows a specific anatomical pattern: typically, ossification begins first in the dorsal, axial, cranial and proximal regions of the body and only later expands into the ventral region, appendicular, caudal and distal regions [8]. Other common features often present in patients with a classic phenotype include malformations in the cervical vertebrae, osteochondromas, thumb malformations, and ear canal changes, probably due to ossification of the middle ear [6]. A small percentage of affected patients show varying clinical characteristics compared to the classic phenotype [9]. Often these patients have a different degree of severity in big toe malformations while others show alterations in normal skeletal development such as the total absence of big toes and/or thumbs [9]. On the other hand, another small percentage of patients present in addition to the two characteristics of the classic phenotype also other clinical manifestations [9]. These atypical features include persistence of adult baby teeth, mild cognitive disability, retinal detachment, cerebellum changes, and cataracts [9]. It is not yet clear to what extent these traits are influenced by genetic or independent causes [9]. In most cases, individuals with FOP appear normal at birth, with the exception of congenital

malformations of the big toes [9]. Typically in early childhood, particular clinical signs are present, although changes in neck movement may frequently be present [9].

During the first decade of life, most patients develop episodic and painful flare-ups in soft tissues [10]; Some of these regress spontaneously, while others lead to the replacement of tissues such as skeletal muscle, ligaments, and tendons with mature heterotopic bone [11]. It has been observed that the biochemical, metabolic and radiological properties of the formed ectopic bone are comparable to those of normal tissue [8] and that diaphragm, tongue, cardiac and smooth muscle tissue are not subject to the formation of ectopic bone [10]. Typically, during a patient's lifetime, minor trauma such as intramuscular injections, mild muscle trauma, bruising, fractures, or influenza virus infections can trigger new flare-ups leading to progressive heterotopic ossification [11].

In addition, bone formations expand over time and join together to form extra-skeletal bone elements that lead to ankylosis of the joints and almost total immobility of the body [11]. In this regard, most patients are in wheelchairs by the third decade of life [6]. The severe disability caused by FOP leads to reduced reproductive fitness, so much so that fewer than ten multigenerational families are known worldwide [12].

Given the rarity of this disease, the diagnosis of FOP is not always immediate; according to the records of the International FOP Association (IFOPA), the average age of onset of first symptoms is 5.4 years while the average age of diagnosis is 7.5 years [13]. FOP is commonly mistaken for other conditions, including aggressive juvenile fibromatosis, lymphedema or soft tissue sarcomas [8]. Because of these errors, patients are subjected to unnecessary biopsies, which contribute to further exacerbating disease progression8. In addition to this, research on FOP has been very slow due to several factors.

In fact, beyond the impossibility of obtaining tissue samples from patients, for a long time no suitable cell or animal models were available for the study of FOP since the causative genetic mutation was still unknown [7]. A turning point in FOP research was the discovery of the causative gene, activin A type I receptor (Acvr1) [14].

1.2.1 FOP genetic background

In 2006, Shore and collaborators identified the Acvr1 gene (also known as Alk2) as the gene responsible for FOP [14]. The Acvr1 gene, ubiquitously expressed in healthy tissues, is located on chromosome 2q23-24 and encodes a Bone Morphogenetic Protein (BMP)

type I receptor, called activin A [14] type I receptor. All FOP patients have an activating heterozygous mutation in the Acvr1 [14] gene.

In most cases, the mutation occurs as a de novo mutation, while only in rare cases are inherited mutations with autosomal dominant inheritance observed [9]. Both genetic and environmental factors influence the phenotype of FOP [15]. A study conducted on three pairs of monozygotic twins showed that, within each pair, the malformations of the big toes were identical. Despite this, heterotopic ossification in postnatal life varied to a large extent depending on the individual's history and the environmental factors to which they were exposed [15]. This study confirmed that genetic determinants strongly influence the disease phenotype during prenatal development and that environmental factors greatly influence the progression of heterotopic ossification in postnatal life [15].

More than 95% of cases have a recurrent heterozygous mutation in which the substitution of a single nucleotide (c.617G>A) presents a missense mutation of codon 206 of the protein, where an arginine is replaced by a histidine (R206H) [14]. This residue is located within a glycine/serine-rich (GS) receptor domain, which is important for downstream intracellular signalling [16]. To date, 14 other different mutations that cause FOP are known, all located in the Acvr1 gene at the level of the GS domain or the protein-kinase (PK) domain of the receptor (Fig. 1) [17].

The R206H mutation has been extensively analysed in different studies, different populations and geographic groups around the world, always invariably leading to a

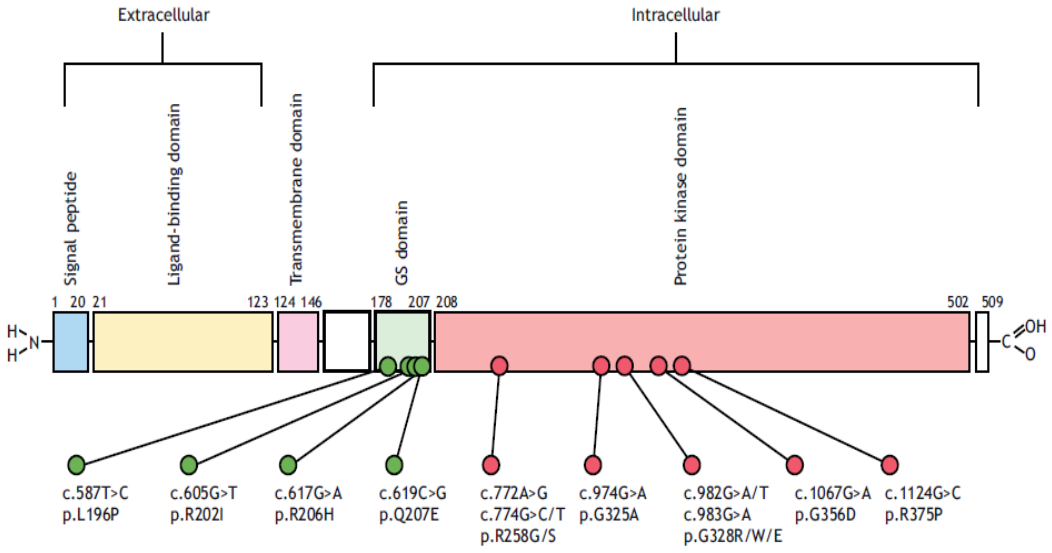

Figure 1. Human Acvr1 gene mutations associated to FOP [99].

correlation with FOP [17]. The high recurrence of R206H has stimulated interest in understanding the reason for this recurrence. In most cases this mutation occurs as a de novo event, probably in germ cells, while only in a small proportion of cases the mutation is hereditary and is transmitted in an autosomal dominant manner [14,18].

1.3 BMPs signalling pathway

Bone Morphogenetic Proteins (BMPs) belong to the TGFβ family, which in mammals includes 33 members; these include TGFβ, activins, nodals and growth differentiation factors (GDFs) [22]. Some members of the TGFβ family have been classified within the BMP [22] subfamily. The latter were originally identified as osteoinductive factors capable of inducing bone and cartilage formation when implanted in ectopic sites [23]. To date, it is known that BMPs are actually involved in a wide range of biological processes both during embryonic development and in tissue homeostasis in adult life [24]. Among the processes regulated by BMPs are differentiation, proliferation, and apoptosis in different cell types [24]. In addition, the BMP signalling pathway is implicated as a key mechanism in the regulation of chondrogenesis and bone formation (including endochondral ossification), both in development and in postnatal life [25]. Several BMPs and GDFs, but not TGFβ and activins, have been shown to induce heterotopic bone formation [26]. Osteogenic and non-osteogenic activity within the TGFβ family depends not only on the ligand type but also on the receptors to which they bind, intracellular signalling cascades and target genes [24]. BMP2, BMP4, BMP7 and BMP9 induce the formation of new bone in muscle tissue while TGFβ, activin A and myostatin do not exhibit this activity *in vivo* [26]. Like the other members of the TGFβ family, BMPs act through two types of transmembrane serine-threonine kinase receptors, type I and type II receptors, distinguished by the presence (type I) or absence (type II) of the GS domain in the juxtamembrane region [24]. In fact, the bioactive ligands of the TGFβ family are dimeric proteins in which each monomer has two binding sites, one for type I receptors and the other for type II receptors [27–29]. Both types of receptors have a short extracellular domain, a transmembrane domain, and an intracellular domain with serine/threonine kinase activity [30]. The kinase domain of type I receptors is inactive in the absence of ligand, while type II receptors have a constitutively active kinase domain [27–29]. Seven type I receptors have been identified in mammals. These were divided into three groups: the

BMPR-I group (ALK3 and ALK6), the ALK-1 group (ALK1 and ALK2) and the TβR-I group (ALK4, ALK5 and ALK7) [24].

As far as type II receptors are concerned, three receptors (BMPR-II, ActR-II and ActR-IIB) are known in mammals that are widely expressed in various tissues [24]. Serine threonine kinase type II receptors are constitutively active and phosphorylate the wisteria and serine (GS)-rich domain on different serine and threonine residues of type I receptors as a result of ligand binding [24]. Following this event, type I receptors undergo a change in the conformation of the GS domain, which in this context acts as a regulatory switch for the enzymatic activity of type I [31] receptors. The latter, once activated, phosphorylate downstream intracellular substrates, including Smad proteins [24]. The type of Smad that is phosphorylated by a particular ligand-receptor complex depends on the type of type I receptor involved.

The receptors of the ALK-1 group and those of the BMPR-I group activate Smad 1/5/8 and transduce similar intracellular signals, while those of the TβR-I group activate Smad 2/3 [24]. Osteogenic ligands of the TGFβ family bind to ALK1, ALK2, ALK3, and/or ALK6 receptors as type I receptors that induce Smad 1 and 5; non-osteogenic ligands bind to ALK4, ALK5 and/or ALK7 which activate Smad 2 and 3 [24]. Unlike type I receptors, the binding capabilities of type II receptors are broader by sharing both osteogenic and non-osteogenic ligands [26].

Generally, in the absence of ligand, type I receptors form inactive oligomeric complexes with type II receptors. To prevent independent activation of receptors, the negative regulator FKBP1A binds to the intracellular GS domain of type I receptors and inhibits the binding of effector molecules [32]. In the presence of ligand, the type II receptor phosphorylates the type I receptor in its GS domain leading to the dissociation of FKBP1A and allowing phosphorylation of intracellular substrates by type I [30] receptors. Type I receptors thus activate the downstream intracellular signalling cascade through Smad-dependent and Smad-independent signalling pathways (such as the p38 MAPK signalling pathway) in order to regulate the transcription of BMP24-responsive target genes.

Like the other members of the BMP/TGFβ receptor family, the Acvr1 protein contains an extracellular N-terminal ligand-binding domain, a transmembrane (TM) domain, a glycine and serine-rich (GS)-rich intracellular domain, and a protein kinase (PK) domain

[33]. The loop located in the HLH (Helix-loop-Helix) of the GS domain contains key residues responsible for the activation of Acvr1 following phosphorylation [34]. Acvr1 is capable of forming heteromeric complexes with type II receptors including BMPR2, ACVR2A, and ACVR2b [35]. Once bound to the receptor, these ligands lead to the activation of Acvr1 according to the mechanisms explained above. The activated Acvr1 receptor phosphorylates the receptor-regulated Smad (R-Smad) Smad1/5/9/(8) [30]. At this point, phosphorylated Smad1/5/8 forms complexes with the co-mediator Smad4 translocating to the nucleus. Here, in association with co-activators and co-repressors, Smad4 regulates the transcription of downstream genes.

In this context, Smad1 and Smad5 activate transcription while Smad9 acts as a transcriptional repressor [36]. In addition to the canonical Smad signalling pathway, Acvr1 can also activate non-canonical signalling pathways such as the p38 MAPK and PI3K/Akt17 signalling pathway (Fig. 2).

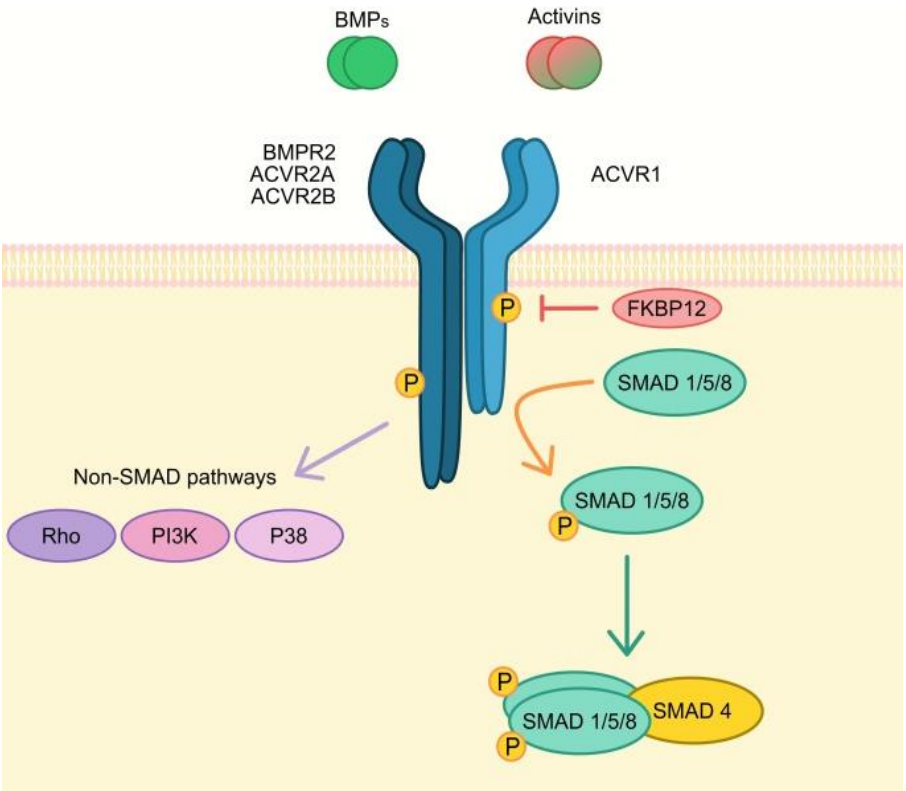

Figure 2. Downstream signal transduction of Acvr1 [17].

