Muscle regeneration: molecular aspects and therapeutic implications

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With respect to diverse clinical applications for muscle regeneration, this paper discusses the latest markers for identifying skeletal muscle precursor cells in regenerating muscle, the implications of alternative sources of myogenic precursor cells and putative stem cells, and the current status of administration of exogenous factors to enhance muscle repair.

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Abbreviations

DMD	Duchenne muscular dystrophy
MTT	myoblast transfer therapy
FGF	fibroblast growth factor
HGF	hepatocyte growth factor
IGF	insulin-like growth factor
RGTA	regenerating agent

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Introduction

This paper outlines clinical interest in muscle regeneration and related research. New molecular markers, which are being used to identify myogenic precursor cells, are examined, mainly with respect to satellite cells; these are mononucleated myogenic reserve cells that lie on the surface of mature myofibres beneath the external lamina. Alternative sources of myogenic precursor cells (originating in interstitial connective tissue or bone marrow) and putative stem cells are now attracting considerable attention, and these are the next topic to be discussed. Finally, recent developments related to exogenous administration of factors *in vivo* with the potential for enhancing skeletal muscle repair are presented.

Clinical interest in muscle regeneration

Therapeutic interest in muscle regeneration (for review [1]) includes the conventional disciplines of increased efficiency of repair in sports medicine [2,3], in myopathies, and after severe injury or muscle transplantation, in addition to recovery of strength in disuse atrophy and space flight [4]. Regeneration also has a novel effect in ablating mitochondrial myopathies, because new muscle is effectively repopulated by satellite cells with 'healthy' mitochondria [5], and this might be exploited clinically. Regeneration is being studied in experimental animal models [6], in which limb muscles are usually examined, but it should be noted that the efficiency of regeneration may differ between various muscle types and the relatively ineffective repair of masseter muscles may account for the development of temporo mandibular disorders after trauma [7•]. (An unusual application of muscle grafts, as acellular scaffolds, is for reconstruction of injured peripheral nerves [8].) Regarding the ageing population, there has been an additional focus on the capacity for repair of old muscle, and this is well reviewed elsewhere [9–11]. In brief, myogenesis is highly efficient in old muscles, although it might be slightly delayed because of adverse changes in the host environment [1] and long-term function is compromised by impaired innervation [12,13]. These studies emphasize the crucial role of host factors (angiogenesis, inflammatory response, innervation) in the efficiency of skeletal muscle regeneration. For clinical purposes, the simplest strategies to improve the efficiency of new muscle formation are increasing the speed and avidity of the macrophage response [14,15•], and stimulating revascularization [1,16,17].

During the past decade there has been increasing interest in the transplantation of isolated cultured myoblasts, for a diverse range of reasons. Initially myoblast transfer therapy (MTT) was developed to replace defective genes in myopathies [e.g dystrophin in Duchenne muscular dystrophy (DMD)] [18]; clinical interest in this topic stimulated much research, although many problems remain to be resolved. (Similarly, gene therapy rather than MTT has replaced sarcoglycans in limb–girdle muscular dystrophy [19•].) MTT has also been used (for review [1]) to enhance muscle repair after damage; for delivery of genes into the bloodstream [20], brain or joints [21]; and to replace cardiac muscle cells in acutely injured myocardium [22]. Cultured myoblasts can form ectopic muscle [23], and are used in the rapidly emerging discipline of tissue engineering to construct potential 'artificial' muscles for transplantation purposes [4,24]. For all of these purposes, stem cells (discussed below) represent a potential powerful new source of myogenic cells.

Myogenic cell markers

The classical view is that new muscle is formed from satellite cells. These are located between the sarcolemma and external lamina of myofibres, and they are normally quiescent in mature muscle, but are activated in response to damage. It should be noted that all mononucleated myogenic cells are often widely referred to as myoblasts, regardless of their origin. Satellite cells can be identified using electron microscopy or by double immunostaining with antibodies to molecules near the sarcolemma (e.g. dystrophin, spectrin) and in the external lamina (e.g. collagen IV, laminin-α_2). Activated satellite cells can move out of this position, and it has now been recognized (discussed below) that myoblasts may be derived from cells other than satellite cells. Thus, identification of early myogenic precursors (regardless of their origin and location) requires the presence of some specific marker; several candidate genes are discussed below.

