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Stem Cells and Heterotopic Ossification: Lessons from Animal Models

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Abstract

Put most simply, heterotopic ossification (HO) is the abnormal formation of bone at extraskeletal sites. HO can be classified into two main subtypes, genetic and acquired. Acquired HO is a common complication of major connective tissue injury, traumatic central nervous system injury, and surgical interventions, where it can cause significant pain and postoperative disability. A particularly devastating form of HO is manifested in the rare genetic disorder, fibrodysplasia ossificans progressiva (FOP), in which progressive heterotopic bone formation occurs throughout life, resulting in painful and disabling cumulative immobility. While the central role of stem/progenitor cell populations in HO is firmly established, the identity of the offending cell type(s) remains to be conclusively determined, and little is known of the mechanisms that direct these progenitor cells to initiate cartilage and bone formation. In this review, we summarize current knowledge of the cells responsible for acquired HO and FOP, highlighting the strengths and weaknesses of animal models used to interrogate the cellular origins of HO.

Keywords

animal models; heterotopic ossification; stem cells; progenitor cells; fibrodysplasia ossificans progressiva; FOP; ACVR1; ALK2; activin; BMP; fibro/adipogenic progenitors; FAPs; satellite cell; endothelium; skeletal muscle; tendon; PDGFR α ; TIE2; scleraxis; Mx1; osteogenesis; cartilage; bone

1. Introduction

Heterotopic ossification (HO) is a debilitating condition that results from genetic disease or traumatic injury. Bone formation in heterotopic ossification can either occur directly (intramembranous ossification), as is the primary mechanism of HO in progressive osseous heteroplasia (POH), or can be preceded by the formation of a cartilage anlagen, termed endochondral ossification (see [1,2], for review). This review focuses on endochondral HO, which appears to be the primary pathogenic mechanism for ossification of the skeletal

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muscle and associated soft tissues[2–4]. Injury and soft tissue inflammation are significant triggers for HO, although the mechanistic role of these triggers has been difficult to discern, as an inflammatory response is typically associated with soft tissue injury. Epidemiological studies have shown a 10–20% incidence of HO following traumatic injury, which can include injuries as varied as soft tissue sports injuries, central nervous system damage, total hip arthroplasty, and burns[2,5,6]. HO is particularly prevalent in residual limbs following amputations related to traumatic combat injuries[3,4]. HO following traumatic injury can cause significant pain and swelling, have a profound effect on muscle structure, result in joint ankylosis, impede rehabilitation, and affect the extent of functional recovery[2–6]. HO reaches its most extreme form in fibrodysplasia ossificans progressiva (FOP), an ultra-rare, catastrophically disabling genetic disease in which seemingly spontaneous HO of the musculoskeletal system—often beginning in early childhood—progressively immobilizes afflicted individuals. Injury is also a trigger for HO in FOP patients; however, in this genetically susceptible background, even mild soft tissue trauma and inflammation (biopsies, immunizations, mandibular blocks for dental procedures, incidental bumps and bruises) can result in severe HO[1,7,8].

Despite the prevalence of HO in the general population and its significant impact on quality of life, treatment modalities are limited to systemic administration of non-steroidal anti-inflammatory medications, compounds that inhibit calcium phosphate deposition, radiation therapy, and surgery[2]. These treatments usually have only modest benefit and are accompanied by significant side effects. Surgical resection, for example, can be indicated in some cases, although post-operative complications and a high recurrence rate limit its application[2]. Although the therapeutic horizon appears promising[9], no current treatments have proven effective in slowing the progression of HO in FOP patients, and surgical intervention is almost never indicated, as a robust new osteogenic response is a likely outcome[2,10–15].

Developing effective and specific strategies to eliminate or reduce the incidence and extent of HO will require basic knowledge of the osteogenic signals, the identity of the cells that respond to such signals, and the relationship of soft tissue trauma and inflammation to the activation of the osteogenic program among competent cells. While the histological pathology of endochondral HO is well documented, the respective roles of specific cell populations in mediating the initiation and growth of HO are only beginning to be understood. This review will first provide an overview of murine HO models, with a focus on those that have shown utility in identifying candidate stem/progenitor populations responsible for HO. We will then summarize and evaluate the relevance of cell populations that have been implicated in acquired HO and FOP.

2. Animal models of heterotopic ossification

HO models for adult stem cell discovery have two major components: mechanisms of eliciting HO and methodological tools to discover and characterize relevant cell populations. Section 2 will focus on experimental paradigms of HO induction, while Section 3 will focus on cell discovery. Several animal models have been developed to explore HO pathogenesis of distinct etiologies. Because underlying proximate causes and pathophysiological

mechanisms are often poorly understood, uncertainties remain, in many cases, as to the accuracy of such models to the corresponding human condition. Yet, even models that elicit HO by non-physiological means have been instrumental in discovering candidate cell populations. For example, induction of HO by intramuscular implantation of large quantities of osteogenic bone morphogenetic proteins (BMPs) have yielded important clues of the cellular origin of HO lesions. With regard to FOP, these data are beginning to be validated using recently developed, genetically accurate models.

