Identifying the Cellular Mechanisms Leading to Heterotopic Ossification

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Abstract

Heterotopic ossification (HO) is a debilitating condition defined by the *de novo* development of bone within non-osseous soft tissues, and can be either hereditary or acquired. The hereditary condition, fibrodysplasia ossificans progressiva (FOP) is rare but life threatening. Acquired HO is more common and results from a severe trauma that produces an environment conducive for the formation of ectopic endochondral bone. Despite continued efforts to identify the cellular and molecular events that lead to HO, the mechanisms of pathogenesis remain elusive. It has been proposed that the formation of ectopic bone requires an osteochondrogenic cell type, the presence of inductive agent(s), and a permissive local environment. To date several lineage-tracing studies have identified potential contributory populations. However, difficulties identifying cells *in vivo* based on the limitations of phenotypic markers, along with the absence of established *in vitro* HO models have made the results difficult to interpret. The purpose of this review is to critically evaluate current literature within the field in an attempt identify the cellular mechanisms required for ectopic bone formation. The major aim is to collate all current data on cell populations that have been shown to possess an osteochondrogenic potential and identify environmental conditions that may contribute to a permissive local environment. This review outlines the pathology of endochondral ossification, which is important for the development of potential HO therapies and to further our understanding of the mechanisms governing bone formation.

Key words

Heterotopic Ossification

Heterotopic ossification (HO) is defined by the *de novo* formation of lamellar bone within non-osseous soft tissues. Tissue formed through the process of HO is intriguing since it is composed of endochondral bone, which contains a central canal filled with bone marrow [1]. This distinguishes the process of HO from other pathological conditions characterised by the deposition of ectopic mineral within soft tissues, such as progressive osseous heteroplasia (lacks endochondral ossification) and dystrophic calcification (associated with the development of kidney stones and lacking the presence of bone marrow) [2,3]. The development of acquired HO is predominantly associated with severe trauma, particularly to muscle or neuronal tissues, such as traumatic brain injury, spinal cord injury, joint arthroplasty, severe burns or combat blast wounds and amputations [4,5]. In fact, of the 80% of war victims who suffer major extremity trauma during combat injury, approximately 64% of these patients go on to develop some degree of HO [6,7]. Aside from physical trauma, HO is also associated with the hereditary condition fibrodysplasia ossificans progressiva (FOP). However, this hereditary form of HO is rare (less than one per million of the population), comparatively well understood, and potentially less pathologically complex when compared with acquired HO. Both hereditary and acquired HO are debilitating conditions that can lead to the atrophy of skin and soft tissue breakdown throughout the residual limb, severe pain, nerve entrapment and impaired joint movement. Current treatments are invasive (surgical removal) or non-specific (radiation therapy or non-steroidal anti-inflammatory drugs), with the likelihood of HO recurrence potentially reaching levels of 25% [6,8]. Therefore, the need to understand the complex pathological mechanisms underlying HO is of great importance if effective therapies are to be devised. This review will provide a clinical and biological evaluation of HO, and provide a current perspective of the cellular and molecular mechanism leading to ectopic bone formation.

Clinical Problems

Patients with HO experience a wide range of problems due to the mechanical effects of hard tissue formation in extra-skeletal sites. These include pain, loss of joint mobility, skin ulceration, overlying skin graft failure, muscle and neurovascular entrapment, and prosthetic limb fitting difficulties [9]. Clinically, HO first presents with pain and swelling in the affected limb [10]. This can cause difficulty with diagnosis as the symptoms overlap with infection and deep vein thrombosis, which are also common diagnoses in these patients. Nerve blocks or ablations, and excisions form the mainstay of surgical treatments [5]. Rest, analgesia, non-steroidal anti-inflammatory drugs (NSAIDs), radiotherapy, bisphosphonates, and physiotherapy are common non-surgical approaches [5]. NSAIDs are used widely to cover a variety of situations where patients are at risk of developing HO, and reports concerning the efficacy of these drugs vary [11]. There is evidence that, in addition to their normal side effect profile, NSAIDs increase the risk of non-union after acetabular fixation and inhibit bone remodelling [12,13]. Additionally, NSAID administration carries the risk of gastric irritation, occasionally preventing (20-37%) patients from completing treatment [14]. Single dose radiotherapy is widely used and there is persuasive clinical evidence demonstrating its effectiveness as a HO prophylactic following surgery [15-17]. Furthermore, radiotherapy and NSAIDs may be given in combination pre- or post-operatively for high-risk patients [18]. This type of combination treatment has perhaps proven most effective for

preventing HO recurrence following surgery (e.g. the excision of ectopic bone) [18]. However, radiotherapy is by no means ideal since it carries a number of potential risks such as malignancy, genetic mutations, and gonadal effects [19,20]. Bisphosphonate use remains controversial after a Cochrane review [21] failed to find conclusive evidence of efficacy. Furthermore, these drugs act to delay the onset of HO rather than prevent it. A second Cochrane review [22] investigating the use of passive movement physiotherapy in a heterogeneous group of patients (including traumatic brain injury and spinal cord injury patients at risk of HO) concluded that there is insufficient evidence to show whether or not this therapy is effective. It is our view that a better understanding of the cellular and molecular pathology underlying HO is required to develop improved prophylactic treatments.