Non-canonical pathways also appear to play an important role in heterotopic ossification and FOP. As an example, mice lacking Acvr1 expression in cartilage have reduced Smad-mediated responses, but also reduced activation of p38 MAPK [37]. In addition, lymphocytes derived from patients with FOP have been observed to exhibit an alteration

in the Acvr1-p38 MAPK signalling pathway that can be blocked by p38 [17] inhibitors. Among the non-canonical pathways, mTOR has also been linked to heterotopic ossification and FOP by several research groups [38–40]. In this regard, it has been observed that inhibitors of mTOR complexes including rapamycin and PI3Kα inhibitors are able to reduce heterotopic ossification both in FOP and in other acquired forms [38,41].

1.3.1 Acvr1R206H mutation effects on BMP signalling pathway

The Acvr1 R206H mutation leads to an activation of the intracellular cascade that is partly independent of BMP, as well as to an increased responsiveness to BMP stimulation in different cell lines, in patient-derived cells and in embryonic models of zebrafish [17]. Moreover, all Acvr1 mutations and in particular the R206H mutation lead to increased activation of Smad1/5/8 downstream, following stimulation of BMP [32,42–44]. In addition, it was observed that cells engineered for mutant forms of Acvr1 in which the coding sequence for the ligand-binding domain had been eliminated showed in any case an increased activation of the BMP signalling pathway [45]. To explain these observations, it was proposed that the increased activation of the signalling pathway was due to an altered interaction between Acvr1 and FKBP12.

This hypothesis was confirmed with crystallographic analyses by demonstrating that several mutations, including R206H, were able to destabilize the inactive form of the receptor and FKBP12-mediated inhibition [46]. This mechanism could further contribute to the altered BMP signalling pathway. In addition, some experiments have shown that FKBP12 overexpression is able to restore the normal BMP signalling pathway with varying efficacy depending on the Acvr1 mutations considered [47]. Despite this, for most mutations, the Acvr1 receptor retains the ability to be inhibited and bound by FKBP1A, suggesting that the mechanism does not fully explain the increased activity of the Acvr1 receptor [46–48].

Interestingly, while the Acvr1 R206H mutation in heterozygosity causes FOP, the complete loss of function of the wild type Acvr1 allele in Acvr1R206H/+ mice leads to a substantial increase in heterotopic bone volume [49]. Together, these results highlight the importance of competition between the wild-type and mutant receptors *in vivo*. This competition for ligand binding and sharing of type II receptors could influence the BMP signalling pathway and highlights the important role of the balance between the

expression levels of type I and type II BMP receptors [17]. Recently, two independent studies have been conducted on the relationship between wild type and mutated Acvr1 receptors both *in vitro* and *in vivo* [50,51]. The use of antibodies against Acvr1 showed inhibition of the BMP signalling pathway induced by BMP-7 and activin A *in vitro*. However, administration of the same antibodies to mouse models of FOP has reported mixed results *in vivo*, leading to a remarkable increase in heterotopic ossification compared to untreated animals [50,51]. These recent findings add a further degree of complexity to the mechanisms underlying the pathogenesis of FOP and indicate that antibodies against Acvr1 should not be used as a possible treatment for FOP [50,51].

1.3.2 Activin A role in FOP

Activin A, another member of the non-osteogenic TGFβ family, is able to bind the Acvr1 receptor, in complex with the type II receptors ACVR2A/B, and to signal via the Smad2/3 signalling pathway [17]. Since activin A shares type II receptors (ACVR2A/B) with BMP ligands, it is able to competitively antagonize the activation of Smad1/5/8 following stimulation by BMP6/9 through binding to ACVR2A/B-ACVR1 complexes [52]. In fact, under normal conditions, activin A binds to the wild-type Acvr1 receptor and the type II receptor forming a complex that does not lead to the signalling normally activated by BMP [53,54].

Studies have shown that the R206H mutation of Acvr1 confers a new function to the receptor; it has been established that activin A is able to induce the activation of Smad1/5/8 both through the Acvr1 R206H receptor and with other mutations [45,54,55]. In response to activin A, the Acvr1 R206H receptor is able to lead to endochondral ossification *in vivo* and to lead to chondrogenesis of mesenchymal stromal cells derived from FOP-iPSCs *in vitro* [55]. In addition, activin A has been shown to promote heterotopic ossification of fibro/adipogenic precursors (FAPs) in Acvr1 R206H mouse models [49]. Also in this study, it was demonstrated that antibodies against activin A (ActA-mAb) are able to prevent heterotopic ossification in transplanted mice with FAP isolated from Acvr1 R206H mice. Spontaneous or trauma-induced ossification was also inhibited or partially reduced in most mice treated with these antibodies [49]. These experiments confirm that activin A plays a central role in promoting heterotopic ossification in FOP. It should also be remembered that activin A is secreted by several cells of the innate immune

system such as neutrophils, monocytes, macrophages and dendritic cells and that its secretion is induced by damage in soft tissues further amplifying the downstream effects of the Acvr1 receptor R206H [56,57].

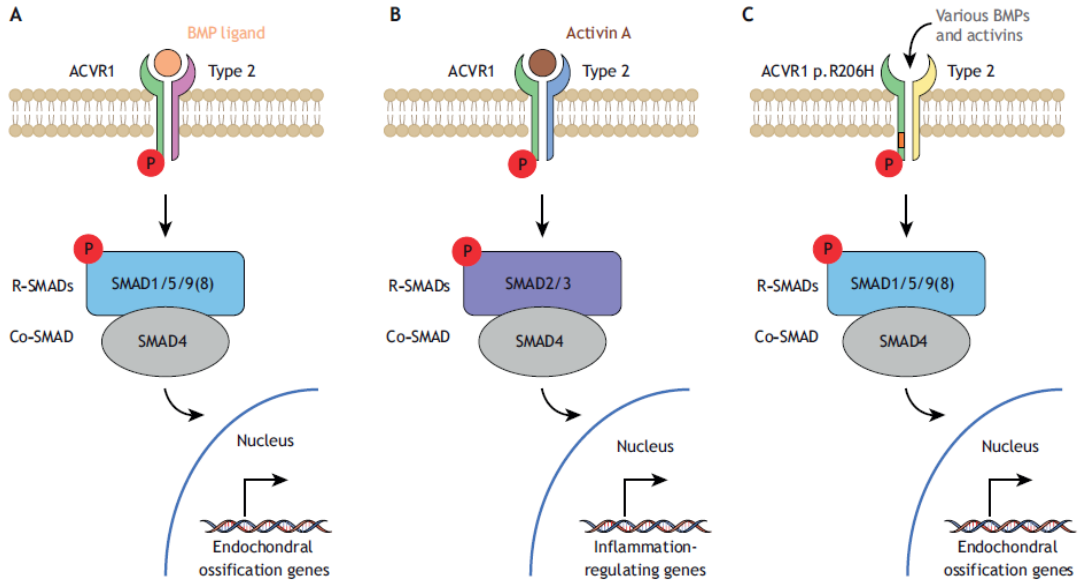

Figure 3. Acvr1 downstream signalling in presence of A) osteogenic BMPs; B) activin A; C) BMPs and activin A in presence of the R206H mutation [99].

1.4 FOP lesions histology

Lesion formation in FOP is a pathological process of metamorphosis in which the structure and function of one tissue, such as skeletal muscle tissue, is destroyed and replaced with that of another tissue, in this case bone [58]. The possibilities to study different stages of lesion formation in FOP patients have been extremely limited due to the impossibility of performing biopsies on patients [7]. Despite this, over time, the collection of data by different research groups has made it possible to lead to an adequately accurate description of the different histological phases that characterize the formation of lesions. Although lesion formation in FOP appears to begin spontaneously, most often these episodes are triggered by soft tissue damage [5].There are two main phases in this process: the catabolic phase and the anabolic phase.

The catabolic phase, triggered by damage, is characterized by cell damage and necrosis as well as the presence of an inflammatory infiltrate composed of mononuclear cells such as lymphocytes, macrophages and mast cells present in the perivascular space of early FOP lesions, between skeletal muscle and connective tissue [58,59]. This initial response is similar to the normal tissue response to damage, although in patients with FOP it is

impaired and excessive [5]. Following the catabolic phase, an anabolic phase occurs characterized by a fibroproliferative response followed by angiogenesis [59]. In this phase, there is an altered production of the extracellular matrix and an increased stiffness of the tissue compared to what normally occurs in a tissue following damage [60,61]. The presence of inflammatory infiltrate is associated not only with muscle damage, but also with a hypoxic microenvironment [5]. Both of these conditions are hypothesized to trigger a fibroproliferative response in early lesions [5]. These early and intermediate stages of the lesion are microscopically indistinguishable from aggressive juvenile fibromatosis, for which FOP is often mistaken [59].

At this point, at the site of inflammation, the fibroproliferative lesion begins to differentiate in response to the intracellular BMP cascade, leading to the transformation of fibroproliferative tissue into cartilage, which will subsequently mature into bone through the process of endochondral ossification, thus completing the metamorphosis process [59]. In addition, hypoxic conditions partially promote chondrocyte differentiation by promoting activation of the BMP signalling pathway via retention of Acvr1 in endosomes [62]. Hypoxia also induces VEGF expression, promoting the formation of blood vessels that can then guide the process of bone formation endochondral [7,62]. It has been observed that mast cells are present in every histological phase and that they are much more abundant when compared to normal skeletal muscle [59]. In addition, all stages of histological development are present in the lesions of FOP patients, indicating that different regions within the lesion mature at different times [59].

1.5 Immune system in FOP

The contribution of the immune system in the pathophysiology of FOP is an important area of research. The environment in which osteogenesis occurs is a complex set of different cell types and molecular components and due to its complexity much still needs to be investigated and elucidated [18]. It is also thought that the mutation in the Acvr1 gene, being present in different cell types, may to some extent influence immune responses, thus increasing the complexity of pathogenic factors involved in disease progression [18]. As described above, the tissue lesions that form are home to many cells of the immune system such as lymphocytes, macrophages, and mast cells. Systemic TGFβ suppression has also been shown to reduce heterotopic ossification in mouse models of FOP, thus

implying that the TGFβ signalling pathway induces and promotes heterotopic ossification [63]. Given that TGFβ is a cytokine secreted by macrophages involved in tissue repair, these results suggest that myeloid cell lineages may play a crucial role in driving the early phase of inflammation in FOP [64].

Macrophages are heterogeneous and multi-functional cells that are critical to tissue functions in both steady state and disease state. Initially, they were identified as immune cells playing their major role in inflammation; nowadays, macrophages are known to have a much wider array of roles ranging from tissue remodelling during organogenesis to tissue homeostasis, injury repair, and immune response to pathogens [65].

In normal conditions, resident macrophages are responsible of maintaining tissue homeostasis and of responding to sudden changes, whether physiological or pathological. Tissue macrophages consist of two classes: resident macrophages and infiltrating macrophages. In adult mammals, while resident macrophages are present in all tissues, infiltrating macrophages are found in a diseased tissue, such as injured tissue. Unlike infiltrating macrophages, which are all derived from blood monocytes originating from bone marrow hematopoietic stem cells (HSCs), tissue-resident macrophages arise from multiple origins during embryonic and adult hematopoiesis.

In a disease state, macrophages activate by displaying several functions thanks to their high adaptability and plasticity when responding to environmental changes. The canonical division of macrophages has always been M1, or classically activated, and M2, or alternatively activated. M1 and M2 macrophages differ in their activation stimuli, cell surface markers, arginine metabolism, and cytokine production profiles [66].

While M1 macrophages, activated by IFN-γ ± LPS, are pro-inflammatory, M2 macrophages, activated by Il-4 ± IL-13, can be anti-inflammatory, pro-regenerative, and/or pro-fibrotic *in vitro*. However, growing evidences demonstrate that the M1/M2 paradigm of macrophage activation is over-simplistic and cannot mimic complex *in vivo* settings, due to many other cell types interacting with macrophages in the different tissues. *In vivo*, M1 and M2 stimuli often co-exist, macrophages can display mixed M1/M2 phenotypes, and they may not expand clonally to maintain phenotype. The phenotype of *in vivo* macrophages may be M1-like or M2-like but not strictly M1 or M2 [67].

We could more precisely assess that macrophages at the early stage of inflammation can be considered as more "pro-inflammatory", while the macrophages at the later stages of inflammation have a more "anti-inflammatory" and "pro-regenerative" phenotype.

Macrophages are also known to have a role in bone remodelling, a process that is finely regulated by the balance between the activity of osteoblasts and osteoclasts. Within this framework, macrophages are known to undergo a process defined as osteoclastogenesis, which can be induced through many inflammatory cytokines which also affect the immune system, such as the predominant cytokine receptor activator of nuclear factor kappa-B ligand (RANKL), macrophage colony stimulating factor (M-CSF), tumor necrosis factor-alpha (TNFα), interleukin-1 (IL-1), IL-6, IL-7, IL-17, IL-23, transforming growth factor-beta (TGFβ), and interferon-gamma (IFNγ) [68].

In the context of bone remodelling, osteoclasts play a fundamental role, since their bone resorption activity allows the turnover of old or damaged osteocytes allowing the formation of new healthy bone [68].

The role of macrophages has been investigated in the environment of HO taking advantage of different *in vivo* models, leading to different results. In fact, in some papers, macrophage depletion showed a reduction in ectopic bone volume, while in others it showed an increase [69,70]. These results could indicate that different types of macrophages are involved in different stages of FOP and therefore should be considered as a more heterogeneous population than has been done so far [7]. Studies of patients have also shown that they are in a constant pro-inflammatory state, even when they have no symptoms [7]. In fact, blood samples from patients with FOP showed significantly elevated levels of cytokines such as interleukin 3 (IL-3), interleukin 7 (IL-7), interleukin 8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [71]. In addition, monocytes isolated from peripheral blood from FOP patients showed increased responsiveness to lipopolysaccharide (LPS) stimulus and prolonged NF-kB activation compared to control samples [63]. A study on mononuclear cells from peripheral blood has shown that in patients with FOP there is an increased level of DNAM-1 in monocytes, suggesting a functional effect on monocyte migration, which could represent a biomarker of the inflammatory state in FOP [72]. Other cell types such as mast cells and lymphoid cells could contribute to inflammation in FOP.