2.1 Osteogenic BMP ligand implantation and overexpression

Implantation and overexpression of osteogenic factors are among the most extensively used animal models of HO. Early models included intramuscular injection of alcoholic extracts of bone, bone marrow, and periosteum[16,17]. Later, the seminal study of Urist et al.[18] demonstrated the osteoinductive properties of demineralized bone implantation, which he termed “autoinduction”. These studies ultimately led to the discovery of osteogenic BMPs[19], and follow-up studies with recombinant BMP proteins demonstrated that injection of a single ligand is sufficient to induce HO[20,21]. Since these initial experiments identifying individual osteogenic BMPs, models based on injection of purified BMPs have been widely used to study HO. BMP ligand implantation sites and delivery methods vary depending on the goals of individual studies (see [22], for review). Regarding intramuscular HO, implantation methods using BMP-loaded gels and sponges are most commonly used, as they provide a means for local ligand delivery with minimal disruption of muscle function.

While the non-physiological nature of implanting large quantities of osteogenic BMPs was noted in the Introduction to this section, these models have several advantages and, in important respects, are a reasonable facsimile for HO in humans. Importantly, increased expression of osteogenic BMP ligands can result from soft tissue damage[23–26] and increased expression of BMP4 has been noted in FOP lesions[27,28]. In addition, BMP implantation causes a highly reproducible sequelae of histological events, including tissue inflammation, muscle necrosis, accumulations of mesenchymal cell populations, and emergence of endochondral bone that resembles histological changes in acquired HO and FOP[2,1,29–32]. Although BMP implantation models typically do not incorporate a muscle injury component (apart from the mild injury imposed by BMP injection), and injury is a trigger for both acquired HO and FOP flares, injection of most osteogenic BMPs elicits a robust inflammatory response[33–36]. Thus, a requirement for tissue inflammation may explain, in part, the injury requirement in some models[34,37,38]. In this regard, injection of BMP9 alone causes minimal inflammation and only induces HO when accompanied by a separate inflammatory trigger[34]. In summary, injection of osteogenic ligands is a simple, reproducible, and extensively used method of inducing HO in animal models. Although not a precise model for either acquired HO or FOP, BMP implantation studies have been instrumental for our emerging understanding of HO in humans.

Transgenic delivery of BMP4 has also proven to be a valuable model for investigating offending cell populations and HO pathogenesis generally. Kan et al.[39] made the fortuitous discovery that ectopic expression of BMP4, under the control of the Neuron-specific enolase (*Nse*) promoter[40], reliably produced intramuscular endochondral HO both

spontaneously and in response to injury[39,41,42]. The progression of spontaneous HO began at approximately 1–2 months of age and followed a progression similar to FOP[39]. HO in this model was unexpected, as the NSE-BMP4 mice were developed to study the role of BMP4 in gliogenesis, and the *Nse* promoter was not known to be active in uninjured adult muscle tissue; the BMP4-expressing cells responsible for induction of HO in this model remain unclear. Although the physiological relevance of BMP4 over-expression in uninjured soft tissues remains uncertain, and recent data indicate that activin ligands are essential for both spontaneous and injury-induced HO[43], the NSE-BMP4 model represented the first model of spontaneous HO and demonstrated that BMP4 over-expression is sufficient to cause HO in the absence of exogenously imposed injury. In addition, as for BMP implantation models, the NSE-BMP4 model has provided valuable insights into cellular origins of HO[39,41,42,44,45].

2.2 Cell-specific hyperactivation of BMP signaling

Despite the potential utility of BMP implantation/over-expression for understanding mechanisms of HO, direct versus indirect effects of osteogenic ligands on target stem/progenitor cell populations cannot be distinguished; this is a considerable limitation for understanding the cellular basis of genetic forms of HO, in which disease is presumably driven by cell-autonomous dysregulation of BMP signaling. In this regard, Fukuda et al.[46] utilized the Cre-loxP system (see [47], for review) to generate a conditionally inducible transgenic mouse model that over-expresses an artificial, constitutively active variant of the type I BMP receptor, ACVR1/ALK2, caused by a single amino acid substitution at position 207 of the receptor (Q207D). Upon Cre-mediated recombination, expression of the mutant form of ACVR1 (referred to as caACVR1 herein), which is driven by the strong synthetic CAG promoter/enhancer[48], was sufficient to induce phosphorylation of Smads 1/5/8[46], direct downstream targets of activated type I BMP receptors.

Although global embryonic expression of caACVR1 results in lethality at midgestation[46], restricting caACVR1 expression to adult stages has proven valuable for HO studies. Thus, induction of caACVR1 expression in adult muscle by intramuscular injection of Cre-expressing adenovirus is sufficient to induce endochondral HO that closely resembles the histological progression of BMP-induced HO[37]. Interestingly, induction of HO required antecedent injury when caACVR1 expression was activated with the tamoxifen-inducible, globally expressed CAGGS-CreER[49] driver, indicating that an inflammatory trigger (in these cases, provided by viral infection or injury) is required for induction of HO. More recently, cell restricted Cre drivers have been used to control caACVR1 expression, providing a valuable tool to evaluate the capacity of candidate cell populations to participate in HO cell-autonomously (see Section 3).