Pathogenesis

Current evidence suggests that the formation of ectopic bone *in vivo* requires three primary conditions: (1) a cell type capable of osteogenic differentiation, (2) the presence of inductive agents, (3) a permissive local environment [23]. However, despite continued efforts to identify the cellular and molecular events leading to HO, the mechanisms of pathogenesis continue to remain elusive. To date many contributory biological factors have been implicated in the etiology (see diagram 1), including the bone morphogenetic proteins (BMPs), inflammation, prostaglandin E2, hypercalcemia, hypoxia, abnormal nerve activity, immobilization and dysregulation of hormones [1,24]. However, in addition to the biological contribution, HO is also associated with a change in local biomechanics, as can be observed following reparative surgeries, such as cervical total disc replacement, where resulting changes in height of the functional segmental unit or increases in range of motion may influence the formation of HO [25]. At the onset of acquired HO an injurious stimulus produces an inflammatory environment conducive to the formation of endochondral bone [26]. This may be accomplished through the local signalling and differentiation of resident cells such as myoblasts or satellite cells within muscle, or through the recruitment of systemic cell types, such as circulating osteoblast progenitors, pericytes, or vascular endothelial cells, that may act either directly or indirectly to promote endochondral ossification. Therefore cells actively involved in the development of HO would have to possess either a residual osteochondrogenic potential or acquire this capacity during the tissues response to injury (i.e. cross-differentiation). Currently, the identity of these cells remains controversial, with previous reports identifying a large number of potential candidates that may contribute to the fibroproliferative and osteochondrogenic phases of HO. The focus of this review will be to provide a critical analysis of current literature within the field in an attempt to elucidate candidate cell populations and identify local and systemic factors leading to acquired HO.
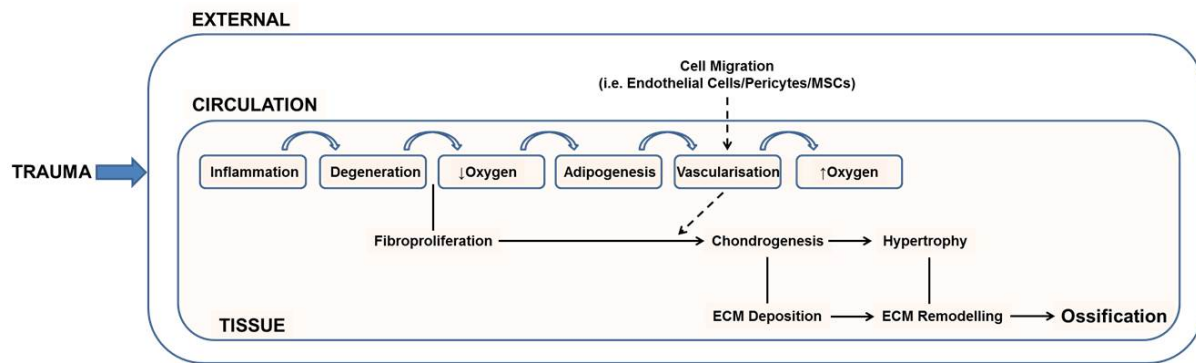
Fig. 1 Hypothetical mechanism for the pathological changes associated with the development of HO following trauma. Tissue damage leads to the infiltration of immunological cells (monocytes, neutrophils and leukocytes) through the local vasculature. Resulting fibro-proliferation of an as yet unknown cell population is accompanied by hypoxia and the generation of brown adipose tissue at the site of damage. The presence of adipose tissue is hypothesised to lower the local oxygen tension leading to the establishment of a chondrogenic environment. Neovascularisation accompanies chondrogenesis and provides an avenue through which systemic cell types (endothelial cells, pericytes etc.) may enter the injury site, and potentially contributed to osteochondrogenic differentiation. A subsequent increase in local oxygen tension promotes chondrocyte maturation and hypertrophy. The collagenous matrix deposited by these cells is then remodelled and ossified to form endochondral bone.

Cellular Origins

Mesoderm

Mesenchymal Stem Cells

The fact that HO leads to the formation of cartilage, which is subsequently remodelled to form lamellar bone has led a number of researchers to postulate a role for mesenchymal stem cells (MSCs) in the pathogenesis. MSCs first isolated by Friedenstein *et al.* (1987) were initially termed bone marrow-derived osteogenic stem cells due to their capacity to form cartilage and bone *in vitro* [27]. MSCs have frequently been shown to form endochondral bone when cultured under appropriate conditions (e.g. under hypoxia and/or in the presence of TGF-β) [28,29]. Furthermore, in some instances the application of MSCs *in vivo* following an initial period of *in vitro* chondrogenic differentiation has led to the formation of endochondral bone that contained a central bone marrow cavity [30]. Bone marrow stromal cells are still considered to be the primary source of all osteoprogenitors found throughout the body, and lineage-tracing studies have indicated that intravenously transplanted bone marrow cells migrate to and settle in skeletal muscle [31]. However, controversially, few of these cells have been shown to migrate to muscle following injury, thereby drawing in to question their potential contribution to HO [32]. This suggests that the regenerative response to trauma is mounted primarily by stem/progenitor cells located at the site of damage, such as muscle precursors.

New evidence suggests that the focus may need to be shifted from migrating bone marrow stromal cells to other available sources of MSCs, such as resident stem/progenitor cells located in the damaged tissue. A clinical study that isolated muscle-derived MSC-like cells from trauma patients that had developed HO and compared them with MSCs from trauma patients lacking HO and uninjured controls showed for the first time that patients with HO had significantly more progenitor cells committed to the osteogenic lineage than traumatised

non-HO patients [33]. Further investigation indicated that these mesenchymal progenitor cells (MPCs) had a comparable osteogenic profile to bone marrow-derived MSCs that had been exposed to osteogenic induction medium [34]. However, these cells were thought to be incapable of terminal osteogenic differentiation, and as such potentially contribute only in part, or only to the initial stages of ectopic bone formation. Interestingly, the number of MSC-like cells isolated from non-HO trauma patients was significantly increased relative to uninjured patients but these cells did not contribute to ectopic bone formation [35]. This suggests that a threshold must be surpassed before trauma induces progenitor cells to become osteogenic. This threshold may be related to several subsequent events associated with tissue damage such as the size of the immunological response, the degree of vascular damage incurred, and/or the resulting local oxygen tension following injury. It may also be hypothesised that a certain concentration of chemokines must be present in order to signal the recruitment of resident or circulating stem cells, and that this is related to the severity of trauma [36]. The need for further work to characterise these cells comprehensively is now of great importance if the true identity of this mesenchymal population and its role in HO is to be elucidated.