Satellite cell protein markers

The cell-surface protein M-cadherin (M-cad) seems consistently useful for identifying satellite cells *in vivo* (for review [25•]) and cultured myoblasts [26], although messenger RNA expression appears to be very low [25•,26]. On isolated myofibres in culture, the great majority of (but probably not all) mouse satellite cells are positive for M-cad protein (Partridge T, personal communication), and M-cad is not detectable on all rat satellite cells [27]. This indicates that M-cad protein may be very low (or absent) in some satellite cells. Isolated myofibres maintained in tissue culture are attracting increasing attention because this represents a 'half-way' situation between in-vivo studies and the extraction and culture of isolated myoblasts [6,28•–30•].

The receptor for hepatocyte growth factor (HGF) c-met is reported to be present on all quiescent and activated satellite cells, and is readily identified by antibodies on frozen tissue sections [6,31••], isolated myofibres [28•] and cultured myoblasts [26]. Although c-met may be an excellent marker for satellite cells on isolated myofibres, the observation that other cells in muscle, possibly fibroblasts, also express c-met transcripts (Yablonka-Reuveni Z, personal communication) indicates that c-met might not be an ideal marker for primary cultures and in-vivo studies.

The skeletal muscle-specific transcription factors MyoD and myf5 are rapidly upregulated in activated myogenic precursor cells [15•,32,33]. There are conflicting data concerning the extent to which one or both of these genes may be expressed in quiescent satellite cells, or in individual myoblasts, and whether different patterns of expression might define two populations of cells; these issues remain to be resolved [6,26]. In-vitro studies on primary mouse muscle cultures and isolated myofibres [29•,30•] have suggested that Myf5-positive cells might represent a 'stem' cell subpopulation of satellite cells.

Expression of the transcription factor myocyte nuclear factor in quiescent and proliferating satellite cells indicates that this might be a useful marker for such cells *in vivo* [34] (for discussion [35•]).

The cytoskeletal protein desmin has been widely used for identifying activated myoblasts because it is present in very low amounts in quiescent satellite cells and is rapidly upregulated in response to damage [3,28•,36]. Because desmin is also expressed in smooth and cardiac muscle, some care may be required in interpreting in-vivo data.

Antibodies to the mitogen-activated protein kinase family members ERK1 and ERK2 clearly distinguish activated satellite cells from myonuclei on isolated myofibres and myoblasts in culture [29•,30•], but they also bind to other cells *in vivo*. They therefore might be less useful for identification of satellite cells in tissue sections.

Reporter genes of transgenic mice as myogenic markers

Another approach to identifying myoblasts *in vivo* is the use of transgenic mice where a reporter gene like *LacZ* is linked to expression of the candidate gene [10] (e.g. desmin [14,37] or *Myf5* [38]). Detection of the *LacZ* gene product (β-galactosidase) by production of a blue colour is often more convenient than immunohistochemical detection of the native protein. Such transgenic mice can be very useful as a source of donor myoblasts that are readily identified after transplantation into normal host mice [37,39••].

Y-chromosome specific probe to track male myogenic cells

Another useful marker for tracking transplanted myoblasts in mice is a Y-chromosome specific probe. This is a permanent nuclear (DNA) marker that is present in all male cells and their progeny; it does not require gene expression and it readily distinguishes male (donor) from female (host) nuclei on tissue sections [23,40,41]. This marker was developed for MTT experiments [23,42•,43,44] and is now being applied to stem-cell studies [40,41].

Alternative sources of myoblasts and myogenic stem cells

Although satellite cells are classically considered the source of myoblasts in postnatal muscle, recent studies (see below) have confirmed that myoblasts can also arise from nonmyogenic sources *in vivo*. There are clinical applications for such alternative sources of myoblasts and for putative myogenic stem cells, and this is now a topic of intense research interest.