Despite the utility of the caACVR1 model, there are distinct differences between this model and accurate genetic models of FOP (see Section 2.4) and mechanisms of HO induction may not be directly applicable to the human condition. For example, the Q207D substitution, which is not found in humans, renders ACVR1 constitutively active[46] and nearly ligand insensitive[46,50], whereas ACVR1(R206H) shows minimal ligand independence and is highly responsive to osteogenic BMPs and activin ligands[43,50–54]. In addition, the

caACVR1 transgene is an over-expression construct that is driven by a strong, constitutive promoter, and the levels and cell specificity of expression is, therefore, not under endogenous control. As such, the caACVR1 model can be an effective tool to facilitate identification of cells with the capacity to undergo osteogenic differentiation, but leaves equivocal the relevance of responsive cells to HO pathogenesis in a physiological setting.

2.3 Tendon injury

Horing 1908[55], Jones 1932[56], and Rothberg 1942[57] presented some of the earliest descriptions of trauma-induced ossification of the human Achilles tendon. Although uncommon in the general population[57–60], tendon/ligament ossification is a significant component of FOP pathogenesis[61,62], and it can be modeled in mice using injury and dysregulated BMP signaling paradigms. In wild-type mice, injury-induced HO of the Achilles tendon is highly penetrant and progresses through the endochondral pathway in a manner that bares close morphological resemblance to BMP implantation, overexpression, and hyperactivation models[63]. By contrast, we have never observed HO following injury of wild-type skeletal muscle, despite the well-documented presence of interstitial osteocompetent cells[38,64,65]. Additionally, tendons of neonatal mice are refractory to injury-induced HO[66]. Whether these differences in HO susceptibility represent key age- and tissue-specific differences in the signaling/inflammatory environments, bioavailability of osteogenic ligands, or susceptibility of distinct stem/progenitor populations remains to be clarified.

2.4 FOP mouse genetic models

The seminal discovery of the FOP-causing heterozygous substitution of c.617G>A (p. R206H) in the *ACVR1* gene[67] provided a much needed genetic target, allowing development of physiological FOP mouse models. Encouragingly, wild-type ACVR1 proteins in humans and mice are virtually identical (>98% identity; NCBI). Thus, there was a strong likelihood that mice engineered to express the ACVR1(R206H) receptor would exhibit classical FOP. Several groups have now produced knockin mice in which the R206H-causing mutation was introduced into exon 5 of the mouse *Acvr1* gene, such that expression of the disease-causing gene is under endogenous transcriptional control. Whereas these new models differ in certain important details, collectively, they represent the most physiologically relevant models for investigations of FOP pathogenesis.

2.4.1 Constitutive *Acvr1*^{R206H} knockin—The first FOP mouse genetic model, an *Acvr1*^{R206H} constitutive knockin, exhibited perinatal lethality in the F1 generation, necessitating analysis of F0 chimeric animals[68]. Lethality of FOP mice was unexpected in light of the mild developmental anomalies exhibited by FOP patients, the most consistent of which is loss of the distal phalanx of the great toes[7,13,62]. Although the dramatically different consequences of embryonic ACVR1(R206H) expression constitutes an important and unexplained difference between FOP mouse models and the human condition, the postnatal phenotype of mice chimeric for the *Acvr1*^{R206H} mutation replicated essentially all features of classic FOP[68]. Importantly, Chakkalakal et al.[68] observed that lesional tissues of chimeric mice were comprised of both *Acvr1*^{R206H/+} and wild-type cells, raising the intriguing possibility that in FOP, ACVR1 non-expressing cells are recruited to sites of

lesion formation initiated by cells expressing ACVR1(R206H). Unfortunately, however, lethality of germline *Acvr1*^{R206H} mice has limited their utility and precluded the use of lineage-tracing strategies to identify the offending cell types.

2.4.2 Conditional *Acvr1*^{R206H} knockin—To overcome the perinatal lethality of the constitutive *Acvr1*^{R206H} mouse, and to define the cell types involved in ACVR1(R206H)-driven HO, Hatsell et al.[43] employed the FLEEx system[69] to establish a knockin mouse line (*Acvr1*^{[R206H]FIEEx}) that conditionally expresses *Acvr1*^{R206H} under control of the endogenous promoter. Briefly, the FLEEx system utilizes Cre-mediated recombination events to turn off the expression of one gene and turn on the expression of another[69]. In this model, loxP and variant lox sites were positioned and oriented such that Cre-mediated DNA excision and inversion events result in the replacement of wild-type exon 5 with the R206H variant, permitting expression of the FOP-causing variant. Although the unrecombined *Acvr1*^{[R206H]FIEEx} allele was designed to function as a wild-type allele, approximately half of the transcripts from the *Acvr1*^{[R206H]FIEEx} allele in ES cells were aberrantly spliced, resulting in the exclusion of exon 5 and a frameshift predicted to encode an inactive product[43]; however, there were no overt phenotypic consequences of reduced *Acvr1* expression from the *Acvr1*^{[R206H]FIEEx} allele in mice[43]. Importantly, when crossed with a globally-expressed, tamoxifen-inducible, Cre driver, *Acvr1*^{[R206H]FIEEx/+} mice developed HO without a requirement for injury, and disease progression and anatomical sites of heterotopic bone resembled that observed in FOP[38,43].