Evidence implying a role for mesenchymal stem/progenitor cells in HO is related to the known age-dependent effects of these cells *in vivo*. Recent evidence has shown that the development of HO following burn injury may be reduced in older subjects [37]. This is significant given that it has been documented for several years that the availability and functional activity of MSCs may be reduced with increasing age or passage [38]. This appears to be particularly true for bone marrow-derived mesenchymal stem cells (BMSCs), with these cells presenting a decreased level of proliferation and differentiation in older subjects [39]. Furthermore, when the angiogenic potential of MSCs was investigated in older subjects the resulting data demonstrated a reduced capacity for adipose-derived stem cells (ADSCs) to form capillary-like networks when compared with ADSCs isolated from younger subjects [40]. A MSCs capacity for vascularisation can be improved following hypoxic pre-conditioning [41]. This is significant given that low oxygen tension can result as a consequence of soft-tissue injury, and may implicate MSCs in ectopic bone formation (see Diagram 1). Hypoxia at the site of injury has been hypothesised to result from the formation of brown adipocytes within the damage site due to the action of local and systemic factors such as BMPs [42]. Currently, no evidence exists to identify the origin of adipose tissue at the site of tissue damage. However, MSCs are defined as multipotent cells capable of adipogenic differentiation, and, as such, their contribution to brown adipose tissue formation during the early stages of HO cannot be ruled out. Brown adipocytes formed at the onset of HO have been shown to express vascular endothelial growth factors, concurrent with endothelial progenitor proliferation [43]. Consequently, the presence of brown adipocytes within the lesion reduces the oxygen tension in the adjacent tissue, due to oxidative metabolism [42]. The presence of hypoxic stress is a requirement for MSC chondrogenesis, which can lead to cell hypertrophy and ossification [42]. MSCs may also contribute to chondrocyte hypertrophy and the progression of HO via their immunomodulatory effects, primarily through the production of anti-inflammatory cytokines and nitric oxide (NO) [44]. The production of NO by MSCs has been shown to impair T cell responsiveness following injury, thereby modulating the overall immune response [45]. Interestingly, nitric oxide is also known to contribute to chondrocyte hypertrophy, which may implicate MSCs and their immunomodulatory effects in the development of HO.

Myoblasts

A myoblast is a proliferative progenitor cell that differentiates to form mature muscle. These cells fuse and align to form multinucleated primary myofibres. Previous studies have shown that skeletal muscle contains populations of osteoprogenitor cells, with ectopic bone formation being achieved experimentally using solubilised factors obtained from bone [46-48]. Evidence of an osteogenic capacity has also been recorded for both rat (L6 cells) and mouse (C2C12 cells) skeletal muscle myoblasts following transfection with osteogenic inducers, such as BMPs [49,50]. Co-culture of C2C12 mouse myoblasts with MSCs has been shown to produce a pro-osteogenic environment, as determined by an increase in the expression of the primary osteogenic regulator Runx2 [51]. A study by Mu and Li (2010) identified TGF-β as a factor capable of inducing myoblast reversion to a multipotent cell type [52]. This is interesting given that several TGF-β isoforms have been identified in both immature and mature ectopic ossifications of HO patients [53]. The study by Mu and Li (2010) showed that incubation of primary myoblasts or C2C12 immortalised myoblasts with a transient and small concentration of TGF-β can lead to the expression of more primitive markers, Pax7 and Sca-1 [52]. However, in the aforementioned study the authors identify the expression of Pax7 and Sca-1 as an indication of reversion to an MSC phenotype, although Pax7 is more commonly used as an indicator of satellite cell phenotype, and Sca-1 has also been identified as a marker of muscle cell progenitors [54]. Therefore, it may be more likely that the presence of inflammatory factors such as TGF-β could lead to a reversion of myoblasts to an earlier muscle progenitor/satellite cell type, which may possess an osteochondrogenic potential. Current, *in vivo* cell tracking studies aiming to define the contribution of skeletal muscle cells to HO have yielded some conflicting results. Liu *et al.* (2012) found that MyoD-Cre^{+} cells contributed to the formation of ectopic bone when induced by the intramuscular delivery of BMP-7 [55]. MyoD is recognised as the master regulatory gene for myogenic differentiation in much the same way as Runx2 and PPARγ are essential for osteogenic and adipogenic differentiation. However, lineage tracing experiments conducted by Lounev *et al.* (2009) identified only a small percentage (5%) of MyoD-Cre cells within ectopic bone formed by BMP-induced osteogenesis [56]. As well as being present on myoblasts, MyoD activation is required for the activation of a population of multipotent stem–like cells localised within skeletal muscle, called satellite cells. These cells are crucial for muscle regeneration and repair, and as such, have been implicated in acquired HO.

Satellite Cells

Satellite cells reside between the sarcolemma and basal lamina of myofibres and are crucial for the regeneration of skeletal muscle. As well as contributing to new myofibre formation, a subset of satellite cells is also capable of self-renewal, a defining property of stem cells. Due to this property, satellite cells are often referred to as muscle stem cells. Much like MSCs, previous reports have identified their potential to form myogenic, adipogenic, and osteogenic tissue [57]. The fact that muscle contains a unique population of adult stem cells has led to them being implicated in ectopic bone formation. Satellite cells derived from both humans and adult mice have been shown to co-express multiple cell fate-determining genes, such as the myogenic gene MyoD and the primary regulator of osteogenesis Runx2 [58]. As such, these cells are commonly referred to as inducible osteoprogenitors, since their osteogenic potential is dependent on the addition of inductive agents such as BMP-2 [59]. Although,

there is some evidence to suggest that human satellite cells may not require BMP-2 induction to reach an osteogenic state [60]. It has been proposed that satellite cells may contribute to the formation of ectopic bone as a result of an inability to restrict their phenotypic plasticity [58]. One study that isolated rat serum following severe burns (40% total surface area) and applied it to satellite cell cultures found enhanced cell proliferation, migration and osteogenic differentiation [61]. However, more recent studies suggest that the image of a satellite cell as a multipotent cell type capable of contributing to osteochondrogenic differentiation may be untrue, with some authors claiming that any non-myogenic differentiation observed in these cultures potentially results from contamination with other stem/progenitor cells obtained during myofibre isolation [62,63]. In fact, a number of studies can be found that claim satellite cells are committed to the myogenic lineage, and as such are crucial for muscle regeneration following injury but unlikely to contribute to the formation of ectopic bone seen in HO [64,65].

