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Learn, simplify and implement: developmental re-engineering strategies for cartilage repair

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Summary

The limited self-healing capacity of cartilage in adult individuals, and its tendency to deteriorate once structurally damaged, makes the search for therapeutic strategies following cartilage-related traumas relevant and urgent. To date, autologous cell-based therapies represent the most advanced treatments, but their clinical success is still hampered by the long-term tendency to form fibrous as opposed to hyaline cartilage tissue. Would the efficiency and robustness of therapies be enhanced if cartilage regeneration approaches were based on the attempt to recapitulate processes occurring during cartilage development ("developmental engineering")? And from this perspective, shouldn't cartilage repair strategies be inspired by development, but adapted to be effective in a context (an injured joint in an adult individual) that is different from the embryo ("developmental re-engineering")? Here, starting from mesenchymal stem/stromal cells (MSCs) as an adult cell source possibly resembling features of the embryonic mesenchyme, we propose a developmental re-engineering roadmap based on the following three steps: (i) *learn* from embryonic cartilage development which are the key pathways involved in MSC differentiation towards stable cartilage, (ii) *simplify* the complex developmental events by approximation to essential molecular pathways, possibly by using *in vitro* high-throughput models and, finally, (iii) *implement* the outcomes at the site of the injury by establishing an appropriate interface between the delivered signals and the recipient environment (e.g., by controlling inflammation and angiogenesis). The proposed redesign of developmental machinery by establishing artificial developmental events may offer a chance for regeneration to those tissues, like cartilage, with limited capacity to recover from injuries.

Key words: *cartilage repair; developmental re-engineering; tissue engineering; mesenchymal stromal cells*

Cartilage regeneration: state of the art

Cartilage is a tissue with poor intrinsic regeneration capacity. Therefore, trauma affecting articular cartilage, if not properly treated, predisposes to osteoarthritis, a pathological condition that provokes joint pain and loss of motility. For this degenerative joint disease, which causes a reduction of the life quality for millions of people world-wide, no effective disease-modifying therapies are available [1]. Therefore there is a need to repair cartilage defects in order to prevent or delay the onset of osteoarthritis. Among the various techniques to heal cartilage traumas [2], cell-

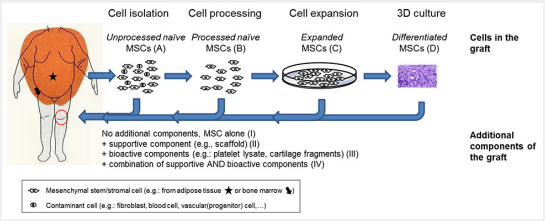

Figure 1

Classification of mesenchymal stromal/stem cell (MSC)-based strategies based on the type and differentiation stage of cells (capital letters in parenthesis) and additional components (Roman numbers in parenthesis) present in the graft. Below are listed examples of MSC-based graft material used in selected clinical trials registered at http://clinicaltrials.gov (keywords used for the research: stem cells AND cartilage repair).

A–II: bone marrow MSC aspirate in a scaffold (identifier NCT00885729).

B–III: MSCs isolated from adipose tissue specimens (liposuction) combined with platelet-rich plasma (identifier NCT01739504).

B–IV: concentrated bone marrow-derived cells on a collagen scaffold, covered with a platelet gel (identifier NCT02005861); MSCs separated from redundant joint tissues (bone marrow or synovium) combined with cartilage fragments in a fibrin gel (identifier NCT01301664).

C–I: expanded adipose tissue-derived MSCs alone (identifier NCT01399749).

C–III: expanded bone marrow MSCs combined with platelet lysate (identifier NCT02118519).

C–IV: expanded bone marrow MSCs combined with chondrocytes in a fibrin gel (identifier NCT02037204).

D–II: chondrogenic pellets derived by bone marrow MSCs embedded in a scaffold (identifier NCT00891501).

based therapy is the most advanced [3, 4]. Autologous chondrocytes expanded *ex vivo* are the favourite source of cells for such therapies. However, clinical outcomes of chondrocyte-based cartilage repair approaches are not predictable [5]. This can be explained by the inability of articular chondrocytes to form cartilage after expansion [6], a step required to increment the initially limited number of cells available from a normal-sized cartilage biopsy [5]. Therefore, the following sources of chondrogenic cells have been proposed: nasal chondrocytes [7]; chondro-progenitors isolated from the superficial zone of articular cartilage [8, 9] or fetal cartilage tissues [10, 11]; mesenchymal stem/stromal cells (MSCs) from various adult [12] or fetal tissues [13, 14]; pluripotent stem cells (embryonic stem cells) [15], or inducible pluripotent stem cells [16]. Table 1 summarises the advantages and disadvantages of using each of these candidate cell sources for cartilage repair.