Using this mouse model[43], as well as induced pluripotent stem cells derived from FOP patients bearing the ACVR1(R206H)-causing mutation[54], it was recently discovered that the ACVR1(R206H) receptor can activate osteogenic BMP signaling in response to activin ligands, an unexpected and fundamental advancement in FOP research. Although initial studies with *Acvr1*^{[R206H]FIEEx} mice were not designed to identify activin-responsive cell populations responsible for skeletal lesions, the capacity for cell type-specific activation of ACVR1(R206H) using Cre/loxP methodologies provides a powerful means of identifying offending cell types with the intrinsic capacity to initiate and contribute to HO in FOP (see Section 3.7). In this regard, we have developed a new FOP allele of *Acvr1* (*Acvr1*^{lnR206H}) that, in addition to allowing cell-specific targeting of *Acvr1*^{R206H} expression, incorporates a fluorescent read-out of recombination status of the FOP allele so that issues of cell-autonomy can be directly addressed[70] (Section 3.7).

3. Progenitors of Heterotopic Ossification

As BMP signaling elicits a range of outcomes depending on developmental and cellular context[71,72], it is reasonable to anticipate that only a small number of adult cell types would be competent to undergo BMP-dependent osteogenic differentiation. Are the major progenitors of HO unique to skeletal muscle and associated soft tissues, thereby explaining the prevalence of HO at these sites? Alternatively, osteocompetent cells may be widely distributed, with the muscle/soft tissue environment providing unique growth, survival, or differentiation factors that are essential for elaboration of the osteogenic phenotype. Although initial studies were restricted to characterizing the cellular composition of lesional tissue using histological and immunohistological methods, in recent years, Cre/LoxP-based

lineage tracing and cell transplantation approaches have provided the technical ability to follow the fates of stably-marked native cell populations and assess their osteogenic capacity. In this section, we present progress to date in identifying stem/progenitor cell populations that directly contribute to heterotopic skeletal tissues.

3.1 Endothelium

Based on lineage labeling with Tie2-Cre (also known as Tek-Cre) transgenic mice[73], endothelial cells were initially suggested to be major contributors to heterotopic cartilage and bone formation after BMP ligand implantation[41,74] and following muscle injury in the NSE-BMP4 mouse[41]. However, as in fracture callus formation[75], TIE2 expression was observed at all stages of HO[41,74], potentially obscuring the lineage relationship between TIE2+ cells in the native limb and lesional tissue. Indeed, *de novo* marker expression is a significant caveat of lineage-tracing studies that employ non-inducible Cre drivers. Further, TIE2 is not uniquely expressed by endothelial cells, and subsequent studies by Wosczyzna et al.[65] identified a Tie2-Cre-lineage-labeled multipotent cell population in the muscle interstitium that is uniquely capable of osteogenic differentiation following intramuscular transplantation of BMP2. This Tie2-Cre lineage-labeled population, which was defined by the marker profile CD45-CD31-PDGFR α +SCA1+[65], is identical to, or a sub-population of, fibro/adipogenic cells (FAPs), first discovered for their adipogenic and fibrogenic capacities[64,76] (see Section 3.6). By contrast, CD31+ endothelial cells did not directly contribute to HO under identical transplantation conditions[65]. Further, no contribution of lineage-labeled cells to BMP-induced HO was detected using a VE-Cadherin-Cre driver[77], which primarily labels endothelium and a subset of hematopoietic cells[65]. The absence of HO when endothelial cells were targeted *in vivo* for expression of caACVR1[38] or ACVR1(R206H)[70] also argues against an endothelial origin of skeletal lesional cells. Notably, however, transfection of human endothelial cells with an ACVR1(R206H) expression construct or treatment with BMP4 or TGF β 2 converted endothelial cells to a mesenchymal, multipotent, cell that exhibited osteogenic differentiation capacity[74]. Whether these apparently conflicting results represent mouse-human species differences or other differences in experimental design requires further investigation.

Interestingly, using a VE-Cadherin-Cre driver, a recent report described the contribution of endothelial cells to HO following combined burn injury and tendonectomy[78]. Transplantation of endothelial cells isolated from the aorta or hindlimb musculature into the tendon injury site further suggested a potential, albeit minor, contribution of endothelium to tendon HO. There are two commercially available VE-Cadherin-Cre strains (B6;129-Tg(Cdh5-cre)1Spe/J[79] and B6.Cg-Tg(Cdh5-cre)7Mlia/J[77]), each exhibiting highly efficient labeling of endothelium, but differential labeling of hematopoietic and other lineages. In comparing the studies of Wosczyzna et al.[65] and Agarwal et al.[78], it is possible that different outcomes resulted from the use of different Cre drivers (not reported in ref.[78]). Alternatively, mechanisms and progenitor cells involved in directing HO following tendonectomy (discussed in Section 3.4) may be distinct from those governing intramuscular HO.