Myoblasts derived from mesenchymal stem cells

The plasticity of mesenchymal cells and the fact that a precursor cell can become a myoblast, adipocyte, chondroblast or osteoblast under different culture conditions has long been recognized. Two populations of mesenchymal stem cells that can be extracted from connective tissue of humans and other species have been described in extensive studies by Young and coworkers [45,46]; these are progenitor cells (with restricted but committed lineages) and pluripotential cells (no committed lineage). It is difficult to distinguish between the possibilities that pluripotential cells in interstitial connective tissue are indeed derived from resident cells, or that they might have originated from bone-marrow derived precursor cells [47•] (see below). That dermal (but not muscle-derived) fibroblasts can give rise to skeletal muscle *in vivo* has been demonstrated [48], and this tissue specificity favours the idea of a truly resident cell rather than a common blood-born precursor. In addition, there is evidence that skeletal muscle precursors can arise from cells in the vasculature (for review [49]), either from cells in the aorta possibly related to endothelial cells and of bone-marrow origin [49], or from vascular smooth muscle cell lines in which it was noted that such myoblasts never expressed Myf5 (Graves DC, *et al.*, unpublished data). A powerful inductive stimulus to convert pluripotent cells into a committed lineage is dexamethasone (and also bone and muscle morphogenic proteins), whereas insulin [or insulin-like growth factor (IGF)-I and IGF-II] is required to induce myogenic expression in progenitor cells. Many months are required for this conversion *in vitro*, however [45]. A soluble factor that induces conversion of the dermal fibroblasts to the myogenic lineage has been identified as a lectin called β-galactosidase-binding protein [50]. It is not known whether similar 'inductive agents' act on mesenchymal stem cells in the interstitial connective tissue of skeletal muscle. The identification of inductive agents to recruit additional cells to the myogenic lineage (that ideally work *in vivo*) is a major challenge, with clinical implications.

Bone-marrow derived myoblasts

The demonstration that muscle nuclei could arise from bone-marrow derived precursor cells [39••,40,41,51] confirmed that this could occur *in vivo*, although it seems to be a rare event. It should be noted that in many (but not all) instances the relatively 'good' contribution of such donor myogenic cells occurred in irradiated muscle [40,41,51], which is an unusually mitogenic environment for donor myoblasts (also see below), and that endogenous regeneration is impaired in irradiated dystrophic mdx muscle. Not only can bone marrow stem cells give rise to skeletal [40,51] and cardiac [40,52] muscle and other cells [47•], but the inverse has been demonstrated with haematopoietic lineages arising from skeletal muscle [41] and neural stem cells [53••]. Thus, it appears that stem cells from diverse tissues may be more similar and totipotential than was previously anticipated. Such bone-marrow derived stem cells might circulate continuously in the bloodstream, or form part of the vasculature, or become resident in interstitial connective tissue (see above). These observations attracted considerable interest, because delivery of muscle precursors through the bloodstream represents an ideal route for distribution to all skeletal muscles. The crucial questions are as follows: can bone-marrow derived cells give rise to large numbers of myoblasts *in vivo*?; can this be exploited as an efficient way to deliver myoblasts to skeletal muscle?; and can this be employed for clinical benefit [41,54]?

Stem cells

The developmental relationships between the various myogenic progenitor and stem cells described above have been reviewed [35•,49]. Ideally, for many myoblast transplantation purposes a 'stem' cell is required; this is defined as a cell that never loses its capacity to replicate – it only divides occasionally to give rise to other cells that undergo extensive replication. This is based on the concept of the Hayflick number that was developed from tissue culture observations, and whether this really applies *in vivo* has been challenged [55]. The quest to isolate such a stem cell has been the holy grail of haematopoietic research for many years [56,57] and is now attracting attention from the perspective of myogenesis.

Experiments with MTT in animals [42•,58,59], in which cultured myoblasts are injected into host skeletal

muscles, suggest that myogenic stem cells can be isolated from skeletal muscle. Myogenic stem cells were identified on the basis that they represent a very slow replicating population of cells in primary cultures and that (after injection) they survived and gave rise to donor myogenic cells with extensive proliferative capacity in the (artificially mitogenic environment of the irradiated) host muscles [42•]. Other experiments [58] concluded that there was a slow and a fast replicating population of satellite cells, and slow and fast replicating populations of satellite cells were also identified on myofibres of growing rats [29•], where it was considered that the slowly dividing satellite cells might represent stem cells. Heterogeneity of cells derived from single myogenic cell clones was demonstrated *in vitro* with the immortalized mouse C2 myogenic cell line [60], and it was suggested that one of the progeny of the initial cells must retain 'stem cell'-like properties. The isolation of four populations of satellite cells from developing human muscle [61] further emphasized the heterogeneity of such precursor cells in muscle tissue. Other in-vivo studies [59] showed that new muscle was formed in muscles that had been heavily pre-irradiated (with 18 Gy, which prevents replication of most cells [62]), and these 'radioresistant' myogenic stem cells were activated only in response to extensive injury provoked by notexin. In all of these situations, the putative resident myogenic stem cells might be a subpopulation of satellite cells, or originate from mesenchymal 'stem' cells, or be of nonmuscle (e.g. bone marrow) origin (discussed above).