Ideally, for efficient cartilage repair the therapeutic cells may have to recapitulate in the adult joint processes occurring during cartilage development. Mesenchymal condensation is a critical transitional stage leading to cartilage formation in the embryo. During this stage, undifferentiated mesenchymal cells migrate from the lateral plate mesenchyme and aggregate, forming a cartilaginous anlage. Within this, two distinct populations of chondrocytes arise: one will differentiate into growth plate chondrocytes (i.e., cells that further mature into hypertrophic chondrocytes, ultimately die and are replaced by bone cells); the other, instead, will differentiate in stable chondrocytes, thus contributing to articular cartilage [17]. For durable cartilage repair, the therapeutic/targeted cells must be capable of efficiently differentiating into articular chondrocytes and not activating the endochondral programme.

Although it is not yet clear to what extent adult MSCs resemble the cells in the condensing embryonic mesenchyme, these cells represent the obvious candidate to recapitulate processes leading to articular cartilage formation. Moreover, MSCs are abundantly available in the human body and have an intrinsic tissue-repair capacity under inflammatory/stress conditions, as well as immunomodulatory effects [18-20]. Indeed, positive structural/functional outcomes of MSC-based cartilage repair have been reported in several clinical case reports and trials of the application of MSCs for cartilage repair [20–22].

MSC-based cartilage repair approaches can be classified on the basis of the type and differentiation stage of cells present in the graft, as follows: cell preparation containing (a) *unprocessed naïve MSCs* together with contaminant cells present in the native tissues (e.g., bone marrow, adipose tissue, synovium), (b) *processed naïve MSCs* (to enrich for MSCs and/or to remove cell contaminants), (c) *expanded MSCs*, (d) *differentiated MSCs* after chondrogenic culture (thus within tissue-engineered cartilage tissue). These cell preparations have been clinically used without (I) or with additional supportive (II) or bioactive (III) components or with combinations of supportive and bioactive components (IV) (fig. 1). It is important to consider that additional bioactive component(s) (matrices and/or growth factors) in association with MSCs on the one hand might allow enhancement of the reparative/regenerative properties of the grafted (and of the resident) MSCs, but on the other hand render the clinical outcome difficult to interpret. In this article the different cell-free approaches investigated for cartilage repair will not be discussed, in order to allow a more focused analysis (for an overview of this topic see the references [23, 24]).

Despite the increased utilisation of MSCs for articular cartilage repair, before these cells can be a widely accepted cell source for the treatment of diseased joints, it will be necessary to identify isolation/culture conditions (and bioactive components) enabling them to trigger orderly and durable cartilage tissue repair. In this review article we discuss the main limitations of the MSC-based approaches and we outline possible innovative strategies to use them to induce cartilage regeneration.

Developmental (re-)engineering: a new paradigm for tissue engineering

Traditional MSC-based tissue engineering (TE) strategies suffer from critical drawbacks, which limit robust and routinely accepted clinical translation [25]. MSCs are indeed intrinsically affected by intra- and inter-donor variability, and currently there is no consensus on common markers to predict their chondrogenic potential or therapeutic effect [26, 27]. Moreover, cartilaginous grafts engineered from MSCs typically undergo hypertrophic differentiation when transplanted ectopically *in vivo* [28]. This finally leads to limited structural and functional similarities between the implanted graft and native cartilage, causing, in most cases, the failure of interface integration [29]. A currently investigated explanation for these issues is the possibility that adult MSCs are intrinsically committed towards terminal, hypertrophic chondrocyte differentiation and cannot stably differentiate into articular chondrocytes. A strategy for “re-programming” the fate of MSCs by exposing them to specific signals may be used to reverse this

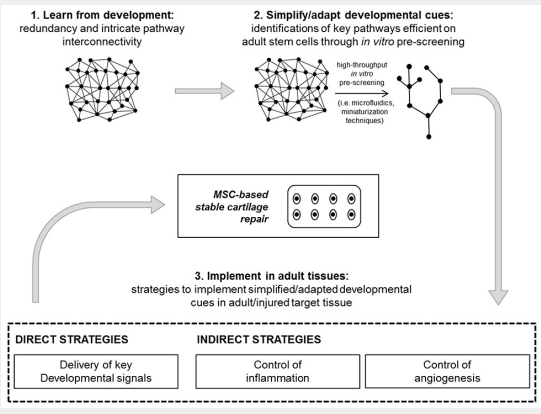

Figure 2

Developmental re-engineering concept: learn, simplify/adapt, implement. After the understanding of pathways involved in the embryonic development of the targeted tissue to repair (1), a simplification/adaptation step is required to extrapolate from the *in-vivo* complexity the key signals that are effective in adult stem cells models. This step (2) could be accelerated by using recently introduced high-throughput *in-vitro* screening tools. The most effective developmentally inspired protocol defined *in vitro* for the acquisition of functional cartilage is then implemented in the adult/injured target tissue. For this aim, different strategies, either direct or indirect, can be identified (3).