3.2 Satellite Cells

Satellite cells are muscle-resident myogenic stem cells that are responsible for skeletal muscle's robust regenerative capacity[80–87]. Named for their anatomical location between the myofiber sarcolemma and basal lamina[88,89], satellite cells are stimulated to re-enter the cell cycle and execute the myogenic program in response to muscle injury. Although classically considered to be committed to the myogenic fate, satellite cells were initially a leading candidate cell-of-origin for muscle HO because they are unique to skeletal muscle and exhibit osteogenic activity in response to BMPs in culture[90–92]. However, lineage tracing[41,42,65,93] and transplantation[65] studies clearly demonstrated that satellite cells do not contribute to BMP-induced HO *in vivo*. Further, targeted expression of caACVR1[38,94] or ACVR1(R206H)[70] in satellite cells is not sufficient to induce HO. Collectively, these studies strongly argue against a direct role for satellite cells in acquired or genetic forms of HO, and illustrate the need for caution when interpreting results from cell culture systems, which may represent relatively permissive environments that are not necessarily reflective of *in vivo* settings. This is particularly relevant here, as most studies concerning the osteogenic action of BMP ligands or dysregulated BMP signaling have been conducted in culture, and using cell types not demonstrably relevant to HO *in vivo*[37,38,43,50,52–54,74,95–99].

3.3 Marrow-derived osteoblast progenitor cells (MOPC)

There are conflicting reports regarding the role of MOPCs in HO. Dysregulated BMP signaling in bone marrow progenitors is neither necessary nor sufficient to initiate HO. Thus, transplantation of caACVR1-expressing bone marrow into wild-type hosts did not result in HO following muscle injury[38]. Similarly, expression of caACVR1 in hematopoietic and lymphoid cells using the Vav1-Cre driver[100] was not sufficient for injury-induced HO of skeletal muscle[38]. Further, bone marrow transplantation (BMT) of normal cells into an FOP patient did not prevent HO[101], arguing against a direct role for ACVR1(R206H)-expressing MOPC in initiating FOP lesion formation. Consistent with these results, BMT studies by Kaplan et al.[101] demonstrated a contribution of β -galactosidase-labeled bone marrow-derived cells to fibroproliferative and marrow-populating stages of BMP4-induced HO, but not to definitive cartilage or bone. By contrast, however, BMT and parabiosis studies of Otsuru et al.[102,103] showed a substantial contribution of bone marrow-derived cells to osteocalcin-producing osteoblasts following BMP2-induced intramuscular HO. Although differences in experimental details make direct comparisons difficult, it should be noted that in the studies by Otsuru et al.[102,103], the vast majority of bone marrow-derived cells were observed lining the heterotopic bone and few were observed embedded in the definitive heterotopic bone. In addition, Otsuru et al.[102,103] analyzed lineage contributions to HO at later time points than did Kaplan et al.[101], well after the initial formation of heterotopic bone. Although the identity of the circulating progenitors is yet to be fully elucidated, murine CD45⁺ hematopoietic cells do not directly contribute to HO[42,102–104]. Interestingly, however, human CD45⁺ cells (derived from blood obtained from patient with FOP during a flare) contributed to heterotopic bone following subcutaneous implantation in a pro-osteogenic matrix[105]. Whether this apparent disparity represents differences in the developmental capacity of mouse and human CD45⁺ cells remains to be determined. Collectively, these data are consistent with a model in which

acquired and genetic HO is initiated locally by tissue-resident progenitors and that circulating progenitors participate in subsequent bone growth and remodeling.

3.4 Tendon and ligament progenitors

Scleraxis (Scx) is a transcription factor selectively expressed by differentiated tendon cells and tendon-resident progenitor populations, which have been evaluated as possible precursors of HO. Howell et al.[66] observed that Scx-expressing cells give rise to cartilage following tendonectomy. Similarly, Scx-lineage-labeled cells accounted for ~40% of definitive cartilage and ~25% of definitive intratendinous bone following burn/tendonectomy[94]. That an apparent minority of Scx+ cells contributed to tendon HO suggests either the involvement of additional progenitor cell populations or lineage labeling of only a fraction of total Scx+ cells.

Cell populations labeled with Scx-Cre drivers are developmentally and molecularly heterogeneous, and there has been some disagreement as to the tissue distribution of relevant Scx+ lineages[38,94]. Dey et al.[38] proposed that Scx-expressing HO progenitors are anatomically restricted to tendons and ligaments, whereas Agarwal et al. reported Scx-Cre-lineage-labeled cells within muscle tissue, and injury-induced, intramuscular HO was observed when caACVR1 expression was induced by a ScxCreERT² driver[94]. Both groups demonstrated spontaneous intratendinous HO when caACVR1 expression was driven by Scx-Cre[38,94]. Interestingly, however, Dey et al. reported that heterotopic cartilage, but not bone, was lineage-labeled[38]. This is in contrast to the contribution of Scx+ cells to intratendinous bone following Achilles injury noted above[94], and additional work is required to determine whether heterotopic bone cells are recruited from other progenitor populations. Scx+ cells have also been implicated in tendon HO in a genetic model of FOP (see Section 3.7).

3.5 Pericytes

Pericytes, also known as mural cells, are a highly heterogeneous population of progenitor cells that are best defined by their anatomical location embedded within the vascular basement membrane. As pericytes can differentiate into many different mesenchymal cell types during tissue repair and in disease pathogenesis[106–109], they have been an obvious candidate precursor of HO. However, no unique markers have been identified for pericytes, which has complicated an assessment of their participation in HO.