Experiments on RAG1-/- mice, which do not have B or T lymphocytes, developed heterotopic bone after damage without delay, and the loss of these specific lineages reduced the spread and total amount of heterotopic bone in heterozygous mice [73,74]. These results indicated that the adaptive immune system is not necessary for the initial formation of heterotopic bone but may be relevant for its expansion [73,74].

Other works have investigated the role of mast cells. Mast cell depletion has been shown to reduce heterotopic bone volume by 50% in an Acvr1 R206H mouse model [69]. Taken together, these data indicate that macrophages, mast cells, and adaptive immune system cells may have different roles in different stages of inflammation in FOP [64].

1.6 Identification of progenitor cell types for heterotopic ossification in FOP

Heterotopic ossification is a complex, multi-step process involving different cell types, although the progenitors that carry out heterotopic ossification have not yet been precisely identified [75]. The different cell types that contribute to heterotopic bone formation in FOP all have an activating mutation in the Acvr1 receptor, which most likely influences and alters their function, thus contributing to the complexity of the factors that lead to the onset and progression of the disease [18]. Several cell populations of muscle tissue-associated progenitor cells have demonstrated osteogenic potential [7].

Satellite cells: Satellite cells are muscle-resident stem cells that are essential for maintaining homeostasis and muscle regeneration following damage [76]. They were among the first candidate cells as possible progenitors of heterotopic bone in muscle as they showed osteogenic activity in response to cultured BMPs [77]. However, *in vivo* lineage tracing studies showed that these cells did not contribute significantly to BMP-induced heterotopic ossification [78]. Moreover, the expression of constitutively active forms of Acvr1 (caACVR1) and the Acvr1 R206H form in satellite cells was sufficient to induce heterotopic ossification [49,79,80], supporting the hypothesis that satellite cells do not play a direct role in the heterotopic ossification process of FOP [81].

Endothelial cells: due to their ability to perform endothelium-mesenchymal transition (EndoMT) and the presence of endothelial markers in lesions of patients with FOP [78,82], endothelial cells were also among the first to be studied as possible progenitors of heterotopic ossification. During the mesenchymal endothelial transition, endothelial cells lose their cell-to-cell adhesions by changing polarity, reducing the expression of

endothelial markers while increasing that of mesenchymal markers. As a result of this transition, the cells are much more invasive and mobile and can contribute to the development of various diseases [83]. In an *in vitro* study, it was shown that endothelial cells that overexpress the Acvr1 receptor or that have been treated with ligands such as BMP4 are able to de-differentiate into stem cells capable of converting into cartilage or bone [82]. Other *in vivo* work has deepened these initial findings, in a mouse model of heterotopic ossification (Nse-BMP4) it has been seen how cartilage and ectopic bone cells express endothelial markers such as vWF, VE-cadherin, Tie1 and Tie2 both after overexpression of Acvr1 and following muscle damage [78,82]. Tie2 and vWF are also expressed in chondrogenic and osteogenic lesions of FOP patients, whereas osteoblasts and chondrocytes of normal cartilage and bone do not express these markers [82].

Lineage tracing studies in Tie-Cre mouse models have shown that 50% of cartilage and heterotopic bone cells were of endothelial origin [78,82]. However, Tie2 is not specific for endothelial cells and more than 90% of the Tie+ cells found in heterotopic bone were also positive for PDGFRα and Sca1, thus indicating a mesenchymal and not endothelial origin [84]. In addition, damage to skeletal muscle tissue is able to induce the expression of endothelial markers such as Tie2, CD31 and VE-cadherin in non-endothelial cells such as, for example, mesenchymal cells [85]. Taken together, these data suggest endothelial cells that undergo EndoMT may give rise to heterotopic ossification, but probably do not play a central role in the process, since the expression of endothelial markers may also emerge from other cell types [86].

Fibro-adipogenic progenitors (FAPs): FAPs are a population of multipotent PDGFRα+ Sca1+ cells found in skeletal muscle and other tissues [84,87]. Muscle-resident FAPs support muscle regeneration but have no myogenic differentiation potential [87,88]. They were initially discovered for their fibrogenic and adipogenic potential, and only later was their osteogenic potential highlighted when stimulated with BMP *in vitro* and *in vivo* [84,87,88]. Wosczyna et al. observed through a lineage tracing experiment of FAPs that they contributed 50% to heterotopic bone and cartilage [84]. It has been proposed that these cells play a fundamental role in the pathophysiology of FOP in humans.

In fact, several studies in mice have shown that the progenitors responsible for intramuscular or tendon heterotopic ossification are often positive for PDGFRα and for cartilage and bone markers [49,79]. Using a mouse model of FOP in which Acvr1 contains

the classical R206H mutation, Dey et al. showed that FAP-like cells could be divided into two subpopulations: tendon-derived Scx+ progenitor cells and interstitial cells residing in Mx1+ muscle [79]. Scx+ progenitors mediated endochondral heterotopic ossification without the need for damage, while the Mx1+ population mediated damage-dependent heterotopic ossification [79]. PDGFRα+ cells in these two populations are only a small subset; however, it has been seen that constitutive activation of the signalling pathway downstream of Acvr1 leads to a greater osteogenic and chondrogenic potential of PDGFRα+ subgroups compared to Scx+ and Mx1+ populations. In another *in vivo* study with a mouse model of BMP2-Matrigel implantation, it was possible to demonstrate that resident tissue FAPs are the primary source of osteogenic cells in post-traumatic heterotopic bone formation [89]. Taken together, these data indicate that FAPs contribute largely to heterotopic ossification due to their wide distribution within skeletal tissue and their documented participation in the process of heterotopic ossification [86].

1.7 Molecular targets for FOP treatment

Currently, there are no specific and effective approved treatments for patients with FOP [7]. The therapy for the symptomatic treatment of flare-ups is based on the use of glucocorticoids and non-steroidal anti-inflammatory drugs [7]. As far as more specific treatments are concerned, the main strategies implemented are based on the inhibition of the altered BMP signalling pathway at various levels [90]. For example, Saracatinib is a kinase inhibitor of the src family originally developed as a treatment for several solid tumours. It is a potent inhibitor of Acvr1 that in preclinical models has demonstrated efficacy in the treatment of heterotopic ossification and has been proposed as a possible candidate for the treatment of FOP, currently in phase II clinical trials [91].

In a mirrored manner, stimulation of Acvr1 by ligands can be prevented. A neutralizing antibody specific to activin A (garetosmab) has been brought into phase II clinical trials after promising preclinical results [92]. Recently, mTOR has been identified as a key factor in the early hypoxic phase and inflammatory phases of heterotopic ossification [64].

In addition to its immunoregulatory function, mTOR is required for the induction of chondrogenesis and osteogenesis [7]. In addition, crosstalk between the mTOR and BMP signalling pathways could amplify heterotopic ossification in FOP [93]. In preclinical studies, rifampicin significantly inhibited heterotopic ossification in a mouse model and

a clinical study is being developed to evaluate its efficacy and safety in patients with FOP [38,93]. Possible targets for the treatment of heterotopic ossification have also been identified downstream of Acvr1.

Palovarotene, a retinoic acid receptor gamma (RAR-γ) agonist, inhibits heterotopic ossification in a mouse model of FOP by blocking chondrogenic progenitor cell differentiation and is currently in several phase II and III trials [80,94]. Other therapies such as VEGF inhibitors, PI3K inhibitors, siRNAs against mutated Acvr1, HIF1-α blockers and TGFβ-activated kinase (TAK1) inhibitors were investigated [7].

1.8 FOP models

The development of accurate models for the study of rare diseases is of fundamental importance both for the understanding of cellular and molecular mechanisms and for the development of translational strategies for the treatment of affected patients. This need is even more urgent for a rare disease such as FOP due to the impossibility of performing biopsies on patients [7]. For this reason, several *in vitro* and *in vivo* models have been developed.

1.8.1 *In vitro* FOP models

Among the *in vitro* models, primary fibroblasts extracted from the dermis of FOP patients can be found. These cells were transdifferentiated into an osteogenic lineage in order to obtain a cellular model for the study of heterotopic ossification induced by flare-ups [95]. In another study, fibroblasts from the periodontal ligament of teeth extracted from patients with FOP were isolated in order to induce osteogenesis and osteoclastogenesis *in vitro* to understand the molecular mechanisms underlying heterotopic ossification [96]. In 2015, induced human pluripotent stem cells (hiPSCs) were obtained by isolating kidney cells from the urine of patients with FOP. These cells were then differentiated into endothelial cells and pericytes that showed a predisposition to mineralization although they did not transform into mature osteoblasts [97]. Although these models have allowed to highlight some aspects of FOP, *in vitro* models are intrinsically limiting and it was therefore necessary to integrate the study of FOP with appropriate animal models.

1.8.2 *In vivo* FOP models

The first animal models were developed even before the discovery of the FOP causative gene and provided information on the role of the BMP signalling pathway in heterotopic ossification [99]. The first animal model that provided a preliminary clue on the possible cause of FOP was the mutant Decapentaplegic (dpp) of Drosophila melanogaster, which allowed to predict the role of the BMP signalling pathway in the pathophysiology of FOP [100]. This signalling pathway has been studied in several animal models, including Drosophila Melanogaster and Dario rerio, allowing a better understanding of the molecular and cellular mechanisms of downstream signalling of Acvr1 as well as the role of this receptor and its orthologs *in vivo* [62]. Recently, the first adult zebrafish model for the study of FOP has been developed [101,102]. The human Acvr1 gene and the ortholog in zebrafish Acvr11 are 69% identical, and the intracellular receptor domains, i.e., the GS and protein-kinase domain of the protein, are 85% identical between humans and zebrafish [103]. This preservation led to the assumption of a good functional preservation of the protein such as to allow its study in this animal model. Since the expression of the mutation of the zebrafish receptor acvr1lQ204D and the human receptor Acvr1R206H is embryonic lethal in zebrafish, a conditional expression system has been developed [101]. A transgenic model was then developed in which the acvr11Q204D allele is under the control of a thermoinducible promoter [101]. In this way, this model is able to terminate embryogenesis normally, thus allowing the expression of the mutated receptor in adults following thermal shock at 38°C once a day for one hour [101]. The induced animals developed some FOP-like features such as small heterotopic ossification lesions and vertebrae fusion [101]. However, inflammatory stimuli such as actvin A injection, cardiotoxin injection, and mechanical damage did not lead to the development of heterotopic bone at the site of damage [101,102]. However, this zebrafish model could have some advantages such as, for example, the possibility of conducting *in vivo* lineage tracing studies with greater ease in order to identify new cell populations that can contribute to heterotopic ossification [98]. To date, however, among the most widely used animal models in FOP research are mouse models. The mouse Acvr1 gene was first cloned in 1993 and characterized by its ability to associate with type II receptors and to bind *in vitro* a large number of ligands belonging to the TGFβ family, including TGFβ itself and activin *in vitro* [104]. Several initial studies have made it possible to determine its importance in different processes of mouse embryonic development; Given its role in

early development, its loss or overexpression has been shown to be embryonic lethal in mice [105]. The human and mouse Acvr1 genes are 98% identical, while at the protein level they have 99.8% homology in the amino acid sequence [31].

Bmp implant models: among the earliest models used for the study of heterotopic ossification and FOP are BMP implantation models. It was known, in fact, that osteogenic ligands of the BMP family were expressed as a result of soft tissue damage [81]. In addition, an increased expression of BMP4 was demonstrated in the lesions of patients with FOP [81,106]. These models had several advantages, for example the implantation of BMP allowed to cause, with high reproducibility, a series of histological events, including tissue inflammation, muscle necrosis, accumulation of mesenchymal cell populations and endochondral bone formation in a very similar way to what happens in humans [81]. Although these models did not involve the administration of muscle damage, the injection of most osteogenic BMPs was able to trigger a robust inflammatory response [81].

In conclusion, these simple and highly reproducible models have been widely used for the study of heterotopic ossification. Although they were not a particularly precise and accurate model for the study of FOP, they proved to be extremely important for the initial understanding of this pathology in humans [81].

Nse-BMP4 model: another model developed even before the discovery of the causative gene of FOP and also based on the possible involvement of the BMP4 protein in the pathophysiology of the disease is the Nse-BMP4 model. Prior to the development of this model, several promoters were investigated that could drive BMP4 overexpression, but many of these did not allow the development of heterotopic ossification, while others led to developmental abnormalities [107,108]. Among these promoters, only the Nse (neuron-specific enolase) promoter has led to the development of an adequate heterotopic ossification model, both in the presence and absence of damage [73]. In this genetic model, in which BMP4 is under the control of the Nse promoter, the mouse develops postnatal endochondral heterotopic bone, thus recapitulating one of the two features descriptive of the classical FOP phenotype in humans [73]. In addition, as in affected patients, the mouse model does not develop bone at sites such as diaphragm, tongue and extraocular muscles, and does not exhibit abnormalities in normal skeletal development [73]. This model has shown that BMP4 overexpression alone is sufficient to trigger the cascade of events leading to endochondral progressive heterotopic ossification [73]. In addition, this model

has been used to better investigate which cells give rise to heterotopic ossification. Histological analysis in early lesions of the Nse-BMP4 model has shown that cells that early begin to proliferate and then give rise to ectopic bone are cells that express markers of mesenchymal stem cells [73]. In addition, mating of Nse-BMP4 mice with mice overexpressing Noggin, a BMP4 inhibitor, leads to the absence of heterotopic ossification in the offspring [109]. These experiments have allowed to highlight a possible therapeutic role of Noggin, which is in fact able to resolve heterotopic ossification in Nse-BMP4 mice if injected locally into the muscle [110]. Prior to the development of transgenic mice for Acvr1, the Nse-BMP4 model was one of the most widely used for the study of BMP overexpression in the pathogenesis of FOP [111]. Subsequent models based on BMP inhibitor knockout or BMP target overexpression did not allow the FOP phenotype to be reproduced; the subsequent models are therefore based on the expression of mutant forms of Acvr1 [112].