Mesenchymal Progenitor Cells

Several progenitor populations, other than satellite cells, have been identified within skeletal muscle. PW1$^+$/Pax7$^-$ interstitial cells have recently been identified as a population of non-satellite myogenic precursors that can adopt a myogenic or vascular fate. These cells are thought to represent a source of postnatal satellite cells, the recruitment of which is dependent on the local environment [66]. However, the osteogenic potential of these cells has yet to be examined and it remains unknown whether these cells contribute either directly or indirectly to HO. A population of PDGFRα$^+$/Sca-1$^+$ cells have also been localised to the muscle interstitium. These cells have demonstrated both an adipogenic and osteogenic potential, but lack the capacity to form skeletal myoblasts [62]. However, due to the fact that PDGFRα/Sca-1 positivity is also a defining feature amongst pericytes/pericyte progenitors and subsets of bone marrow progenitor cells the exact identity and derivation of these muscle progenitors as well as their potential role in HO remains unclear [67]. CD31$^-$/CD45$^-$ side population cells are a minor subset of MSC-like cells present in uninjured muscle that are known to proliferate in response to muscle damage [68]. These cells are thought to participate indirectly in muscle regeneration following injury, since they exhibit only a limited myogenic differentiation potential *in vivo*. These cells have been shown to be capable of osteogenic differentiation *in vitro* but *in vivo* lineage tracing experiments are required to determine the potential role of these cells during HO [69]. Skeletal muscle also contains a population of haematopoietic stem cells (HSCs) that have been identified as CD45$^+$/Sca-1$^+$ [70]. The osteogenic potential of HSCs isolated from muscle tissue has yet to be studied. However, a similar primitive side population of HSCs isolated from the bone marrow was shown to be capable of giving rise to osteoblasts through an intermediate mesenchymal phase [71]. Studies have indicated that haematopoietic progenitor cells can be split into CD90-positive and CD90-negative fractions [72]. Both fractions appear capable of osteogenesis; however only the CD90-negative fraction has the capacity to form bone containing a central bone marrow filled cavity. These data may imply that the formation of ectopic bone and the physiologically accurate endochondral bone found in HO patients have distinct pathologies that are linked to similar yet unique cell subsets. It is also possible that these haematopoietic progenitors are related to another cell type implicated in HO, the circulating bone marrow-derived osteoblast progenitor cells (MOPCs) that have been shown to be mobilised in the circulating blood following implantation of BMP-2 [73]. Finally, in a study by Jackson *et al*.

(2009) a population of cells termed mesenchymal progenitor cells (MPCs) were isolated from traumatised muscle during surgical debridement [34]. This study showed that following muscle damage a population of cells could be extracted that exhibited an osteogenic differentiation potential comparable to bone marrow-derived mesenchymal stem cells (BMSCs). However, one difference between MPCs and BMSCs was the inability of MPCs to reach a terminally differentiated state, as identified by the expression of the end-point osteoblast marker osteocalcin (OC) [34].
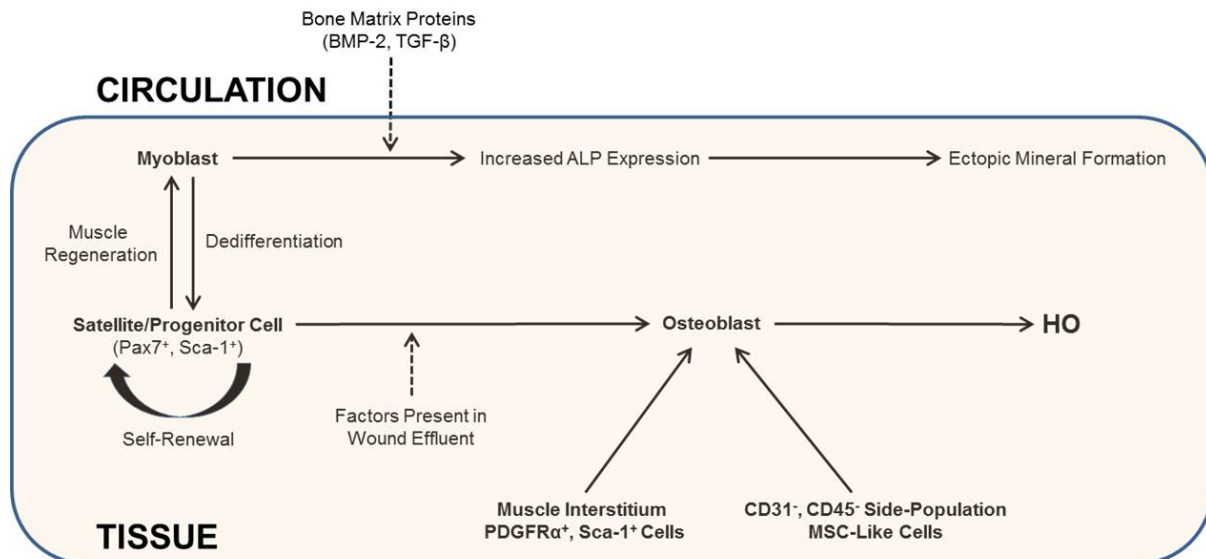

Fig. 2 Based on current evidence, a hypothetical role for cells found within skeletal muscle in the formation of ectopic bone. The diagram highlights the potential direct and indirect contribution of well characterised cells such as myoblasts and satellite/progenitor cells. It also identifies the potential involvement of under-characterised resident cells such as muscle interstitium cells and side population MSC-like cells.

Ectoderm

Epithelial Cells

Embryonic skeletogenesis is initiated when epidermal cells undergo an epidermal to mesenchymal transition. These mesenchymal cells are then able to differentiate towards chondrogenic and osteogenic lineages, in the process of endochondral ossification. Currently, the cross-differentiation from cells of one germ layer to another is gaining much attention as a process involved in the initiation of HO. However, the osteogenic potential of epithelial cells is subject to much debate, with some studies demonstrating an osteogenic potential while others refute it [74, 75]. Early experiments looking into the effects of transplanting urinary transitional epithelium into the connective tissues of dogs and rabbits demonstrated the formation of mineralised tissue [76], while more recent studies have shown that epithelial cells over-express factors required for osteochondrogenic differentiation such as TGF-β and BMP-2 during their proliferative phase, and as such may exert a paracrine effect during HO pathogenesis [77]. Furthermore, the application of a pulsed electromagnetic field (PEMF) has been shown to induce osteogenesis in a population of amniotic epithelial cells [78]. This result is particularly interesting given that acquired HO is frequently associated with victims of blast injury, and shows that certain populations of epithelial cells