Lineage tracing has shown that both glutamate aspartate transporter+ (GLAST) cells[44] and α -smooth muscle actin+ (α SMA) cells[93] substantially contribute to all stages of BMP-induced HO. Relevant here is the observation that a subfraction of lineage-labeled cells using either the GLAST-CreERT[110] or α SMA-CreERT2[111] drivers express the pericyte marker PDGFR β , raising the possibility that pericytes were a responsible cell type. However, because a number of cell types were labeled with each Cre driver[44,93,110,111], and since the precise anatomical location of lineage-labeled cells was not evaluated, these studies could not conclusively include or exclude pericytes as a cell-of-origin for HO[44,93].

That the offending cell population in these studies may be distinct from pericytes is suggested by the recent demonstration that caACVR1 expression driven by SM22 α -Cre[112] or Cspg4-CreERT² [113], both of which target pericytes (as well as other cell types), did not result in injury-induced HO[38]. Interestingly, a subset of intramuscular GLAST-CreERT-labeled or α SMA-CreERT2-labeled cells express PDGFR α [44], the single best marker for FAPs (see Sections 3.1 and 3.6). It is possible, therefore, that FAPs are a major contributor to HO in these models.

3.6 Fibro/adipogenic progenitors

Fibro/adipogenic progenitors (FAPs) are a recently described population of PDGFR α +SCA1+ multipotent cells that reside within the skeletal muscle interstitium but are not exclusive to muscle tissue[64,65,76]. FAPs are often observed in close association with the vasculature, but are distinct from pericytes, as they are negative for NG2 and do not share a basal lamina with the adjacent endothelium[64,65]. Although muscle-resident FAPs appear to have a supportive role in normal muscle regeneration[76], they lack the capacity to directly contribute to myofiber formation. This apparent lack of myogenic capacity, together with their distinct marker profile, distinguishes FAPs from other known muscle resident progenitors[64,76,114–117].

Following injury, FAPs rapidly proliferate, and failure to reduce their numbers to preinjury levels is associated with pathogenic accumulation of fatty and fibrotic tissues, which alter muscle architecture and impair function[64,76,118,119]. Whereas FAPs[76] were initially named for their fibrogenic and adipogenic potential[64,76], FAPs also exhibit osteogenic potential when stimulated by BMP ligands in culture[64,65] and, as noted above (Section 3.1), FAPs are a major contributor to BMP-induced HO in vivo[65]; Woszczyna et al.[65] observed that Tie2-Cre[73] lineage-labeled FAPs accounted for ~50% of heterotopic cartilage and bone. The labeling of approximately half of heterotopic skeletal tissue raised the possibility either that multiple populations participate in HO or that inefficiency in Cre-mediated recombination accounted for the contribution of unlabeled cells to HO lesions. Similarities in marker expression, developmental potential, and anatomical location of lineage-labeled and unlabeled PDGFR α +SCA1+ cells are consistent with the suggestion that Cre inefficiency accounted for at least some, if not all, of the unlabeled cells in skeletal lesions[65]. Importantly, FAP-like cells exist in humans[64,65,120,121], and recent findings[38,70] strongly support a major role for FAPs in FOP (see Section 3.7).

Although cumulative evidence points to a significant role for FAPs in BMP-induced HO and HO in FOP, the complexity of progenitor populations that contribute to HO remains uncertain. Thus, in addition to the involvement of GLAST+, α SMA+, and Scx+ cells noted above, Dey et al.[38] used the caACVR1 model and lineage tracing to show that cells that express Mx1 (Interferon-induced GTP binding protein) contribute to HO. Notably, Mx1-lineage-labeled cells participated in muscle-injury-induced HO but not tendon HO; the latter was attributed to a distinct, tendon-resident, Scx+ progenitor (Section 3.4). As the resulting HO phenotypes and expression domains of Mx1+ and Scx+ lineages were non-overlapping, the authors concluded that two distinct populations of progenitor cells induce regionally restricted HO in response to dysregulated BMP signaling. In the current context, however, it

is important to note that PDGFR α + cells represented a small subfraction of both Mx1+ and Scx+ lineages[38]. In fact, one underlying commonality among most lineage-tracing studies that have identified contributors to intramuscular and intratendinous HO is the presence of PDGFR α + cells (presumptive FAPs) within the heterogeneous composition of labeled cells[38,65,94,122,123]. In addition, in the burn/tendonectomy model, large aggregates of highly proliferative PDGFR α + mesenchymal-like cells accumulated at the injury site immediately prior to the onset of HO[122,123]. These PDGFR α + cells were positive for the cartilage marker SOX9, and reappeared during recurrent HO resulting from partial surgical excision of the initial HO[123]. Thus, cumulative data from several independent labs using diverse experimental paradigms suggest that FAP involvement may represent a common mechanistic feature of heterotopic skeletogenesis, irrespective of the proximate genetic or physical trigger. It is important to emphasize that FAPs, as defined by marker profile, multipotency, and bone-forming capacity, are not restricted to muscle but are, instead, widely distributed in tissues and organs, even those not known to be susceptible to HO[65]. This raises the intriguing possibility that skeletal muscle, tendons, and associated soft tissues provide instructive or permissive signaling environments required for FAP-directed HO.