Acvr1^{Q207D} mouse model: The first constitutively expressing mouse model, Acvr1 Q207D, was developed using the inducible Cre-Lox system even before the discovery of the FOP causative gene [113]. The Q207D mutation causes the substitution of a glutamine with aspartic acid in the GS domain of the receptor, and although it is not present in patients with FOP, it is still able to confer constitutive activity on the Acvr1 receptor in a similar, though more severe, manner than FOP-associated mutations such as the R206H mutation and the Q207E mutation [114,115]. In the initial characterization of this mouse model, it was noted that even a mild and ubiquitous overexpression of Acvr1 Q207D was able to promote the signalling pathway downstream of BMP and to impair embryonic development [113]. To curb this problem, the model was subsequently refined in order to avoid embryonic lethality [116]. This was done by two methods: the first method consisted of an adenoviral vector injection for Cre recombinase in the lower extremities in order to induce local expression of Acvr1 Q207D, while the second consisted of crossing these Acvr1Q207D models with mice that ubiquitously express an inducible tamoxifen-activated Cre (CAGGCreERT) [113,116]. The first method resulted in heterotopic ossification and immobility of the treated limb with a penetrance of 100% within 30 days of injection, while the second method did not develop heterotopic bone within 60 days of induction [113,116]. Interestingly, the latter mice when injected with a control adenovirus were able to develop heterotopic ossification. These data suggest that the expression of Acvr1Q207D

alone is not sufficient to lead to ectopic bone and that the presence of an inflammatory environment or damage such as a viral infection that can stimulate bone formation is also required [116]. Despite the limitations of this model, it has been useful for the preclinical development of drugs for the treatment of FOP, such as Palovarotene, a retinoic acid receptor gamma agonist (RARγ) [80] and to establish the indispensable role of HIF-1α-mediated hypoxia in early FOP lesions, thus identifying a further possible therapeutic target for the treatment of this disease [62]. Despite the usefulness of this model, it is not a totally accurate model. In fact, the Q207D substitution does not exist in humans and makes Acvr1 constitutively active and almost insensitive to the ligand, in contrast to Acvr1 R206H which shows only minimal ligand independence and is highly responsive to osteogenic BMPs and activins [113,114]. Subsequent studies have also shown that this protein variant of Acvr1 Q207D is distinct from FOP-associated mutations and only marginally appropriate for the development of animal models [114]. Moreover, the Acvr1 transgene expressed in this model is found in an overexpressed construct that is driven by a strong promoter, and the level and specificity of expression is not under endogenous control [113].

$Acvr1^{R206H}$ mouse model: in an attempt to obtain more relevant models in reproducing the FOP phenotype, more recently mouse models with the classical FOP mutation (R206H) in the Acvr1 receptor have been developed. The first model was obtained by inserting the sequence of the Acvr1R206H gene into the endogenous locus, at the level of exon 5 of the Acvr1 receptor [105]. Since this mutation is embryonic lethal in mice, 70%-90% chimeras for this mutation have been studied [105]. Chimeric mice developed the two classic features of FOP, namely congenital malformation of the fingers of the lower limbs and the development of heterotopic bone [105]. In addition, these models developed ossification in a significant way following muscle damage and also showed variable features of FOP such as rib and vertebral malformations, osteochondroma of the tibia and other bones [105]. It should be noted that in the damaged tissues of these mice there were both cells with the R206H mutation in Acvr1 and wild type cells [105]. This has raised the hypothesis that in FOP cells that do not express the mutation are recruited to the site of damage by Acvr1 cells R206H [81,105]. Subsequently, further expressing from a line of mesenchymal cells derived from this chimeric mouse was conducted [117]. Using these cells, it has been shown that the Acvr1 R206H receptor is necessary but not sufficient on its own to promote

chondrogenesis *in vitro* and heterotopic bone formation *in vivo* [117]. In fact, BMP stimulation is required to promote the initial activation of Acvr1 R206H, suggesting that this cell line is initially ligand-dependent in the activation of the downstream signalling pathway and then converts to ligand-independent constitutive activation [117].

Despite the good reproducibility of the disease phenotype in these chimeras, the problem of embryonic lethality has greatly limited the use of this model in *in vivo* studies. To this end, models have been developed to overcome the problem of embryonic lethality. A Cre recombinase-dependent conditional expression (Acvr1[R206H]/FlEx) knock-in model was created [54]. In this model, the mouse exon containing the R206H mutation is located on the antisense strand and is therefore not expressed at birth by mice until it is moved into the sense strand by Cre recombinase.

This rearrangement is made possible by the use of FlEx arrays from the system. In this system, lox sites and lox site variants are positioned and oriented in such a way as to lead to Cre-mediated DNA excision and inversion resulting in the replacement of wild type exon 5 with the variant containing the R206H mutation [54]. Although the unrecombinant Acvr1[R206H]/FlEx allele was designed to function as a wild-type allele, approximately half of the transcripts of the Acvr1[R206H]/FlEx alleles in ES cells were subject to altered splicing, resulting in exon 5 exclusion and frameshift leading to the encoding of an inactive product [54]. Despite this, there were no clear phenotypic consequences due to the reduced expression of Acvr1 from the Acvr1[R206H]/FlEx allele of mice [54].

These mice were then crossed with a Gt(ROSA26)SorCreERT2/+ line in order to obtain Acvr1[R206H]FlEx/+ heterozygous mice; Gt(ROSA26)SorCreERT2/+ that globally expressed Tamoxifen-inducible Cre recombinase.

In these models, once the activity of Cre recombinase and therefore the expression of Acvr1R206H has been induced following the administration of Tamoxifen, it is possible to observe the development of heterotopic bone between 2 and 4 weeks after induction, without the need to induce damage [54]. Disease progression and anatomical sites affected by heterotopic ossification resemble what is observed in patients with FOP [54]. Histologically, heterotopic bone lesions also showed evidence of muscle damage, inflammatory infiltrate, fibroblast proliferation, cartilage and bone formation in a similar way to lesions found in patients [54]. In addition, this model was useful in identifying the role of activin A in aberrant activation of the Acvr1 receptor, a finding that is critical to

advances in FOP research [54]. The same Acvr1[R206H]/FlEx transgenic line was used to develop a new mouse model based on doxycycline-induced recombination [69]. These mice, in which endochondral heterotopic bone formation was observed starting 2 weeks after cardiotoxin (CTX) damage induction, were used to study the role of immune system cells in skeletal muscle following damage [69]. This study showed that different populations of immune cells (such as neutrophils, monocytes/macrophages, and mast cells) that normally participate in tissue repair were increased in mice expressing Acvr1R206H and that they persisted at high levels during heterotopic bone formation instead of returning to baseline levels as occurs during normal tissue repair [69]. This increased cellular response is accompanied by an increase in pro-inflammatory factors such as TNFα, IL-6, and IL1β [69]. To underline the *in vivo* importance of the immune system, in particular mast cells and macrophages, in the formation of heterotopic bone, depletion experiments of these two cell populations were performed in the mouse model Acvr1R206H [69]. This model, together with another independently created mouse model, in which the expression of Acvr1R206H is Cre-dependent and under the control of the endogenous Acvr1 locus (Acvr1tnR206H), have also been used to characterize the contribution of other cell populations to heterotopic ossification, in particular the contribution of fibroadipogenic precursors (FAPs) [49,79].

1.8.3 In silico FOP models

In addition to animal models, a new approach is the development of computational models of diseases. These in silico models have already been developed for the study of endochondral ossification and have allowed to investigate the interaction between different factors, including growth factors, angiogenesis, oxygen recruitment, proliferation and differentiation of osteoprogenitors [118,119]. These models could be adapted in the future to simulate the process of endochondral ossification in FOP in order to provide an alternative way to study potential drugs for the treatment of this disease [99].

2. Results

2.1 Tamoxifen-induced Acvr1R206H$^{lox/lox;}$Gt(ROSA26)SorCreERT2 mice develop ectopic bone after muscle injury

We initially focussed on establishing a consistent ossification protocol, to assess how accurately this mouse model could reproduce the features of FOP.

After activating the R206H mutation with tamoxifen administration, we decided to initiate a localized inflammatory response by inducing a mechanical muscle injury by pinching the gastrocnemius. This approach recreates a condition comparable to the flare-ups observed in FOP patients. Following the injury, we monitored the animals for 21 days and assessed the presence of ectopic bone in the injured muscles by computerized axial tomography (CAT) at both 14 and 21 days (Fig. 1A).

As a result, FOP mice showed HO in the gastrocnemius at both at 14 and 21 days after muscle injury. Tomography also revealed the progressive nature of ossification, with bone volume increasing by approximately 50% between day 14 to day 21 after the injury (Fig. 1B).

Consequently, due to HO, mice displayed a gradual decline in hind limbs mobility.

We also performed histological analyses on muscle sections of the injured muscles of FOP mice. Hematoxilin and eosin (H&E) staining revealed increased immune infiltrate over course of time following the injury, in contrast to control mice, which regenerate their muscles and display normal muscular morphology (Fig. 1C).

Additionally we explored whether muscle regeneration was impaired in FOP mice by analysing the cross-sectional area (CSA) of the muscle fibres (Fig. 1D). We observed a significant decrease of the CSA average in the FOP mice at 21 days after the injury, indicating that muscle regeneration remained severely impaired up to 21 days after pinch injury.

2.2 Macrophage depletion impairs HO in clodronate-treated mice

To assess the impact of the immune system on heterotopic ossification, we performed monocyte/macrophage depletion using clodronate liposomes in FOP mice. Clodronate, at high intracellular concentrations, is able to induce apoptosis. Administered via liposomes, it can be internalized by circulating phagocytes, specifically by monocytes and

macrophages. It is important to note that clodronate liposomes can only achieve a partial depletion of circulating phagocytes and multiple injections are required, due to the rapid turnover of infiltrating macrophages [120] (Fig. 2A).

We administered clodronate liposomes through four intravenous injections, starting one day prior to muscle injury and continuing every other day afterwards. For the control group, we injected liposomes containing PBS in FOP mice. Subsequently we monitored the control and the clodronate-treated groups for 21 days (Fig. 2A).

CAT revealed a significant reduction of the ectopic bone volume in the injured legs of clodronate-treated mice both at 14 and 21 days after the injury (Fig. 2B).

Remarkably, while we could already detect fully mineralized ectopic bone in control mice trhough histological stainings after 14 days, clodronate-treated mice consistently display the presence of cartilage in the injured muscles (Fig. 2C).

At 21 days after muscle injury, cartilage was no longer detected in in clodronate-treated mice, as intramuscular bone was mineralized (Fig. 2D). However, the total volume of ectopic bone was decreased by almost 75% at both the timepoints in the clodronate-treated group (Fig. 2E).

These findings suggest that macrophage depletion, albeit partial, delays the endochondral ossification process, resulting in decreased ectopic bone volume. Moreover, clodronate treatment underscored the pivotal role played by macrophages in the HO process described in FOP mice.

2.3 Single-cell RNA sequencing reveals altered cell clusters in response to muscle injury in FOP mice

Our previous findings confirmed that, once Cre is activated by Tamoxifen administration, Acvr1R206H$^{lox/lox}$;Gt(ROSA26)SorCreERT2 mice consistently develop ectopic bone after muscle injury, shedding light on the immune system's impact on HO. With consistent HO observed at later timepoints after triggering inflammation through muscle injury, our aim was to investigate the early events that led to subsequent ossification.

Thus, we conducted single-cell RNA sequencing on whole injured gastrocnemius muscles from control and FOP mice at 5 and 7 days after pinch injury, to compare their transcriptional profiles (Fig. 3A).

Initially we defined cell clusters and populations based onto their canonical markers expression, noting changes in the percentages of cells within several clusters (Fig. 3B).
Remarkably, FOP mice exhibited an increase in inflammatory monocytes while controls had a higher percentage of anti-inflammatory macrophages. This difference, particularly prominent at 7 days after injury, suggests a delayed transition of macrophages towards an anti-inflammatory phenotype in the KI mice.
Regarding other cell populations, FOP mice exhibited a higher percentage of fibro-adipogenic progenitors (FAPs) compared to controls. Among FAPs, subpopulations included adipogenic and pro-remodelling cells (associated with fibrogenesis), with both of these subpopulations increased in FOP mice.
Control mice, conversely, had higher percentages of endothelial cells and B cells, known for their role in orchestrating tissue regeneration by recruiting immune cells and turning off inflammation, respectively.
Altogether, these findings suggest that FOP mice experience an impaired muscle regeneration from the early stages of injury response, due to the establishment of a prolonged pro-inflammatory environment that may hinder damage resolution. Prolonged inflammation has also been associated with increased adipogenesis and fibrosis [121], as evident from the higher percentages of pro-adipogenic and pro-fibrogenic cells pobserved in FOP muscles following injury.
Furthermore, these observations align with histological findings at 21 days post-injury, where muscle fibre size was reduced, and inflammatory infiltrate was still detectable, collectively suggesting an altered microenvironment within the regenerating muscle cellular niche.

2.4 FOP macrophages contribute to HO by displaying an upregulated inflammatory profile and expressing osteoclast markers after muscle injury

Having defined the clusters, we carried on a differential gene expression (DGE) analysis, focusing on macrophages and FAP.
While macrophages' role in HO remains unclear and has received limited investigation in the context of FOP, FAPs are directly involved in bone growth and differentiation due to their osteogenic potential.[122].

We identified highly expressed genes in macrophages at each timepoint, revealing upregulated genes like Spp1, Sparc, and Postn (Fig. 4A) encoding Osteopontin, Osteonectin, and Periostin, respectively. These secreted proteins are involved in extracellular matrix remodelling and are related to ossification.[123].

Hypoxia-related genes, Hif1a and Hilpda, were also upregulated in macrophages at 5 days post-injury (Fig. 4B, with that hypoxia, combined with inflammation known to promote HO [124].

At 7 days post injury, macrophages exhibited upregulation of collagen genes (Col1a1, Col1a2, Col3a1 (Fig. 4C). Type I collagen has been associated with endochondral ossification, since it promotes the formation of the cartilagineous scaffold necessary for the subsequent ossification [125].