may have the potential to revert to a mesenchymal phenotype following mechanical stimulus [79]. However, more recent data examining the role of epithelial cells in the development of HO have shown that these cells are likely to have only a minor and indirect role in HO. For instance, when cultured with mesenchymal cells of the mouse thigh or intramuscularly implanted, osteochondrogenic differentiation was observed [74]. However, in this study it was not reported whether it was the epithelial cell population or local mesenchymal cells within the muscle that contributed to the resultant osteogenic population. Therefore, to more accurately determine whether epithelial cells or mesenchymal cells were responsible for the production of ectopic mineral a study was designed in which BMP-7-transduced human oral keratinocytes and mesenchymal cells were co-cultured using a diffusion chamber [80]. This experimental design allowed the investigators to distinguish whether epithelial cells possessed direct osteogenic potential or indirectly affected mesenchymal cells through the release of paracrine factors [80]. The results of this experiment confirmed that osteogenic material was only formed close to the implanted diffusion chamber on which the mesenchymal cells were located, indicating that the mesenchymal cells formed mineral and not the epithelial cells.

Endoderm

Endothelial Cells

Recent evidence points to the involvement of endothelial cells in HO. Tissue vascularisation represents a highly important step during the transition from hypertrophic cartilage to bone during the endochondral ossification of cartilage growth plates [81]. The formation of neo-vasculature is also likely to be of key importance for the progression of HO, since the formation of bone requires a rich vascular network. Neo-vascularisation occurs within damaged tissue as a result of low oxygen tension produced by local formation of brown adipose tissue. Low oxygen tension leads to the stabilisation of hypoxia-inducible factor-1α (HIF-1α), which has been shown to promote transcription of the key vasculogenic factor VEGF [42, 82]. Vascularisation is further promoted by hypertrophic chondrocytes located within the predominantly avascular cartilage matrix at the onset of endochondral ossification. After becoming hypertrophic these cells alter their gene expression profile and produce signalling molecules such as VEGF and transferrin that initiate endothelial cell migration from the circulation and the accumulation of these cells to the site of calcification [83, 84].

Endothelial cells have been shown to have a definite indirect contribution to HO, with the production of paracrine factors leading to chondrocyte hypertrophy and matrix ossification [81]. Recent evidence published by Kim *et al*. (2013) identified that Ang1/Tie2 signalling in MSC cultures led to a strongly osteochondrogenic phenotype, further identifying a link between endothelial cells and the pathological process of ectopic bone formation [85]. Furthermore, there is evidence to indicate that the expression of the endothelial Tie2 receptor is increased during osteogenesis and that treatment with angiopoietin 1 (Ang1), a growth factor involved in the production of stable vasculature, enhanced osteogenesis by potentiating the effects of BMP2 on MSCs [86]. The coupling of angiogenesis and osteogenesis is not a newly identified phenomenon, and links between the two processes are found during embryogenesis and tissue regeneration [87]. Additionally, much like epithelial cells during embryonic skeletogenesis, endothelial cells have been shown to make a transition to a mesenchymal cell in a process termed endothelial-mesenchymal transition (EMT) [88]. *In*

vivo studies tracing the lineage of osteochondrogenic cells during HO have identified the involvement of endothelial cells by the presence of endothelial Tie2-Cre expression on chondrocytes and osteoblasts present in the resulting ossified tissue [89]. Further investigation identified that endothelial-mesenchymal transition (EMT) could be attributed to mutations in ALK2, a type I BMP receptor linked with the development of the hereditary condition FOP [89]. The cross-differentiation of endothelial cells to a mesenchymal phenotype has been demonstrated by the expression of several identifying surface markers, such as Stro-1, CD44, and CD90, among others. These markers are among several distinguishing features of MSCs, potentially indicating that EMT associated with HO results in the formation of multipotent stem/progenitor cells that have the capacity to differentiate towards an osteochondrogenic cell type. It would be interesting to further characterise the mesenchymal cells formed as a result of EMT to examine their multi-potential. This would help determine whether the mesenchymal cells formed during EMT represented authentic MSCs with the potential to form osteogenic, chondrogenic, adipogenic, and potentially neurogenic tissues, or more simply a type of endothelial progenitor.

Further evidence for the potential involvement of endothelial cells in HO is the intermediate presence of brown adipose tissue during pathogenesis [42]. Currently, the origin of this adipose tissue has yet to be confirmed. A recent study examining the adipogenic potential of extracted muscle fibres *in vitro* found that adipogenic differentiation could only be achieved by Tie-2 labelled cells (possible endothelial precursors) present within the heterogeneous muscle fibre [65]. This is a highly significant finding given that HO is perhaps most commonly observed in skeletal muscle [90]. A derivational link between endothelial cells and adipocytes has also been identified, with capillary sprouts present in adipose tissue being found to express both endothelial (VE-Cadherin) and pre-adipocyte (Zfp423) markers [91]. Furthermore, the formation and expansion of adipose tissue is reliant on the presence of a rich capillary network. This data suggests that adipocytes can be of endothelial origin and may provide a model for the formation of brown fat during HO [92]. Furthermore, a study by Lounev *et al.* (2009) identified the presence of Tie2-cre labelled cells within ectopic osteochondrogenic tissue of HO [56]. Together, this data supports a role for endothelial progenitor cells in throughout the fibroproliferative, adipogenic and osteochondrogenic stages of HO (see Diagram 3)
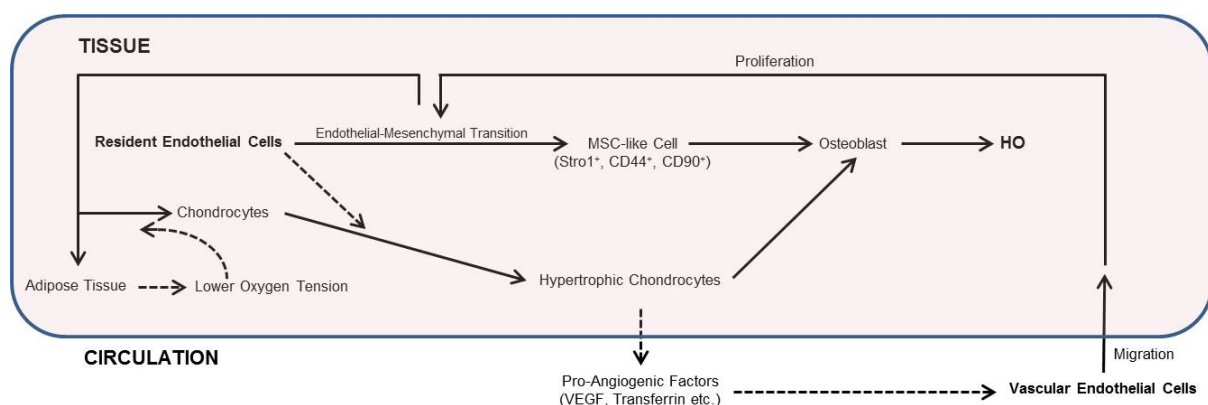