3.7 HO progenitor cells in FOP

Although data summarized above and parsimony would suggest a common progenitor for both acquired and genetic forms of HO, the recent development of accurate genetic models of FOP now allows the “common progenitor cell hypothesis” to be rigorously assessed. In the chimeric *Acvr1^{R206H}* mouse model[68], TIE2-expressing cells participated in all stages of heterotopic endochondral bone formation. Although the analysis was not designed to distinguish whether TIE2 expression defined a specific cell population in the native limb or represented *de novo* TIE2 expression among lesional cells, these data are at least consistent with the involvement of FAPs in FOP. Direct evidence for the involvement of FAPs in FOP has come from studies in which specific cell populations were targeted for expression of *Acvr1^{R206H}*. Before discussing these data, the consequences of broadly targeting *Acvr1^{R206H}* expression to limb bud mesenchyme[124] will be briefly described, as these studies constituted the first proof-of-principle that targeting *Acvr1^{R206H}* expression to specific cell types/anatomical regions during development could avoid the perinatal lethality of the original *Acvr1^{R206H}* constitutive knockin allele and phenocopy key aspects of FOP.

In a study to test the therapeutic efficacy of the RAR γ -agonist, Palovarotene, Chakkalakal et al.[124] used *Acvr1^{[R206H]FlEx/+}*[43] mice and the Prx1-Cre driver [125] to broadly target expression of *Acvr1^{R206H}* to limb bud mesenchyme. *Acvr1^{[R206H]FlEx/+};Prx1-Cre* mice showed severe, spontaneous intramuscular and intratendinous HO of the limbs, beginning by postnatal day 7, which was severe by 1-month-of-age[124]. In addition, these mice exhibited congenital defects of digits I and V, reminiscent of the great toe malformation that is a universal, albeit benign, developmental anomaly in FOP patients.

The involvement of Scx+ and Mx1+ cells in HO, and their relationship to FAPs, was described above (Sections 3.4 and 3.6) and this section will emphasize key findings and implications specifically relevant to FOP pathogenesis or those warranting further discussion. Targeting *Acvr1^{R206H}* expression to Scx+ and Mx1+ cells yielded results similar

to those of caACVR1 overexpression, although the penetrance and severity of HO was reduced, and the age-of-onset was delayed, for *Acvr1^{R206H}*-induced HO. In contrast to the *Acvr1^{R206H}/FIE^{x/+}*;Prx1-Cre model, *Acvr1^{R206H}* expression in Scx+ populations did not result in overt digital defects, indicating that great toe anomalies in FOP result from *Acvr1^{R206H}* expression in distinct embryonic progenitors not targeted by the Scx-Cre driver. We note, however, that Cre-dependent recombination efficiency can substantially affect phenotypic outcomes, both quantitatively and qualitatively, so caution should be used in ruling out particular progenitor populations for specific disease manifestations. As in caACVR1 mice, *Acvr1^{R206H}* expression in Scx+ lineages resulted in spontaneous HO of the tendons, which approached complete penetrance by 1-year-of-age. Interestingly, cardiotoxin-mediated muscle injury of *Acvr1^{R206H}/FIE^{x/+}*;Scx-Cre mice did not induce intramuscular HO, indicating that tendon/ligament-restricted Scx+ progenitors[38] are not sufficiently migratory to induce HO in juxtaposed muscle tissue.

In contrast to Scx+ cells, Mx1+ lineage-labeled cells were abundant in the muscle interstitium, but not detected in tendons and ligaments[38]. Not surprisingly then, *Acvr1^{R206H}/FIE^{x/+}*;Mx1-Cre mice did not exhibit HO of tendons/ligaments. Interestingly, however, whereas these mice exhibited intramuscular injury-induced HO, albeit with reduced penetrance (reported at 30–50%) compared to caACVR1 mice, spontaneous HO of skeletal muscle was not observed[38]. The low penetrance of injury-induced HO and absence of spontaneous HO in *Acvr1^{R206H}/FIE^{x/+}*;Mx1-Cre mice is perhaps surprising given that close to 50% of muscle interstitial cells were reported to be lineage labeled in *Rosa26-YFP*;Mx1-Cre mice[38], and that spontaneous intramuscular HO was observed in ubiquitously recombined *Acvr1^{R206H}/FIE^{x/+}* mice[38,43] and *Acvr1^{R206H}* chimeric mice[68]. However, the recombination status of the *Acvr1^{R206H}/FIE^x* locus was not determined by Dey et al.[38], and the modest phenotypes observed could have resulted from low recombination efficiency of FAPs, which were postulated by Dey et al. to be the relevant HO-causing progenitors within the heterogeneous cell population labeled with the Mx1-Cre driver[38].

Our recent lineage tracing and cell transplantation studies using a new genetic mouse model of FOP strongly support a prominent role for FAPs in FOP[70]. Indeed, mice in which *Acvr1^{R206H}* expression is targeted to FAPs recapitulate all major aspects of HO pathogenesis in FOP, including both injury-induced and spontaneous disease, and the occurrence of HO in appendicular and back musculature, tendons/ligaments, major joints, and the jaw. Although our studies do not rule out the involvement of other cell types in skeletal lesion formation, no additional cell types need to be invoked to explain the broad anatomical distribution of HO in FOP. The presence of a PDGFR α + cellular fraction (presumptive FAPs) in both Scx+ and Mx1+ populations[38] (see Section 3.6) is consistent with the view that FAPs were responsible for both tendon and muscle HO in these models, as postulated by Dey et al.[38] for muscle HO.