Another gene that appeared among the most upregulated in macrophages at 7 days after injury is Acp5 (Fig. 4D). This gene encodes for the tartrate-resistant acid phosphatase (TRAP), which is a metalloprotease involved in bone resorption and is typically found in active osteoclasts [126]. At this time point FOP macrophages also upregulate genes encoding for different isoforms of cathepsins (Ctsl, Ctsd, Ctsz), which are typically expressed in osteoclasts and contribute to bone resorption by degrading the organic phase of bone (Fig. 4E).

Enrichment analysis confirmed an upregulation of the HIF-1 signalling pathway at 5 days post-injury and an enrichment in the glycolysis pathway, associated with pro-inflammatory responses [127], maintained through the subsequent timepoint (Fig. 4F). Genes related to osteoclast signalling were also increased at the later timepoint (Fig. 4G).

We further characterized this cluster using Monocle [128], performing a trajectory analysis, to define transcriptomic profiles driving macrophage differentiation in response to injury in control and FOP mice (Fig. 5A).

We set monocytes as the starting point and followed the differentiation into pro-inflammatory and anti-inflammatory macrophages. We defined four clusters and then we analysed their biological functions. The analysis confirmed that control mice exhibited higher percentages of anti-inflammatory macrophages, while FOP mice maintained elevated inflammatory monocytes at 7 days post injury (Fig. 5B).

Analyses of gene expression in different branches of the trajectory revealed distinct patterns (Fig. 5C). We focused on the two clusters generated after branch 1, where inflammatory monocytes and anti-inflammatory macrophages diverge. We found that the left branch, referred to as "Cell fate 1", exhibited an upregulation of genes associated with TNF signalling and osteoclast differentiation (Fig. 5D), while in the other branch, called "Cell fate 2", showed an upregulation of genes related to oxidative phosphorylation and arginine metabolism, commonly linked with anti-inflammatory macrophages [127] (Fig. 5D).

2.5 FAPs actively contribute to bone formation by upregulating ossification genes in FOP mice

Differential gene expression analysis of FAPs unveiled upregulation of Sox9 and Runx2 in FOP mice, master regulators of endochondral ossification, (Fig. 6A) indicating an ongoing ossification process in response to early inflammatory stimuli.

Activin A expression significantly increased in FAPs at 5 days post-injury (Fig. 6B), along with genes linked to pro-fibrogenic development, including Acta2, Fn1, and Timp1 (Fig. 6C). This suggests that FOP mice tend to form more fibrotic tissue in response to muscle injury compared to controls

Transgelin (Tagln) previously associated with FAPs osteoblast and adipocyte differentiation in response to TGF-β stimulation [129], was also upregulated in FOP FAPs (Fig. 6D).

Gene enrichment analysis using Gene Ontology revealed upregulation of HIF-1 and inflammatory response pathways at 5 days post-injury in FOP mice (Fig.6E), followed by increased expression of genes involved in osteoblast differentiation at the subsequent timepoint (Fig. 6F).

Overall, macrophages and FAPs appear to be the primary contributors to HO, creating a niche that supports bone growth as a consequence of muscle inflammation.

2.6 FOP mice display altered cell-cell interactions during muscle regeneration

We conducted a deeper analysis to study cell interactions and cell-cell communication using CellChat. We assessed how pathways varied between cell populations at different

timepoints and conditions by identifying the source and receiver clusters of signalling interactions.

We first analysed cell interactions by timepoint (Fig. 7A). At day 5, control mice showed interactions primarily between endothelial cells, FAPs, and the smooth muscle & pericytes cluster, while FOP mice displayed interactions involving muscle satellite cells (MuSCs) and neural cells.

At the subsequent timepoint, FOP mice exhibited increased interactions in FAPs, with strong intracluster interactions and moderate interactions with MuSCs and neural cells. Control mice, in contrast, mainly demonstrated strong intracluster interactions within the smooth muscle and pericytes cluster.

We also assessed alterations in cell interactions between timepoints (Fig. 7B).

Control mice primarily featured smooth muscle & pericytes, dendritic cells, neural cells, and endothelial cell clusters as the main signalling contributors. Macrophages in control mice predominantly engaged with other immune populations such as T cells and neutrophils.

In contrast, FOP mice exhibited an overall increase in cluster communication from day 5 to day 7 post-injury. The most robust interactions were driven by fibro-adipogenic progenitors (FAPs), with significant interactions between FAPs themselves and endothelial cells. Intriguingly, FOP macrophages displayed reduced interactions with T cells and neutrophils but increased interactions with FAPs compared to controls.

Furthermore, we conducted a comprehensive analysis of significantly increased signalling pathways across all clusters to identify those heightened in FOP mice (Fig. 7C). At 5 days after the injury, we observed a significant upregulation in pathways related to L1CAM, SPP1, ANGPTL, TWEAK, PTN, CCL, CD52 and TENASCIN. At the subsequent timepoint, FOP mice maintained the upregulation of signalling pathways related to SPP1, ANGPTL, PTN and TENASCIN, while they also displayed a significant increase in the pathways related to PERIOSTIN, VISFATIN, FN1, THBS and COLLAGEN.

Interestingly, the upregulated pathways in FOP mice are mainly involved in the processes such as endochondral ossification, angiogenesis and fibrosis, corroborating our previous finding from DGE and gene enrichment analyses.

Furthermore, CellChat analysis provided us significant insights into cell-cell interactions in response to a muscle injury. Control mice display more ordered dynamics of skeletal muscle regeneration, with FAPs, MuSCs and endothelial cells initiating interactions at day 5 after the injury, followed by pericytes, T cells and neutrophils at day 7. Conversely, FOP mice showed similar interactions but at a delayed timepoint, with FAPs emerging as major signalling contributors and interacting with various cell types.

These results, coupled with the distinctive pattern of pathway activation, provide valuable insights into how the muscular regenerating microenvironment in FOP diverges from the normal response.

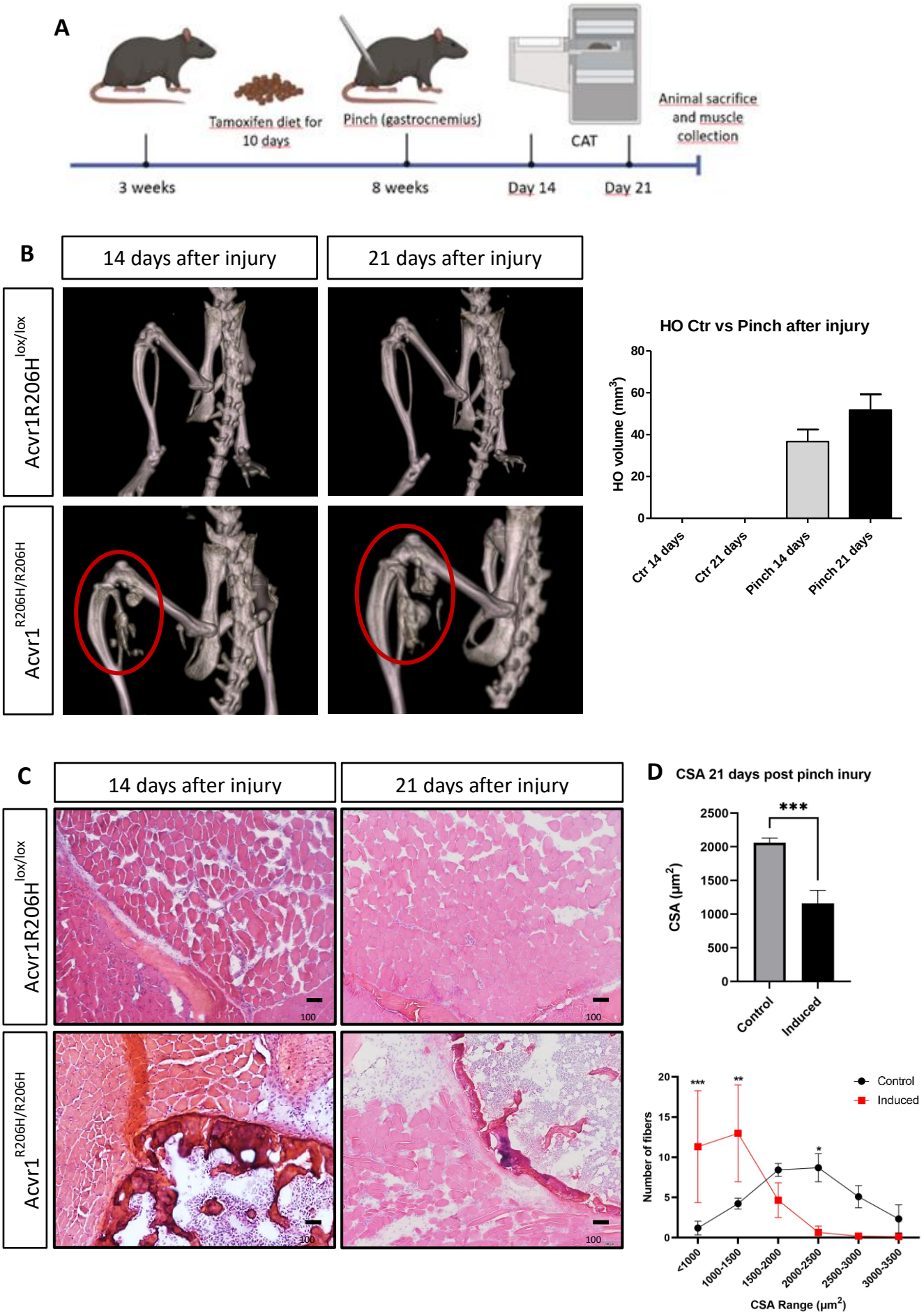

Fig. 1 A) Experimental design of the ossification protocol. B) CAT scans reveal HO in FOP mice (left) and ectopic ossification volume in control and FOP mice (right). C) Muscle sections stained with H&E of control and FOP mice gastrocnemius 14 and 21 days after pinch injury (right), evaluation of muscles CSA 21 days after the injury.

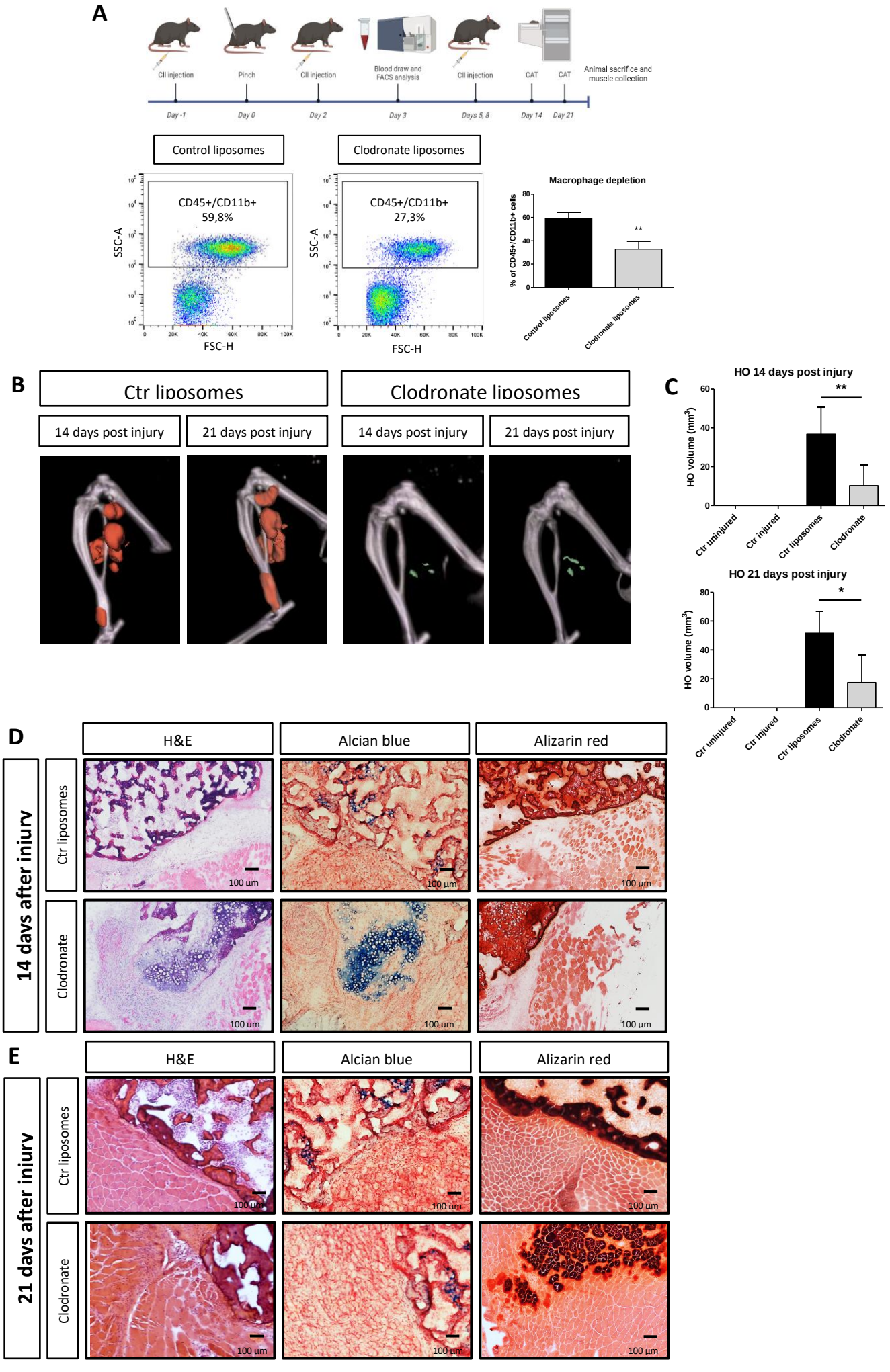

Fig. 2 A) Experimental design for clodronate treatment and estimate of macrophage depletion by FACS analysis. B) CAT scans of control and clodronate-treated FOP mice. C) Ectopic ossification volume in control and clodronate-treated FOP mice. D-E) Muscle sections stained with H&E, Alcian Blue and Alizarin Red of control and clodronate-treated FOP mice gastrocnemius 14 and 21 days after pinch injury.