Fig. 3 Hypothetical role for resident and vascular endothelial cells in the formation of ectopic bone. The diagram highlights the paracrine roles of endothelial cells, as well as the potential direct contribution of these cells through endothelial–mesenchymal transition (EMT). Additionally, we would like to propose a potential contribution of endothelial cells to the formation of brown adipose tissue during the initial stages of HO.

However, the role of endothelial cells/progenitors was recently called into question following the publication of data showing that the Tie2 positive cells found in endochondral bone lacked the expression of VE-Cadherin, a principal endothelial marker protein [93]. Furthermore, Tie2^{+} cells only accounted for approximately one half of the cells present within the ectopic bone mass [56]. Taken together these data allude to the presence of a non-endothelial Tie2^{+} VE-Cadherin[-] cell type. This data has proven highly interesting given that Tie2 is known to be expressed almost exclusively by endothelial cells, quiescent haematopoietic stem cells, monocytes and pericytes. Therefore, we suggest that blood vessels formed within the developing ectopic bone mass may provide some insight to understanding the pathogenesis of HO, but that endothelial cells themselves may not be directly responsible for the formation of ectopic bone but rather as a yet unidentified vascular cell type. Therefore, the role of cells associated with the vasculature, such as newly discovered PDGFRβ[+] MSC-like or multipotent cells such as pericytes, needs to be discussed [87]. Currently, data examining the osteogenic potential of PDGFRβ[+] MSC-like cells is lacking. These cells have been shown to be associated with a newly discovered and specialised blood vessel subtype termed 'type H vessels' that are unique to bone neo-angiogenesis [87]. The presence of PDGF receptors as a marker of mesenchymal cells closely associated with the vasculature correlates with work conducted by Wosczyna *et al.* (2012). This work showed that a population of non-endothelial Tie2^{+} PDGFRα[+] Sca-1^{+} cells in the muscle interstitium was found to be consistently incorporated into chondrogenic and osteogenic lesions [94]. However, the expression of PDGFα also draws a direct comparison with bone marrow-derived pericyte progenitors (PDGFRα[+] Sca-1^{+}) previously identified by Uezumi *et al.* (2010) [62] (see Table 1). What is most interesting about these reports is that both PDGFR and Sca-1 are defined as MSC/progenitor cell markers [95]. This suggests that the cells responsible for HO may be associated with the vasculature but mesenchymal in origin.

Neural Crest

Pericytes

Pericytes are contractile cells that envelope the surface of the vascular wall of blood vessels, where they communicate with endothelial cells. These cells have been defined as PDGFRβ[+], and therefore could be linked with the population of MSC-like cells found in blood vessels [67,87]. However, confusion regarding the identity, progeny and ontogeny of pericytes abounds, and the relationship between these cells and other mesenchymal cells such as MSCs and vascular smooth muscle cells (vSMCs) remains elusive [96]. Pericytes have been shown to have an osteogenic potential when cultured both *in vitro* and *in vivo*, and consequently could be implicated in HO [97]. In fact, conjecture relating to the aberrant differentiation of pericytes along osteogenic, chondrogenic and adipogenic lineages has implicated these cells in a number of similar pathological conditions, such as atherosclerosis and ectopic calcification [98]. One current opinion is that pericytes are in fact multipotent cells capable of forming fat, cartilage and bone, but that these differentiation pathways are repressed except in cases of pathogenesis [96]. For example, a study by Kirton *et al.* (2007) has shown that the chondrogenic differentiation pathway of pericytes can be activated by Wnt/beta-catenin signalling in the presence of TGF-β3 [98]. Additionally, a recent study by Kan *et al.* (2013) identified that cells presenting the glutamate transporter GLAST were found to contribute to the formation of ectopic bone, and that these GLAST^{+} cells appeared to be

distinct from the Tie2$^+$ population identified by Woscyzna *et al*. (2012) [94]. Pericyte populations have been shown to express GLAST [99], and consequently this information further highlighted the potential role of these cells during HO. However, due to a lack of novel surface markers that can be used for the definitive phenotypic characterisation of pericytes *in vivo*, the role of these cells in HO remains elusive. For instance a study presented by Armulik *et al*. (2011) identified pericytes based on the positive expression of α-smooth muscle actin (α-SMA) and a lack of expression of the endothelial marker von Willebrand factor (vWF) [100]. However, this method of identification fails to provide a means of distinguishing between pericytes, vascular smooth muscle cells, myofibroblasts and MSCs, which are all α-SMA$^+$ vWF$^-$ [101]. Furthermore, the fact that purified perivascular cells exhibit multipotency and become indistinguishable from traditional MSCs when expanded in culture has fuelled continued debate over the relationship between pericytes and MSCs [96]. However, due to a lack of unique cell surface markers, limitations exist concerning the phenotypic characterisation of these cells *in vivo*, and the precise relationship between these cells and MSCs remains elusive. Therefore, further *in vivo* cell tracking experiments are required that accurately identify pericytes based on an established CD146$^+$/CD34$^-$ phenotype in order to determine the contribution of these cells to the ectopic bone mass [96].