An important question related to the stem cell basis of FOP concerns the capacity of ACVR1(R206H)-non-expressing cells to participate in heterotopic skeletogenesis, once initiated by ACVR1(R206H)+ cells. As noted in Section 2.4.1, analysis of *Acvr1^{R206H}* chimeric mice, while not designed to identify specific cell populations, demonstrated the

contribution of wild-type cells to all lesional stages[68]. Although lineage tracing of Scx+ and Mx1+ lineages was not undertaken with *Acvr1^{[R206H]/FIE^x}* mice, parallel experiments with caACVR1 mice indicated that essentially 100% of lesional cartilage was derived from Scx- or Mx1-lineage-labeled cells, suggesting the exclusive participation of mutant cells to the cartilaginous phase of HO[38]. However, it is important to interpret these findings cautiously, as recombination of the *Rosa26-YFP* locus is not a definitive indicator of recombination of the caACVR1 transgene and recruitment of unrecombined cells could not be ruled out. Surprisingly, lineage-labeled heterotopic bone cells were either undetected (Scx-Cre) or rare (Mx1-Cre), indicating that heterotopic bone in these models was almost entirely derived from alternative, unknown, lineages. Further, although not directly assessed, the near-complete lack of lineage labeling implies that heterotopic bone originated from cells that did not express caACVR1. These observations are in contradistinction to results from other models of HO described in this review, which demonstrated a contribution of specific lineage-labeled cell populations to both cartilage and bone stages. In addition, using an *Acvr1^{R206H}* allele that incorporates a fluorescent marker such that the recombination status of individual lesional cells can be assessed, we have shown that ACVR1(R206H)-expressing FAPs give rise to the great majority of both cartilage and bone[70]. Although seemingly at odds with the findings of Dey et al.[38], it remains formally possible that FAPs are a heterogeneous population comprised of chondrogenic and osteogenic fractions, with only the chondrogenic fraction being targeted by the Scx-Cre and Mx1-Cre drivers. Single cell transcriptomics and comprehensive fractionation/transplantation studies should address this issue.

4. Concluding remarks

The identity and biology of stem/progenitor cell populations responsible for the ossification of soft tissues has been intensively investigated and debated. Whereas early studies necessarily relied exclusively on histological analyses, leading Urist[18], for example, to describe the cell induced by demineralized bone matrix to be “..... a fixed histiocyte or perivascular young connective-tissue cell,” the development and implementation of technological advancements in cell analyses has led to recent progress in identifying cells responsible for HO. Yet, many questions remain, resolution of which will require rigorous application of existing technologies, and further development of animal models that best approximate HO-causing conditions in humans. In FOP, the seminal discoveries of the genetic basis of the disease[67] and of the requirement for activin ligands in disease initiation[43,54] has allowed development of accurate genetic models of FOP that will prove invaluable for understanding underlying pathogenic disease mechanisms, including precise definition of offending cell populations. By comparison, less is known mechanistically of the causes of acquired HO in the general population, and how accurately models based on BMP implantation and other paradigms approximate these conditions remains unclear. Less still is known of the causes of neurological HO, in which central nervous system damage can cause HO at distant sites, although a recently described spinal cord injury model[126] appears to be an attractive system to investigate this phenomenon.

The identification of HO progenitors in an *in vivo* setting has relied heavily on lineage-tracing methods based on Cre/loxP recombination technologies. Despite the importance of

this approach, such studies are often limited by the unavailability of highly specific Cre drivers, and it is often easier to exclude the involvement of lineage-labeled cell populations than to ascribe relevance to a particular cell type. Cre expression during lesion formation poses additional challenges when non-inducible Cre drivers are used, as ongoing Cre expression obscures possible lineage relationships between cells labeled in the native limb and those comprising the heterotopic skeleton. As such, the development and characterization of additional inducible, cell-restricted Cre mouse lines will be important tools for future studies aimed at defining cells-of-origin of HO. However, regardless of Cre-driver, rigorous *in vitro* testing and transplantation of highly purified FACS-fractionated sub-populations should be more extensively employed to unambiguously identify relevant cell types. Using these methodologies, it may soon be possible to determine whether HO of distinct etiologies (genetic, traumatic soft tissue and CNS injury, burn) is driven by the same progenitor populations or by distinct cell types with the same or overlapping developmental capacities.

It should also be emphasized that identification of stem/progenitor cells responsible for HO is only the first step in understanding cell-related pathophysiological mechanisms. In fact, understanding how FAP (and other implicated cell populations) lineage choices are developmentally regulated and the role of the tissue environment in driving or permitting these lineage choices remains a central unresolved issue. Molecular characterization of offending cell populations, knowledge of their normal functions and a detailed understanding of the signaling mechanisms that subvert their physiological functions toward pathological bone formation, may lead to cell-specific therapeutic strategies to prevent or treat HO caused by injury or disease.

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Highlights

- The utility and limitations of current animal models of acquired heterotopic ossification (HO) and fibrodysplasia ossificans progressiva are described
- The role of BMP and activin ligands in HO etiology are discussed
- Multiple stem/progenitor cell populations have been proposed to contribute to HO
- Fibro/adipogenic progenitors exhibit robust osteogenic potential and are implicated as a key cell-of-origin for both acquired and genetic forms of HO