The characterization of myogenic 'stem cells' is of considerable interest and attention is now focusing on cell surface markers that might identify and enable purification of such cells [41,46]. Similar markers were assessed for human bone-marrow derived mesenchymal stem cells by Pittenger *et al.* [47•], but those investigators did not test for myogenic capacity; these cells do manifest a skeletal muscle lineage, however (Pittenger MF, personal communication). The expression of Bcl-2 (an apoptosis-inhibiting protein) has been also been suggested as a marker for myogenic stem cells, although skeletal muscle is formed in Bcl-2 null mice [63]. The isolation of embryonic stem cells from the human blastocyst [64] provided another potential source of human 'myogenic stem cells' for clinical MTT and tissue engineering [65].

Clinical implications

Such an 'alternative' source of myoblasts has particular clinical merit in the potential treatment of DMD patients by an ex-vivo gene therapy approach [54,66]. In this situation the patient's own myoblasts are extracted, genetically corrected, and the autologous myoblasts are implanted back into the patient's own muscles. This approach is a combination of gene replacement and MTT, and the use of autologous cells is designed to overcome potential problems of immune rejection. Because satellite cells from the skeletal muscle of DMD boys probably have a limited capacity for replication, an alternative source of autologous myogenic cells (e.g. from dermal fibroblast or bone-marrow stem cells) is ideal for such ex-vivo gene therapy. Even for conventional MTT (or in tissue engineering to produce 'artificial' muscles), deriving donor myoblasts from dermal fibroblasts, mesenchymal, or bone-marrow stem cells might be preferable to using muscle biopsies.

Growth factors and myogenesis *in vivo*

Because of the complexity of in-vivo events [6] and the pleiotrophic action of many factors [17], it can be difficult to determine the precise cellular mechanism of action of a treatment regimen or factor unless samples at different time points are analyzed. The necessity for comprehensive examination of the total pattern of regenerative events has been emphasized by studies on injured muscles [3,15•] in which a marked stimulation of satellite cell proliferation had no overall effect on regeneration; and in whole muscle grafts in MyoD null mice in which myoblast proliferation was sustained and myotube formation delayed by 2 days, although overall regeneration was not impaired (White J, *et al.*, unpublished data). Many well-designed in-vivo studies are now being undertaken as indicated below.

Can administration of exogenous factors enhance muscle repair?

A huge range of growth factors and other molecules are known to influence muscle regeneration (for review [6,11,67]). Whether administration of exogenous growth factors can significantly enhance clinical muscle function or repair remains to be seen. One approach for in-vivo studies is to administer the growth factor (or an inhibitor/blocking agent) directly and assess the impact on muscle regeneration. This has been done using leukaemia inhibitory factor [17,67], fibroblast growth factor (FGF), interferon-α, chemically substituted dextrans [68•], dexamethasone, triiodothyronine, propranolol, clenbuterol and isoprenaline (for review [1]). Factors that have attracted much interest recently are HGF, and the FGF and IGF families. These and other factors relating to recent in-vivo experiments are discussed below. Before discussing these factors it is pertinent to comment on the emerging use of genetic engineering, which will have a huge impact on generating information regarding the critical importance of various factors during regeneration of adult muscle *in vivo*.

Genetically engineered mice

Genetic manipulation of gene expression can be achieved by injecting the gene of interest directly into the muscle [69•,70,71], or by generating null or

transgenic mice [10]. Such new mice are wonderful models for the study of factors that control the crucial events of muscle regeneration after experimental injury or muscle transplantation [26,67,72••,73,74] (White J, *et al.*, unpublished data). A convenient way to assess the impact of modified gene expression on muscle regeneration is to cross such null or transgenic mice with dystrophic *mdx* (or *dy/dy*) mice, in which there is endogenous repeated muscle injury and regeneration. Using this approach it was demonstrated that the absence of MyoD in *mdx* mice resulted in impaired regeneration and a more severe dystrophy [72••] and the dystrophic process was also exacerbated by a lack of FGF-6 [74].