Pericyte Progenitor Cells

It is interesting to note that at least three populations of bone marrow-derived cells are known to give rise to pericytes [67]. For instance studies have identified bone marrow-derived PDGFR-β$^+$/Sca-1$^+$/CD11b$^+$ pericyte progenitors [102], bone marrow-derived CD45$^+$/CD11b$^+$ progenitors [103], and Sca-1$^+$/Tie2$^+$/CD13$^+$ pericyte progenitors that have been associated with tumour development [104]. To our knowledge, there is no information on the osteochondrogenic capacity of these cells and as such their potential roles during HO remains unknown. However, based on knowledge of the phenotypic profile of these cells and the osteochondrogenic potential of mature pericytes, it could be postulated that the Tie2$^+$ cells identified in endochondral bone by Lounev *et al*. (2009) represent a population of pericyte progenitors rather than endothelial cells, as first proposed [56]. This hypothesis would account for the fact that the cell population identified within ectopic bone by Lounev *et al*. (2009) was Tie2$^+$ but lacked the expression of VE-Cadherin, a principal endothelial marker [56]. Furthermore, the fact that pericyte progenitors are a heterogeneous population with differing immunophenotypes could account for the fact that only approximately 60% of the cells found in endochondral bone were Tie2$^+$ [56]. Therefore, we identify the need for further studies to determine the presence of other common markers of pericyte progenitor cells such as Sca-1.

Cell Type	Immunophenotype	Chondrogenic	Osteogenic	References
MSC	CD73$^+$/CD90$^+$/CD105$^+$	Y	Y	28, 29, 30
Myoblast	MyoD$^+$/Myf5$^+$	N	Y	46, 49, 50, 51, 52, 55
Satellite Cell	Pax7$^+$/Sca-1$^+$/CD34$^+$	Y/N	Y/N	57, 60, 65
Interstitial Cell	PW1$^+$/Pax7$^-$	?	?	66
Interstitial Cell	PDGFRα$^+$/Sca-1$^+$	?	Y	62, 94
Muscle Side Population Cells	CD31$^-$/CD45$^-$	?	Y	68, 70
Muscle Haematopoietic Stem Cells (HSC)	CD45$^+$/Sca-1$^+$	?	?	70
Bone Marrow HSC Side Population	Lin$^-$/Sca-1$^+$/cKit$^+$/CD45$^+$	?	Y	71
Circulating Osteogenic Precursor (MOPC)	CD44$^+$/CXCR4$^+$/SDF-1$^+$	?	Y	73
Mesenchymal Precursor Cell (MPC)	CD44$^+$/CD49e$^+$/CD73$^+$/CD90$^+$/CD105$^+$	Y	Y	34
Pericyte	CD146$^+$/α-SMA$^+$, vWF$^-$/PDGFRβ$^+$	Y	Y	94, 97, 98
Bone Marrow-Derived Pericyte Progenitors	PDGFRβ$^+$/Sca-1$^+$/CD11b$^+$ CD45$^+$/CD11b$^+$ Sca-1$^+$/Tie2$^+$/CD13$^+$	?	?	102, 103, 104
Epithelial Cells	E-Cadherin$^+$/KL1$^+$/EpCAM$^+$	Y/N	Y/N	76, 77, 78, 79
Endothelial Cells	Tie2$^+$/VE-Cadherin$^+$	Y	Y	89
PDGFβ$^+$ MSC-like Cells	PDGFβ$^+$	?	?	87, 89

The table shows the immunophenotype of these cells as well as their ability to differentiate towards chondrogenic and osteogenic lineages, as would be required for endochondral ossification

Abbreviations 50-Nucleotidase (CD73), Thymocyte antigen 1 (CD90), endoglin (CD105), myogenic differentiation 1 (MyoD), myogenic factor 5 (Myf5), paternally expressed gene 3 (PW1/*Peg3*), paired box protein 7 (Pax7), stem cell antigen-1 (Sca-1), hematopoietic progenitor cell antigen (CD34), platelet-derived growth factor receptor alpha (PDGFRa), platelet endothelial cell adhesion molecule (CD31/*PECAM-1*), leukocyte common antigen (CD45/*LCA*), lineage cocktail (Lin), mast/stem cell growth factor receptor (cKit), homing cell adhesion molecule (CD44), integrin alpha-2 (CD49e), melanoma Cell adhesion molecule (CD146/*MCAM*), C-X-C chemokine receptor type 4 (CXCR4), stromal cell-derived factor 1 (SDF-1), alpha smooth muscle actin (a-SMA), von Willebrand Factor (vWF), Integrin Alpha M (CD11b/*ITGAM*), Tie receptor tyrosine kinase (Tie2), Aminopeptidase N (CD13), epithelial cadherin (E-cadherin), Cytokeratin 1 (KL1), Epithelial cell adhesion molecule (EpCAM), vascular endothelial cadherin (VE-Cadherin), Platelet-derived growth factor beta (PDGFb). *Italics* denote other commonly used abbreviations for each marker.

Future Treatment Strategies

Prediction and Diagnosis

Significant benefits may be gained by early identification of patients who will go on to develop HO. Reliable risk stratification and prediction allows clinicians to target prophylactic therapies at the right patients, thus reducing the risk of harmful side effects in those who are not at risk of developing HO. Several techniques are being developed to achieve this goal. In one example, the risk of a patient developing HO is identified by measuring key local and systemic inflammatory biomarkers at the time of first surgical debridement [26]. However, it

must be noted that these studies have not been validated in a civilian population and the authors' stress that the complex interrelationship between the inflammatory markers remains incompletely understood. The same group has also looked upstream at osteogenic gene expression as a predictor of HO in combat wounds [105]. They report a significant upregulation in transcriptional activity in key osteogenesis-related genes (ALPL, BMP-2, BMP-3, COL2A1, COLL10A1, COL11A1, COMP, CSF2, CSF3, MMP8, MMP9, SMAD1, VEGFA) in patients that developed HO compared to those who did not. This study may, however, be confounded by the finding that the HO group had a significantly higher injury burden, more bacterial colonisation, bigger wounds, and more amputations than the non-HO group.

The predictive ability of advanced non-invasive imaging techniques has also been the subject of recent research activity. *In vivo* Raman spectroscopy has been employed to detect bone mineral deposition in a rodent HO model as soon as 5 days post injury [106]. In a similar manner, the same group have developed a near infra-red optical imaging system that can demonstrate HO formation 5 days after injury [107]. Not only are these techniques able to detect HO sooner after injury than any other modality but they are non-invasive and do not rely on ionising radiation.