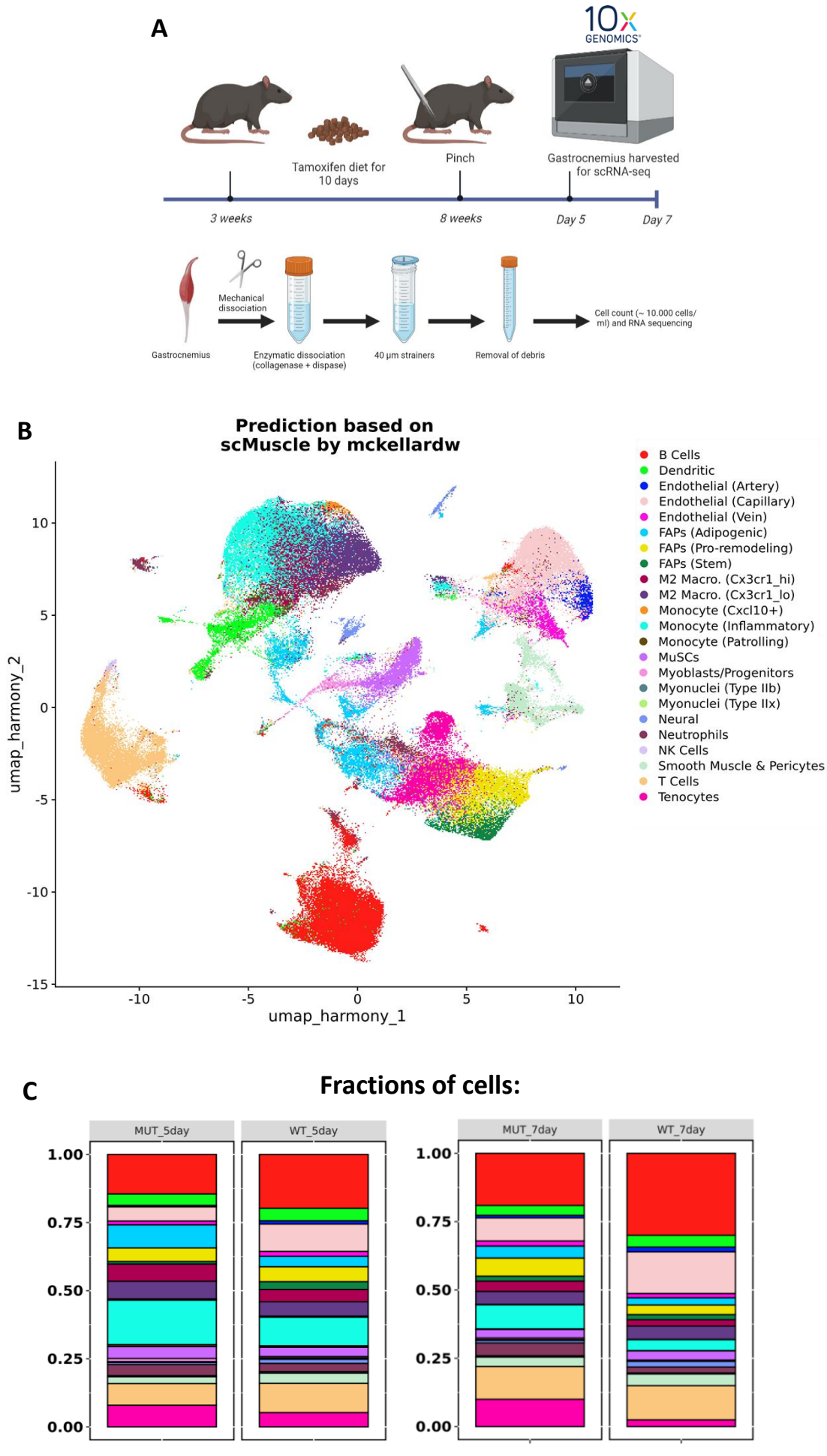

Fig. 3 A) Experimental design of single-cell RNA sequencing. B) Cell clusters definition after data integration. C) Variation of cell populations 5 and 7 days after the injury in control (WT) and FOP mice (MUT).

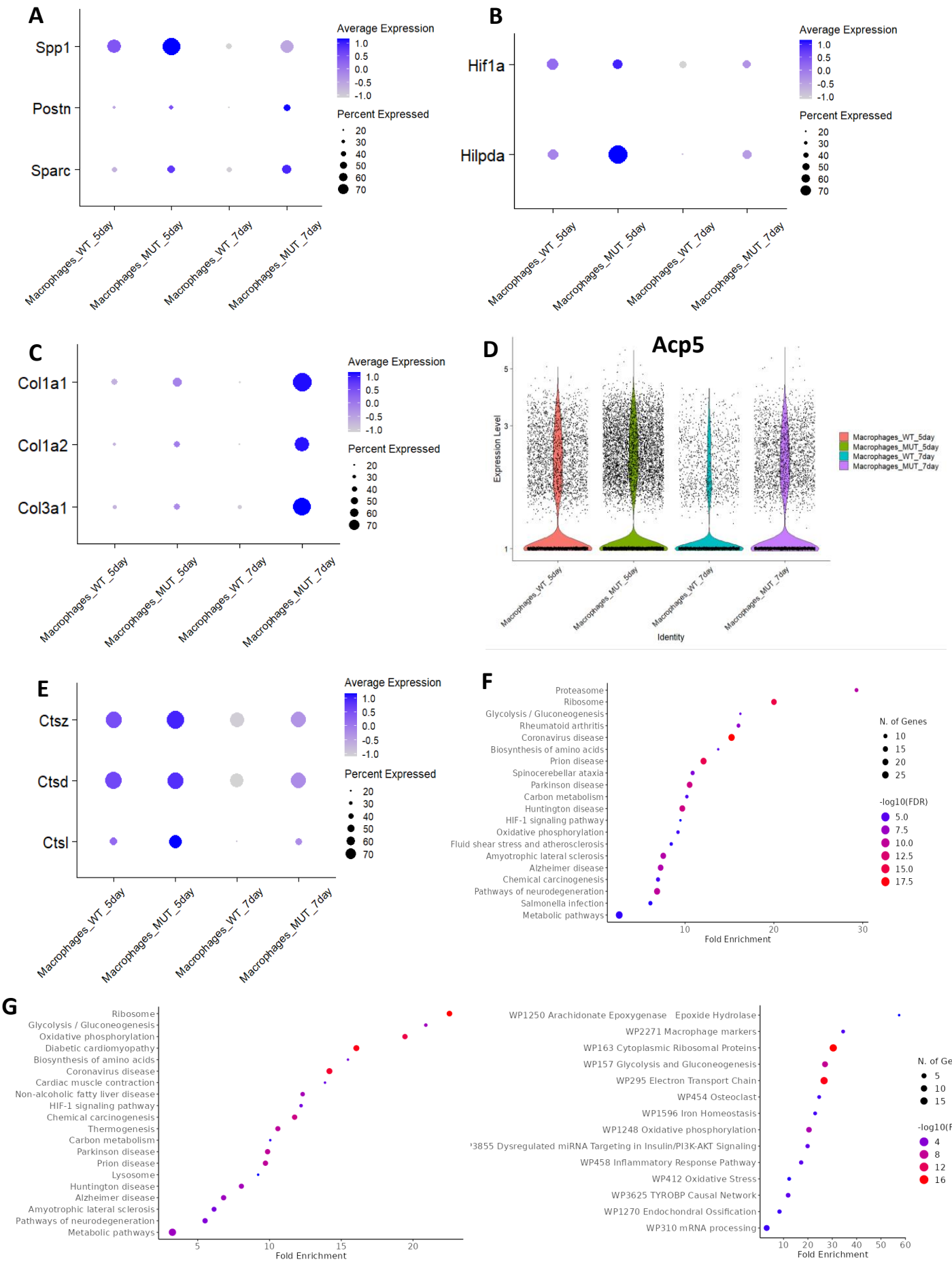

Fig. 4 A) Expression of matrix remodelling genes in control and FOP macrophages after muscle injury. B) Expression of hypoxia genes in control and FOP macrophages after muscle injury. C) Expression of collagen genes in control and FOP macrophages after muscle injury. D) Expression of Acp5 in control and FOP macrophages after muscle injury. E) Expression of cathepsins genes in control and FOP macrophages after muscle injury. F-G) enriched pathways in macrophages of FOP mice vs. control mice at 5 (left) and 7 days (right) after muscle injury.

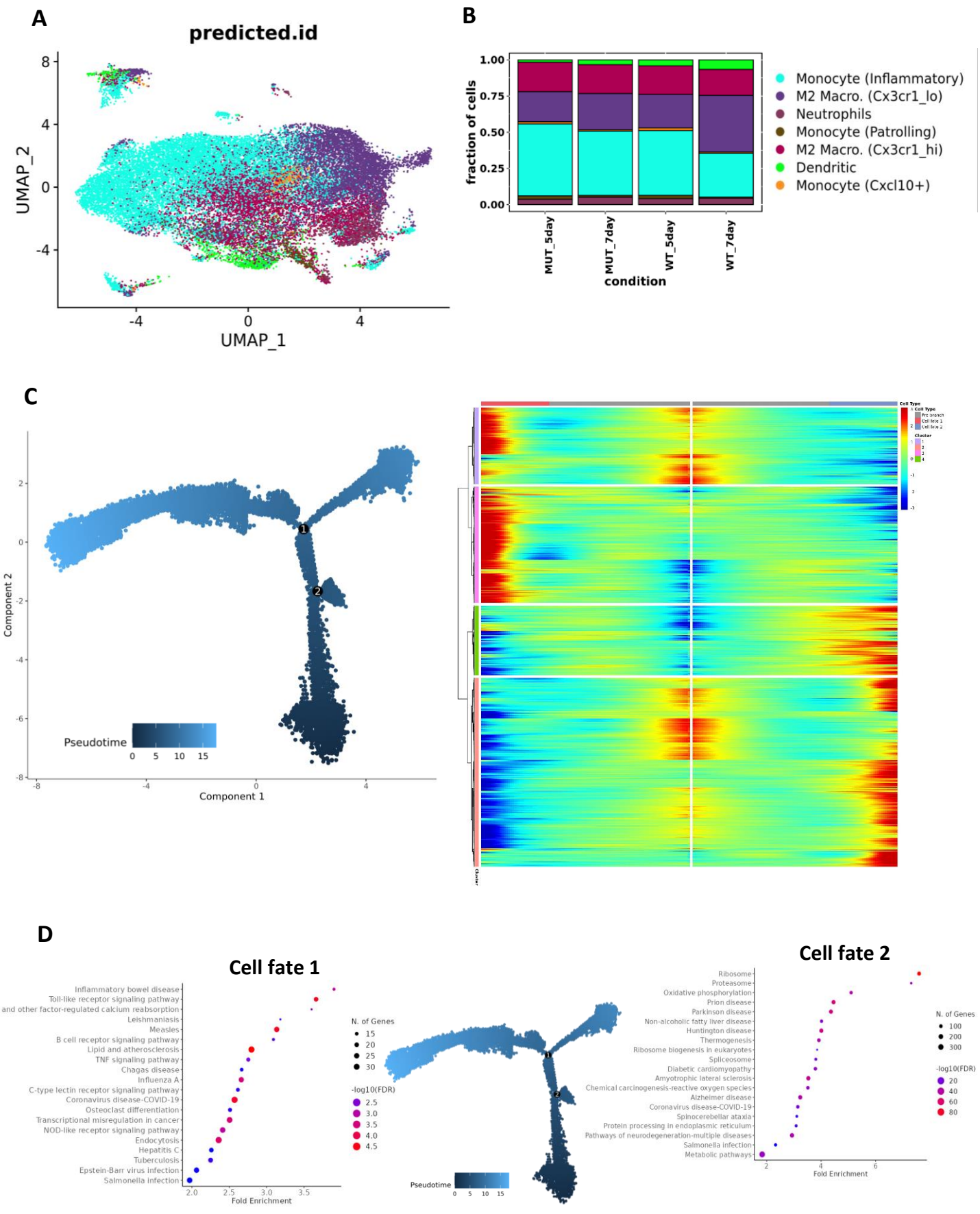

Fig. 5 A) Clustering of monocytes and macrophages. B) Variations of monocyte and macrophage subpopulations after muscle injury. C) Trajectory differentiation of the monocyte and macrophage cluster (right), gene expression profile of clusters as they diverge after the branching (left). D) Enriched pathways in the two main branches after muscle injury.