Further sophisticated genetic engineering theoretically enables selected genes to be knocked out or induced in a tissue-specific manner (e.g. in skeletal muscle only) using Cre-loxP recombination technology [75] combined with constructing genes with an inducible enhancer that responds to an exogenous substance [75–78]. Such 'designer' transgenics can be used to study the effects of enhanced or ablated expression of a specific gene in a specific tissue (regenerating muscles) at a particular time. Another application is in the study of MTT or myogenic stem cells (see above), to assess the effect of an engineered pattern of gene expression on the numbers or function of such cells.

Hepatocyte growth factor

One of the hot candidates to stimulate regeneration *in vivo* is HGF. The receptor for HGF, c-met, is expressed by quiescent satellite cells and HGF is present in myotubes *in vitro* [79] and adult myofibres *in vivo* [31••]. HGF is able to activate quiescent satellite cells and is a potent mitogen for myoblasts [79], but not fibroblasts (in this way it differs from FGF-2), therefore making it a very attractive factor to stimulate myogenesis preferentially without fibrosis *in vivo* [31••]. Once activated, the satellite cells are kept proliferating and are prevented from differentiating by HGF [31••] and these effects are mediated through the basic helix loop helix (bHLH) protein Twist and the cyclin-dependent kinase inhibitor p27 (Leshem Y, *et al.*, unpublished data). The intriguing mitogen extracted from damaged muscle now appears to be HGF [31••], which strongly supports the idea that HGF plays a major role during muscle regeneration. Furthermore, HGF is produced by myofibres and myoblasts and the messenger RNA is upregulated by 12 h in rat satellite cell cultures (Shannon M, *et al.*, unpublished data). Assessment in tissue culture of the proliferation of myoblasts extracted from muscles sampled 16 h after in-vivo administration of HGF and other growth factors further supported a crucial role for HGF in stimulating satellite cell activation [31••]. The pattern of proliferation of satellite cells on isolated rat myofibres in culture did not support the notion that HGF is more critical than FGF-2 for satellite cell activation, however [29•]. The complexity of in-vivo administration has been demonstrated by studies in mice [80] that showed increased myoblast proliferation when HGF was injected intramuscularly at the time of cold injury, although overall regeneration was not improved. Furthermore, sustained HGF administration inhibited myoblast differentiation, leading to impaired regeneration; this effect was more severe if HGF was given from early in the regenerative process and the tissue recovered if HGF administration was stopped [80].

Fibroblast growth factors

FGF-2 is well recognized as a potent mitogen for myoblasts, and it causes satellite cells on isolated myofibres from adult rats to proliferate [29•]. Conflicting results were obtained with in-vivo administration of FGF-2 (for review [67]), and this may be because availability of the receptors [81,82] and critical proteoglycans, rather than FGF-2 itself, might be a limiting factor *in vivo*. A recent study on isolated cultured myofibres from adult rats (Kastner S, *et al.*, unpublished data) showed that FGF-1, FGF-2, FGF-4, FGF-6 and HGF all enhance satellite cell proliferation to a similar extent, whereas FGF-5 and FGF-7 had no effect. This study also showed that myofibres express more FGF-6 messenger RNA than the surrounding connective tissue cells. Hence, FGF-6 seems to play a particularly important role in myogenesis of adult muscle, as was demonstrated by impaired regeneration in FGF-6 null mice [74] and the association of *Fgf6* gene expression with extended myoblast replication and muscle fibre hyperplasia in trout [83]. Another recent study on rat satellite cells in culture [82] similarly showed a mitogenic effect of FGF-1, FGF-2, FGF-4, FGF-6 and FGF-9, and no effect with FGF-5, FGF-7 and FGF-8, and HGF further enhanced the mitogenic effects of FGF-2, FGF-4, FGF-6 and FGF-9. The mitogenic effects of HGF and the FGFs appear to be critical for satellite cell proliferation, but their precise interactions and roles are yet to be clarified *in vivo*.