Treatment

In order to exploit earlier diagnosis, novel anti-HO treatments are being developed. One strategy that has shown great promise is retinoic acid receptor gamma (RARγ) agonism to inhibit chondrogenesis [108]. Without a cartilage scaffold, the endochondral processes that form HO are blocked and no mineral can be deposited. It is worth noting that, in the rodent model, there was a transient prolongation of fracture healing associated with RARγ agonism. However, the mechanism was so effective that the RARγ agonist Palovarotene has been taken forward into clinical trials (NCT02279095) as a treatment for the genetic form of HO known as fibrodysplasia ossificans progressive (FOP) [109].

Another novel strategy has been demonstrated in a burn-tenotomy rodent HO model [37] whereby hydrolysis of ATP at the burn site lead to reduced HO formation at a distant site. In addition to revealing a potential therapeutic strategy, this "remote ATP hydrolysis" method provides a further mechanistic insight into the role of phosphorylated SMAD proteins in the development of HO. In another rodent tenotomy model (without burn injury), treatment with the antibiotic echinomycin inhibited HO formation [110]. The proposed mechanism is that echinomycin inhibits hypoxia-induced factor 1-α (HIF-1-α), a signalling molecule thought to be crucial to the process of chondrogenic differentiation of mesenchymal stem cells in HO.

Heterotopic ossification research has diversified massively in the last few years. Rather than simply optimising the well-understood treatment modalities, researchers are exploiting breakthroughs in the understanding of the pathogenesis of this condition to diagnose it earlier and develop completely novel therapeutic strategies. However, a better understanding of the cellular and molecular mechanisms leading to HO is required for the development of novel therapeutics.

Conclusion

HO is likely to result from a highly complex interplay between a number of cell types that are either resident in the damaged tissue or recruited from the circulation following trauma and inflammation. The majority of current evidence practically excludes cells of ectodermal origin as primary contributors to HO, however the paracrine effects of these cells cannot be ruled out. Perhaps the most promising evidence so far links the onset of HO with the release of pro-angiogenic factors following injury, and therapies that utilise anti-angiogenic molecules may be significant for the prevention of HO. This review identifies a potential role of endothelial cells during the onset of HO but concludes that non-endothelial cells closely associated with the vasculature, such as pericytes or multipotent cells of mesenchymal origin are likely to be directly involved in the pathogenesis. Throughout this review, we have identified that limitations in phenotyping, and overlap between the identities of different populations of mesenchymal progenitors/stem cells limit the power of current *in vivo* methods. In fact, the lack of unique sets of cell surface markers together with significant overlap and confusion regarding the relationships between different subsets of stem/progenitor cells is perhaps one of the fundamental issues delaying progress in HO research. Therefore, the need for a more comprehensive understanding of stem/progenitor subpopulations, and the hierarchies that exist between them, is required before HO contributors can be accurately identified.

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Table and Figures

Table 1.

Comprehensive list of cells potentially involved in HO. The table shows the immunophenotype of these cells as well as their ability to differentiate towards chondrogenic and osteogenic lineages, as would be required for endochondral ossification. Abbreviations: 5'-Nucleotidase (CD73), Thymocyte antigen 1 (CD90), Endoglin (CD105), Myogenic differentiation 1 (MyoD), Myogenic factor 5 (Myf5), Paternally expressed gene 3 (PW1/*Peg3*), Paired box protein 7 (Pax7), Stem cell antigen-1 (Sca-1), Hematopoietic progenitor cell antigen (CD34), Platelet-Derived Growth Factor Receptor alpha (PDGFRα), Platelet Endothelial Cell Adhesion Molecule (CD31/*PECAM-1*), Leukocyte Common Antigen (CD45/*LCA*), Lineage cocktail (Lin), Mast/Stem cell growth factor receptor (cKit), Homing cell adhesion molecule (CD44), Integrin alpha-2 (CD49e), Melanoma Cell Adhesion Molecule (CD146/*MCAM*), C-X-C chemokine receptor type 4 (CXCR4), Stromal Cell-Derived Factor 1 (SDF-1), Alpha Smooth Muscle Actin (α-SMA), von Willebrand Factor (vWF), Integrin Alpha M (CD11b/*ITGAM*), Tie receptor tyrosine kinase (Tie2), Aminopeptidase N (CD13), Epitheial Cadherin (E-Cadherin), Cytokeratin 1 (KL1), Epithelial Cell Adhesion Molecule (EpCAM), Vascular Endothelial Cadherin (VE-Cadherin), Platelet-Derived Growth Factor Beta (PDGFβ). *Italics* denote other commonly used abbreviations for each marker.

Diagram 1.

Hypothetical mechanism for the pathological changes associated with the development of HO following trauma. Tissue damage leads to the infiltration of immunological cells (monocytes, neutrophils, leukocytes) through the local vasculature. Resulting fibro-proliferation of an as yet unknown cell population is accompanied by hypoxia and the generation of brown adipose tissue at the site of damage. The presence of adipose tissue is hypothesised to lower the local oxygen tension leading to the establishment of a chondrogenic environment. Neovascularisation accompanies chondrogenesis and provides an avenue through which systemic cell types (endothelial cells, pericytes etc.) may enter the injury site, and potentially contributed to osteochondrogenic differentiation. A subsequent increase in local oxygen

tension promotes chondrocyte maturation and hypertrophy. The collagenous matrix deposited by these cells is then remodelled and ossified to form endochondral bone.

Diagram 2.

Based on current evidence, a hypothetical role for cells found within skeletal muscle in the formation of ectopic bone. The diagram highlights the potential direct and indirect contribution of well characterised cells such as myoblasts and satellite/progenitor cells. It also identifies the potential involvement of under-characterised resident cells such as muscle interstitium cells and side-population MSC-like cells.

Diagram 3.

Hypothetical role for resident and vascular endothelial cells in the formation of ectopic bone. The diagram highlights the paracrine roles of endothelial cells, as well as the potential direct contribution of these cells through endothelial-mesenchymal transition (EMT). Additionally, we would like to propose a potential contribution of endothelial cells to the formation of brown adipose tissue during the initial stages of HO.