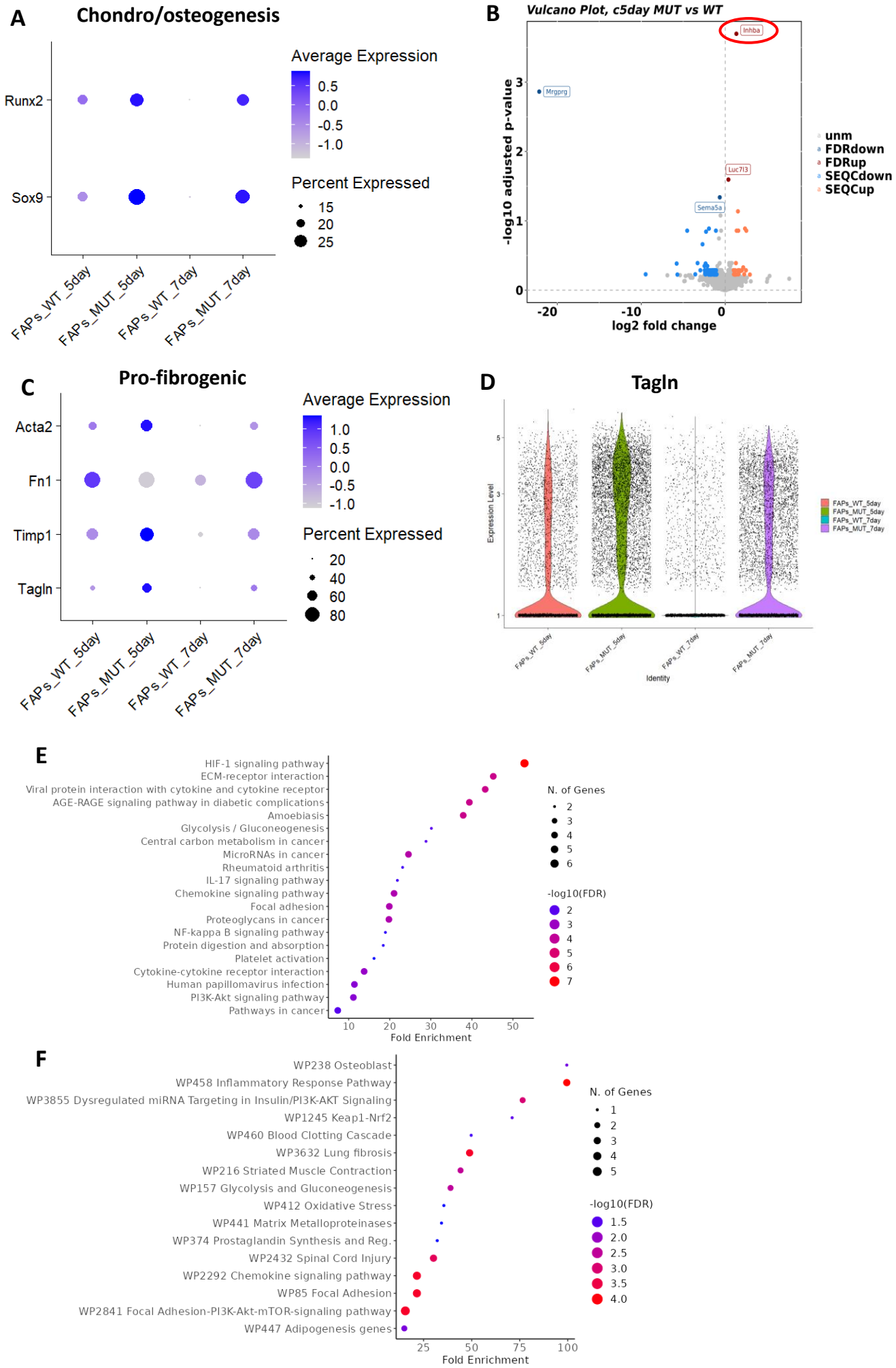

Fig. 6 A) Expression of chondro/osteogenic genes in control and FOP FAPs after muscle injury. B) Activin A upregulation at 5 days after injury in FOP FAPs. C) Expression of pro-fibrogenic genes in control and FOP FAPs after muscle injury. D) Expression of Tagln in control and FOP FAPs after muscle injury. E-F) enriched pathways in FAPs of FOP mice vs. control mice at 5 (E) and 7 days (F) after muscle injury.

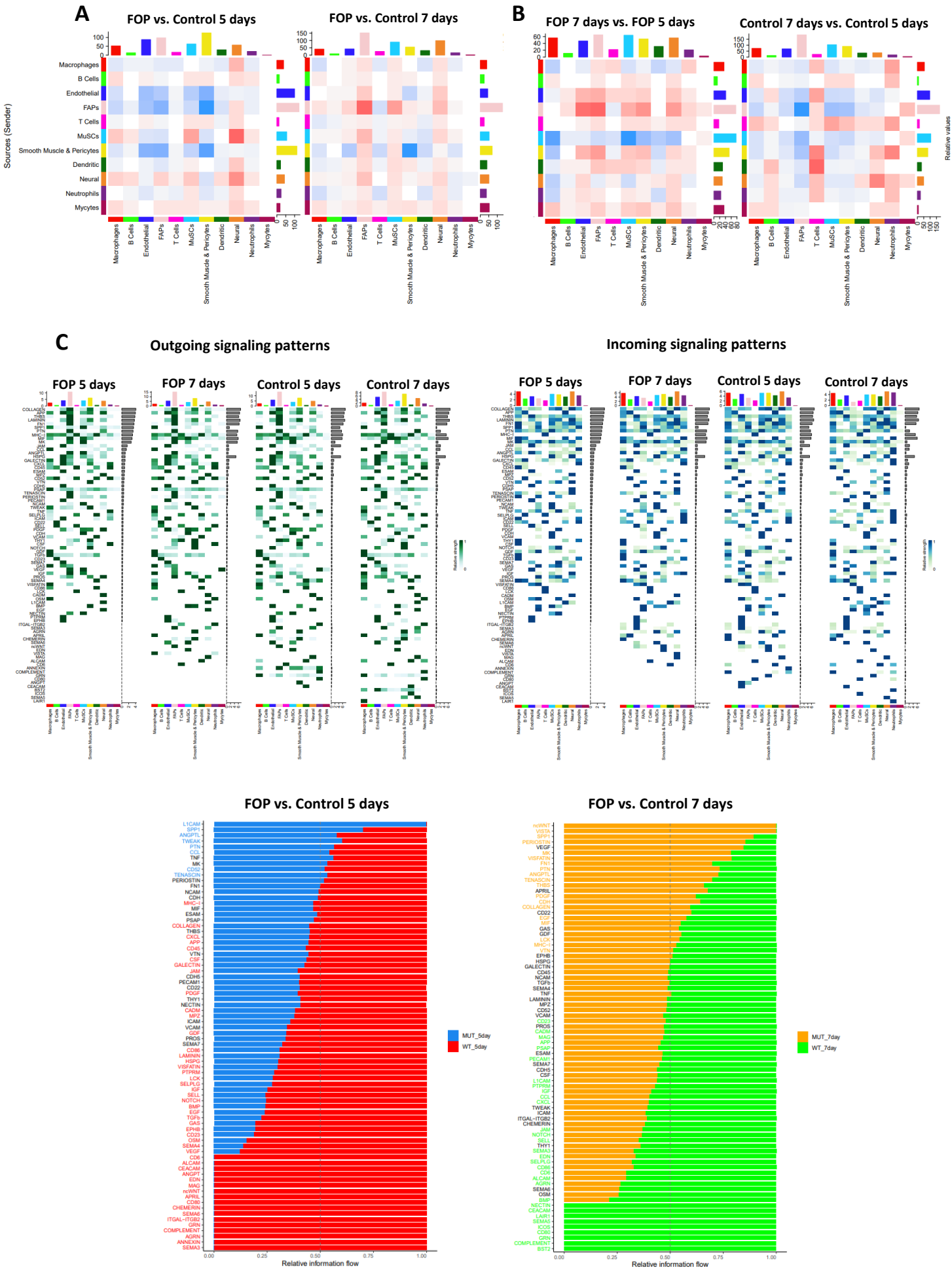

Fig. 6 A) Cell clusters interactions in control vs. FOP mice 5 and 7 days after the injury. B) Cell clusters interactions in FOP mice (7 days vs. 5 days) and control mice (7 days vs. 5 days). C) Variations of signalling pathways at different timepoints in control and FOP mice.

3. Discussion

Fibrodysplasia Ossificans Progressiva is an extremely rare disease characterized by extraskeletal bone formation, primarily in soft tissues. Extensive efforts have been made to discover a treatment for FOP in clinical trials, but a conclusive cure remains elusive. Moreover, acquiring specimens from patients proves highly challenging due to the potential induction of HO through surgery. Therefore, the development of animal models is essential for in-depth research into this pathology. Given the high perinatal lethality observed in FOP mice with constitutive expression of the mutated ACVR1, it is imperative to rely on mouse models where the expression of the mutated ACVR1 is conditionally controlled [105]. For this study, we utilized the Acvr1R206Hlox/lox;R26CreERT2 mouse strain, provided by Dr. Daniel Perrien and IFOPA at Emory University. This conditional FOP mouse model ensures widespread expression of the human mutant ACVR1 receptor upon Cre-mediated recombination. By deactivating the mutated receptor at 3 weeks of age, approximately equivalent to 2.3 years in humans, we achieve early induction while circumventing the issue of perinatal lethality [130]. Another significant advantage of this model lies in the utilization of the human variant of the mutated receptor. This enabled a direct examination of the role played by the mutated human receptor in murine cells, given the striking similarity in protein sequences between the two receptors. Additionally, previous reports indicate that mice tolerate the expression of the human receptor quite well [131].

A primary aim of this study was to establish a protocol for inducing HO in adulthood in a consistent and reliable way. To this extent, we induced a mechanical injury in the gastrocnemius muscle by pinching it with a pair of forceps. We chose this protocol because we observed a higher reproducibility in terms of HO compared to cardiotoxin injury. It has been shown that that cardiotoxin injection mediated injury trigger a stronger but shorter inflammatory stimulus compared to a mechanical injury [132]. However, our goal was to maintain a consistent level of inflammation in the injured muscles. Additionally, we opted to steer clear of needle-based injury, as even a minor trauma could introduce a potential bias regarding the extent of ossification. This approach enabled us to stimulate heterotopic ossification through an inflammatory stimulus, mirroring the natural course observed in FOP patients following spontaneous flare-ups.

After establishing the experimental model, we started to investigate the role of the immune system in FOP. Current understanding indicates that the immune system intricately regulates remodelling and regeneration of every tissues. This has been particularly investigated in the context of the skeletal muscle where upon injury, a first inflammatory phase is required to allow the clearance of dead cells and debris. This is followed by a pro-regenerative phase [133]. Amongst the different immune populations, macrophages play a fundamental role in regulating the inflammatory environment [134].

Several studies have examined macrophage role in ossification. It was demonstrated that macrophage depletion led to delayed bone regeneration in a bone fracture mouse model [135]. The unbalance between M1 and M2 macrophages led to the persistence of an endochondral intermediate and to delayed bone regeneration [135]. The role of macrophages was also studied in mouse models of acquired HO, with BMP-2 promoting ossification after muscle injury with cardiotoxin. An increase of ectopic bone volume was observed when macrophage where depleted mice after the injury [70]. Conversely, macrophage depletion in a FOP mouse model taking advantage of the Acvr1[R206H]FlEx/+;Gt(ROSA)26Sortm1(rtTA*M2)Jae;Tg(tetO-Cre)1Jaw strain led to a decrease of HO volume [69]. Given the divergence of outcomes observed in these studies, the role of macrophages in HO still remains enigmatic.

Since we were interested in understanding the actual impact of macrophages in the our FOP mouse model, we applied a similar experimental strategy. When we depleted the macrophages with multiple clodronate liposomes injections and injured the muscles by mechanical stimuli, we observed a significant decrease (almost 75%) of ectopic bone volume in the injured muscles of FOP mice.

It is evident that clodronate treatment yields distinct effects based on the specific condition under consideration. In instances like a bone fracture [135] and the BMP2-induced HO model, where macrophage depletion occurs in a wild-type background following an injury, there is an increase in ectopic bone formation likely due to the essential role macrophages play in the initial stages of injury, and the deficiency of M1 macrophages hampers the transition to the M2 phenotype, which is responsible for proper tissue healing.

The different outcomes observed in FOP mice highlight the effect of the Acvr1R206H mutation not only in osteogenic cells, but also in all cells of the niche and raised the

question about which macrophage population predominantly drives heterotopic ossification in a FOP genetic context.
We therefore opted to utilize single-cell RNA sequencing to probe into the inflammatory condition of the injured muscles.

Combining our observations from clodronate treatments and the examination of cell clusters derived from single-cell trascriptomic analysis, we formulated the hypothesis that M1 macrophages may have a significant role in the initial stages of heterotopic ossification in FOP.
It has been described that injured FOP and wild-type muscles are similar in terms of immune infiltrate during the first days after the injury [69]. However, FOP lesions remain more inflamed as time passes.
Analysis of the monocytes/macrophages clusters, showed indeed that inflammatory monocytes remained higher in FOP mice only starting from 7 days after the injury.
Examining the transcriptomic profile of this cell population provided us with deeper insights into the molecular aspect of inflammation.
Our initial observation was an upregulation of hypoxic genes in FOP macrophages. Hypoxia usually arises in response to injuries, since damaged tissues are characterized by blood vessels disruption, causing a lack of oxygen perfusion and of nutrients. In this sense, hypoxic stress works as a signal that stimulates tissue regeneration by favouring the recruitment of endothelial cells, fibroblasts and immune cells [136]. However, if the whole process of revascularisation fails and a prolonged hypoxic environment is established, this eventually leads to impaired muscle regeneration and functional loss of tissue [137].
Hypoxia is also strictly linked to inflammation [121]. Studies have indicated that the activation of Hypoxia-inducible Factor-1α (HIF-1α) leads to enhanced aggregation, invasion, and motility in macrophages. This, in turn, promotes the initial inflammatory response[138].
Hypoxia could be particularly relevant in our study since we also found that FAPs presented an upregulation of hypoxic genes in FOP mice. It has been reported that at low oxygen conditions, FAPs show increased levels of HIF-1α, which controls the transcription of Vegf, Bmps and Nrp-1, controlling mesenchymal cells differentiation and

angiogenesis [139]. Furthermore, it was shown that a hypoxic microenvironment drives the differentiation of FAPs into osteogenic cells, making them sensitive to ossification factors, such as BMPs [140]. Additionally, research has shown that chondrocytes lacking HIF-1α exhibit a marked reduction in collagen and aggrecan expression, both at the transcriptional and protein levels. Concurrently, HIF-1α has been shown to enhance chondrocyte survival and proliferation by regulating glycolysis metabolism under both aerobic and anaerobic conditions [141].

Our study substantiated this finding through the observed upregulation of master genes Sox9 and Runx2, which govern chondro/osteogenesis. Additionally, the heightened presence of genes associated with osteoblast differentiation unequivocally indicates that these cells possess an osteogenic fate in FOP mice.

The associations between macrophages and hypoxia might extend even deeper. Our bioinformatics assessments emphasized the heightened expression of osteoclast markers in FOP macrophages. The significance of osteoclasts, particularly in heterotopic ossification, may have broader implications in bone development and may not solely pertain to basic bone degradation. In fact, prior studies have demonstrated the efficacy of bisphosphonates in countering heterotopic ossification through osteoclast depletion. [142].

Nonetheless, their viability as potential targets for HO treatment in FOP patients remains a subject of intense debate. Other studies have indeed shown that administering bisphosphonates to inhibit osteoclasts does not impede ectopic bone formation. This is attributed to the inherent limitations of naïve osteoclasts, which face constraints in effectively removing ectopic calcification due to restricted activity, limited migration, and inadequate adhesion to sites of ectopic calcification. [143].

Whether they may not have an impact on newly formed ectopic bone, they may have an impact on pre-existing bones. Even if this aspect still needs to be better elucidated, we did not only observe heterotopic ossification in isolated centres inside the injured gastrocnemius, but we also detected new bone forming upon long bones such as the tibia and the femur.