Heparan sulphate proteoglycans and extracellular matrix

Many factors including HGF and FGF bind to heparan sulphate proteoglycans in the extracellular matrix, and such proteoglycans play an important role during myogenesis [84–86]. The administrations of synthetic polymers called regenerating agents (RGTAs) that imitate the heparan sulphates appear to have a remarkable effect on the healing of many tissues. In crush-injured skeletal muscle, RGTAs accelerated both regeneration and reinnervation, and prevented some of the damage resulting from ischaemia in denervated muscle [68•], thus presenting new therapeutic ap-

proaches in various clinical situations. Clearly extracellular matrix molecules play a major role during muscle development, in many myopathies and in regeneration [87,88,89•], but this topic is too large to be discussed here.

Insulin-like growth factors, myostatin and muscle hypertrophy

It is well documented that the IGFs have potent effects on myoblast proliferation and differentiation, and they have recently attracted particular interest due to their anabolic effects, which lead to muscle hypertrophy. This has led to suggestions that IGF-I administration might prevent age-associated myofibre loss, necrosis of dystrophic myofibres [69•] and myofibre atrophy resulting from space travel or disuse [4]. The role of IGF-I in hypertrophy of myotubes was clearly demonstrated *in vitro* by overexpression of IGF-I in transfected cultures of mouse and rat muscle cell lines [33,90,91]. Infusion of IGF-I in rats [92] and enhanced IGF-I expression in type-2 (fast) myofibres (delivered in a viral construct) in *mdx* mice [69•] also resulted in myofibre hypertrophy. Conversely, decreased IGF-I and IGF-II in rats treated with massive doses of corticosteroids may account for the atrophy of diaphragm muscle and be responsible for the diaphragmatic changes seen clinically after steroid treatment [93]. The interest in factors that control hypertrophy and atrophy was excited by the massive hypertrophy seen in mice and cattle when myostatin (a member of the transforming growth factor super family [94]) is not expressed [95]. In humans, increased myostatin levels were found in human immunodeficiency virus-infected men with weight loss and correlate inversely with fat-free mass index [96]. It is possible that there is some inverse relationship between myostatin and perhaps IGF-II [90]. Other anabolic agents that have been studied *in vivo* are clenbuterol [97], and the anabolic steroid nandrolene decanoate [98], which had no effect on the cellular events but a beneficial effect on muscle strength recovery at 2 weeks after injury [88].

Other treatments

Some treatments that traditionally improve muscle recovery or function after soft tissue injury have now been evaluated at the cellular level. Detailed in-vivo experiments show that ultrasound produces a marked stimulation of satellite cell proliferation, but no overall effect on myotube formation or regeneration [3], and the nonsteroidal anti-inflammatory agent naproxen has no overall benefit on muscle repair [2]. Low-energy laser irradiation stimulates satellite cell proliferation and differentiation *in vitro* [99•], and there is a report in Russian of an in-vivo study [100]. Because of the tremendous interest in prescribing corticosteroids such as presnisolone or deflazacort to ameliorate DMD and the controversy that surrounds this, it seems critical to define the effects of these drugs on all aspects of skeletal muscle necrosis and repair. From studies of experimental injury it was concluded that deflazacort promoted muscle repair [101], whereas prednisolone decreased the inflammatory cell response and had an adverse effect on new muscle formation [98].

Finally, strikingly enhanced skeletal muscle regeneration was observed when curcumin, an inhibitor of the transcription factor nuclear factor-κB was administered intraperitoneally in mice [15•]. Such systemic delivery of a pharmacological agent or cytokine [17] is the ideal approach for potential clinical intervention in muscle repair.

Conclusion

There is a renaissance of research interest in postnatal myogenesis, fuelled by the availability of new strains of genetically engineered mice. Useful markers for satellite cells and myoblasts include M-cad, c-met, MyoD, Myf5 and desmin. However, these markers do not address the early identification of 'myogenic' stem cells derived from nonmyogenic sources, and this is a new quest. An even greater challenge is to identify factors that induce the conversion of such nonmyogenic cells into the myogenic lineage *in vivo*. Many in-vivo studies are now critically assessing factors that might enhance the success of muscle regeneration.

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