This may raise the hypothesis that FAPs could be responsible for the formation of isolated ossification centres in the muscles by differentiating into osteoblasts, while macrophages may mediate the fusion with pre-existing bones by differentiating into osteoclasts and thus promoting bone remodelling.

Finally, hypoxia is also responsible for metabolic changes. Oxygen availability significantly impacts metabolism and exerts a crucial influence on macrophage polarization. Studies have indicated that pro-inflammatory macrophages display elevated glycolytic activity and moderate levels of oxidative phosphorylation, whereas anti-inflammatory macrophages exhibit contrasting metabolic characteristics. [144]. Metabolic reprogramming is indeed required to favour the correct switch between macrophages phenotypes [144]. Our data showed that FOP macrophages exhibit increased glycolytic metabolism that, combined with an upregulation of hypoxia-inducible genes, describe an upregulated pro-inflammatory profile and a decreased capacity of switching to the anti-inflammatory phenotype. Not only macrophages, but also osteoclasts metabolism changes according to their state. Indeed, studies have shown that metabolically, osteoclasts depend on oxidative phosphorylation during differentiation, but switch to glycolysis for energy production during bone resorption. [145].

Activin A plays a central role in promoting heterotopic ossification in FOP [14]. Our transcriptomic analysis suggest that FAPs may be one of the sources of activin A in the early phases of HO, since we detected an upregulated expression of Inhba in this cluster at 5 days after the injury. FAPs have recently been described as a source of activin A in human cholesteatoma and it was reported that they can promote osteoclast differentiation [146]. Interestingly, even if the reported case is completely different from FOP, we observed a similar upregulation of several genes belonging to osteoclastogenesis, such as Sparc and different collagen isoforms, in our FOP mice too [146].

This could potentially align with our prior discoveries regarding macrophages, suggesting that FAPs might have the capacity to prompt infiltrating monocytes to undergo differentiation into osteoclasts. Additional in vitro experiments, such as co-cultures, will be instrumental in gaining deeper insights into the potential interactions between macrophages and FAPs.

Combining our findings regarding cell clusters percentages and the enrichment analyses, our data provides substantial evidence that FOP lesions exhibit pronounced inflammation. Within this context, it is plausible that hypoxia plays a pivotal role in fostering this enduring inflammatory profile, while simultaneously steering cell differentiation towards

the creation of a specialized environment that facilitates and sustains heterotopic ossification in FOP mice.

4. Materials and methods

Animals

We used homozigous Acvr1R206H$^{lox/lox}$;Gt(ROSA26)SorCreERT2 (C57BL/6 background) mice for the purpose of this project. This mouse model was kindly provided by the International Fibrodisplasia Ossificants Progressiva Association (IFOPA) and was created thanks to a collaboration between Daniel Perrien (Vanderbilt University), Aris Economides (Regeneron), Yuji Mishina (University of Michigan), Maurizio Pacifici (Children's Hospital of Philadelphia) and Eileen Shore (University of Pennsylvania).
Acvr1R206H$^{lox/lox}$;R26SorCreERT2 mice were housed in the SPF facility at San Raffaele Scientific Institute (Milan, Italy) and treated with the approval of the Institutional Animal Care and Use Committee (IACUC 725).

Ossification protocol

Cre activation in Acvr1R206H$^{lox/lox}$;R26SorCreERT2 mice was induced with tamoxifen diet (Tamoxifen Diet TAM400/CreER, Envigo) for 10 days and eventually checked by PCR. At 8 weeks of age, mice were injured in the gastrocnemius of only one hind limb. Before injuring them, mice were anesthetized with isoflurane. For the injury, the muscles were compressed using a pair of tweezers for 15 seconds, leaving a space of roughly 1 mm between the ends of the tweezers. After all the experimental procedures, mice were euthanized and dissected for the collection of the injured muscles for further procedures.

Macrophage depletion and FACS analysis

At the age of 8 weeks, Acvr1R206H$^{lox/lox}$;R26SorCreERT2 mice were injected intravenously with clodronate (1.8 mg/mouse) or PBS (control) liposomes (Liposoma BV). The injections were performed 1 day before the muscle injury and every 3 days afterwards (at 2, 5 and 8 days after muscle injury).
Depletion of circulating monocytes was checked by FACS analysis. Few µl of blood were taken from the tail of the animals and collected in tubes with EDTA. Samples were

incubated for 15 minutes with red cell lysis buffer to eliminate erythrocytes; after the incubation, we added PBS to block the buffer. Cells were centrifuged at 1'200 rpm for 5 minutes at rt and supernatant was discarded. The pellet was then resuspended in CMF with the following antibody mix: CD45 (1:200 PE-conjugated, Biolegend), CD11b (1:400, Pe-Cy7, Biolegend); samples were let incubating for 15 minutes at 4°C. Finally, Hoechst was added (1:1000 in CMF, Invitrogen). Flow cytometry was performed to check monocytes depletion (MoFlo Astrios EQ, Beckmann Coulter Life Sciences); monocytes were gated as single live CD45+/CD11b+ cells.

In vivo computerized tomography

Micro-computerized tomography (μCT) was performed at day 14 and 21 to monitor ectopic bone formation after muscle injury. *In vivo* μCT imaging was performed using the IVIS SpectrumCT Pre-clinical *in Vivo* Imaging System (Perkin-Elmer, Waltham, MA, USA). μCT images were acquired without any contrast medium, with the following parameters: x-ray tube voltage = 50 kV, tube current = 1 mA, x-ray focal spot size = 50 μm. The μCT images calibrated in Hounsfield unit (HU) were reconstructed with a voxel size of 75 μm^3. Threshold-based image segmentation was performed to obtain a 3D reconstruction and quantification of the ossification.

CT scans visualization, rendering and quantification of the ectopic bone volume of in the injured muscles was done on Slicer 3.0.

Histology

The injured gastrocnemius were frozen for histological analysis by snap freezing. A small amount of tragacanth gum (Thermo Scientific) on a slice of cork. The gastrocnemius muscles were placed onto the tragacanth gum leaving about 3/4ths of the muscle outside, making sure to have the muscle in a perpendicular position with respect to the cork.

After that, an aluminium can was filled with isopentane and suspended in a tank containing liquid nitrogen. Once isopentane had reached the proper temperature, white solid particles form at the bottom of the can. At this point, we rapidly dipped the cork with the muscle into isopentane, keeping the muscle position downwards for 30 seconds. Frozen muscles were then kept in dry ice until storage at -80°C.

To perform subsequent immunohistochemistry stainings, muscle sections were cut on a cryostat with a 10 µm thickness. Hemtaoxylin & eosin, alcian blue and alizarin red stainings were subsequently performed.

Hematoxylin & eosin: glass slides were thawed and quickly hydrated in distilled H_2O; sections were then fixed with neutral buffered 10% formalin solution (Sigma-Aldrich) for 10 minutes at room temperature and quickly washed in deionized water.

Sections were stained in hematoxylin (ScyTek Laboratories) for 30 seconds, rinsed under tap water and stained in eosin (ScyTek Laboratories) for 1 minute. Dehydration was performed through graded alcohols (70%, 90% and 100%) for 3 minutes each. Finally, slides were cleared in xylene (Electron Microscopy Sciences) for 1 minute and mounted with Eukitt Quick-hardening mounting medium (Sigma-Aldrich).

Alcian blue: sections were thawed and hydrated in distilled water. The staining was performed according to the protocol booklet provided by Abcam (Alcian Blue Stain Kit, pH 2.5, Mucin Stain).

Slides were incubated in acetic acid solution for 3 minutes before incubation in Alcian Blue (pH 2.5) solution for 30 minutes at room temperature. Slides were then briefly rinsed in acetic acid solution to remove excess alcian blue. Sections were rinsed for 2 minutes in running tap water followed by 2 changes of distilled water. Nuclear Fast Red Solution was then applied on the slides for 5 minutes, followed by a rinse in running tap water for 2 minutes and 2 changes of distilled water. Dehydration was performed through graded alcohols (70%, 90% and 100%) for 3 minutes each.

Finally, slides were cleared in xylene for 1 minute and mounted with Eukitt Quick-hardening mounting medium (Sigma-Aldrich).

Alizarin red: sections were thawed and hydrated in distilled water. Alizarin red solution 2% was prepared dissolving Alizarin red S powder (Sigma-Aldrich) in distilled water and then filtered with 0.22 µm strainers. Slides were stained with the alizarin red solution for 2 minutes. Excess dye was removed by quickly rinsing the slides under tap water. Dehydration was performed through graded alcohols (70%, 90% and 100%) for 3 minutes each.

Finally, slides were cleared in xylene for 1 minute and mounted with Eukitt Quick-hardening mounting medium (Sigma-Aldrich).

Sample preparation for Single-cell RNA sequencing

For single-cell RNA sequencing, Acvr1R206H$^{lox/lox}$;R26SorCreERT2 and Acvr1$^{R206H/R206H}$;R26SorCreERT2 mice were used respectively as controls and FOP mice. We used three mice for each genotype in order to have enough biological replicates to perform statistical analyses. The sequencing was performed both at 5 and 7 days after the injury, for a total of 4 experimental conditions (control 5 days post injury, control 7 days post injury, FOP 5 days post injury and FOP 7 days post injury).

Muscles were harvested and minced on a petri dish with a pair of scissors, incubated on a shaker at 37°C adding 2 ml of PBS mixed with collagenase (1:15, Sigma-Aldrich) and dispase (1:10, Gibco) for 30 minutes. After the first incubation, a part of the supernatant was removed, 2 ml of PBS with collagenase and dispase were added and the samples were left incubating on a shaker at 37°C for 30 minutes. After the incubation, the suspension was resuspended using a syringe with a 27g needle and then with a 16g needle, until the suspension looked uniform without floating pieces of muscles. The suspension was then filtered through 40 µm cell strainers and 3 ml of CMF (Calcium/Magnesium-Free PBS, 10% FBS, 5% Pen/Strep, 2 mM EDTA) were added to block the enzymes.

After centrifugation at 250xg for 10 minutes at rt, the supernatant was removed and the pellet was resuspended in 200 µl of red cell lysis buffer and left incubating for 10 minutes at rt. After the incubation, the red cell lysis buffer was blocked adding 1 ml of CMF.

To remove cell debris, the samples were processed with the Debris Removal Solution (Miltenyi). Cell suspension was centrifuged at 300xg for 10 minutes at 4°C, then supernatant was aspirated and the pellet was resuspended in 4 ml of cold PBS and transfered in a 15 ml tube. Cold Debris Removal Solution (900 µl) was added and mixed by pipetting 10 times, then overlayed with 4 ml of cold PBS without mixing the two phases. Cells were then centrifuged at 3000xg for 10 minutes at 4°C.

Supernatant was slowly aspirated and discarded and 15 ml of PBS were added, followed by centrifugation at 1000xg for 10 minutes at 4°C. Finally, supernatant was aspirated completely and cells were resuspended in cold PBS. We counted the cells and confirmed that cell viability was higher than 90%.

Single-cell RNA Sequencing

Roughly 10’000 single live cells per sample were counted and processed on the Chromium platform (10x Genomics) using the Chromium Single Cell 3′ Library & Gel Bead Kit v3 kit (10x Genomics). We applied the standard pre-processing workflow for scRNA-seq data in Seurat within R environment (version 4.2.1): selection and filtration of cells based on QC metrics, data normalization and scaling, and detection of highly variable features.

We applied the following filters: number of unique genes detected in each cell > 200 (to exclude low-quality cells or empty droplets) and < 6000 (to exclude possible multiplexes); expressed features: genes expressed in at least in 5 cells; percentage of reads that map to the mitochondrial genome < 10%. The output of the filter stage is a total of 19’493 cells and 26,500 genes, out of those features 2000 were identified as variable features.

Seurat v4 was used to merge the 12 samples of this experiment and Harmony was used to perform the integration of single cell genomics datasets. Starting from a merged object scaled and normalized according to the default Seurat pipeline. We opted for Harmony to remove the influence of sample-of-origin from the embedding.

The aim of the integration is to transform datasets into a shared space to compare them and harmonize differences due to batch effects. PCA, UMAP and clustering are performed on the integrated object.

Cell clusters were then built by using a shared nearest neighbour (SNN) modularity optimization based clustering algorithm which groups cells depending on the differences between values on their Principal Components (PC) and a resolution parameter [147].

The number of PC has been chosen according to the PCA analysis presented in SI. Here we have used 30 PC and a resolution of 0.2 and detected 19 clusters.

To investigate the identity of the obtained clusters we assess the cluster’s Marker Genes (MG). MG are computed by performing Differential Genes Expression (DGE) analysis between the cluster under examination and all the other ones, considering all the expressed genes in the cell. Differential Gene Expression analysis was performed with DGE Seurat approach, which identifies differentially expressed genes between two groups of cells using a Wilcoxon Rank Sum test (via the FindMarkers function). To estimate the identity of the cells, we used the data set proposed by McKellar et al [148].

We perform enrichment analysis of the genes differentially expressed in each cell type with the CRAN enrichR package (v.2.1), which provides an R interface to the enrichr databases and statistics (GO_Biological_Process_2021, GO_Cellular_Component_2021, GO_Molecular_Function_2021, Reactome_2016, KEGG_2019_Mouse, WikiPathways_2019_Mouse, BioCarta _2016).

We used the Monocle2 R package [149] to perform a trajectory analysis of macrophages subpopulations post-injury. First, we subsetted the cells labelled as macrophages and monocytes from the Seurat dataset and across all time points and samples. Second, we performed unsupervised SNN clustering in order to identify new subpopulations in the data, from which we then used the Seurat FindAllClusters function to find differentially expressed genes that characterize the subpopulations. We selected the most expressed genes based on fold-change expression with a minimum of log2(0.25) and adjusted p value of 0.05. This list of differentially expressed genes was then used for clustering and ordering cells using the DDRTree method and reverse graph embedding. To identify genes that are differentially expressed across Monocle branches (states), we transferred the labels back to the Harmony dataset and performed differential expression analysis as described above.

To identify conserved and altered communication networks in control and FOP mice, intra- and intercellular communication networks were modelled based on the abundance of known ligand-receptor transcript pairs with CellChat (version 1.5.0).

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