tendency and guide these cells towards differentiation into stable cartilage. From this perspective, defining an effective, and possibly temporally staged, combination of stimuli represents an important challenge for strengthening MSC-based TE protocols for cartilage repair.

Here, we introduce the concept of "developmental re-engineering" [30] as a strategy for stable cartilage repair. This term describes a combination of the controlled recapitulation of embryonic morphogenetic events as an approach for engineering adult tissues ("developmental engineering"), with the recognition that such events need to be implemented in the different adult environment, where *in-vivo* repair processes take place and thus require a possible "re-engineering" of processes. We propose that the paradigm of developmental re-engineering is summarised in three main steps: learn, simplify/adapt and implement (fig. 2). The first requirement for engineering a functional tissue is to understand the processes and pathways involved in its embryonic development [31] ("learn") (fig. 2 step 1). However, before successfully applying concepts learnt from developmental biology to TE strategies, a further simplification and adaptation step is required ("simplify") (fig. 2 step 2). Indeed, developmental morphoregulatory systems feature an inherent redundancy and intricate pathway interconnectivity, finally contributing to their robustness. This eventually makes them probably too complex to be faithfully reproduced *in vitro*, especially with the perspective of a clinical translation [30]. Key pathways necessary and sufficient for guiding early progenitors commitment *in vitro* have thus to be extrapolated from the intricate redundancy found *in vivo,* and eventually adapted according to the level of commitment of MSCs [32]. To accomplish this aim, reliable and high-throughput *in-vitro* models, mainly based on microfluidics [33–36] or miniaturisation techniques [37–39], have been recently introduced, with the potential to speed up the screening process. Finally, key pathways selected from developmental events need to be correctly interfaced at the site of the injury, thanks to either direct or indirect strategies ("implement") (fig. 2 step 3).

Developmental re-engineering strategies for cartilage repair

In this section we provide a more detailed description of our vision for exploiting the paradigm of developmental re-engineering towards MSC-based approaches for cartilage regeneration and repair.

Step 1: Learn – development of articular cartilage

During limb development, two different chondrogenic phenotypes are formed, (i) the stable articular cartilage, which acts as a crucial joint component throughout life and (ii) the transient cartilage of the cartilage anlage and the growth plate responsible for long bone growth that eventually is replaced by bone tissue [17]. In order to design a successful cartilage repair strategy it is essential to understand the genesis and maintenance of articular cartilage. Its development is linked to joint formation, which manifests first with the creation of the so-called interzone interrupting the cartilage anlage of the skeletal elements at prospective joint sites [40–42]. The interzone is demarcated by down-regulation of chondrogenic genes such as type II collagen and Sox9, as well as the prominent expression of growth differentiation factor-5 (GDF-5, a member of the bone morphogenetic protein [BMP] superfamily), wing-less type proteins (Wnt) such as Wnt9a, Wnt4, chordin and noggin (specific antagonists of BMPs). The precise molecular mechanisms governing the induction of interzone formation are not yet completely known. Clearly, Wnt signalling, in particular Wnt9a secreted from interzone cells, plays an important role since its ectopic application

Table 1: Cell sources for cartilage repair.

Cell source		Advantage	Disadvantage
Differentiated chondrocytes	Adult articular chondrocytes	Competent to produce cartilage matrix	Limited amount from cartilage biopsy Age-related difference in the differentiation capacity Possible phenotypic alteration in post-traumatic/ pre-osteoarthritis joints
	Adult nasal chondrocytes	High proliferation and chondrogenic capacity	Limited amount from cartilage biopsy
	Neonatal, juvenile articular chondrocytes	High proliferation and chondrogenic capacity Immune-privileged cells	Limited tissue availability Use of the cells associated with ethical concerns
Chondrocyte progenitors	Chondrocyte progenitors from adult joint Epiphyseal chondroprogenitors Fetal cartilage-derived progenitor cells	High proliferation capacity Cells maintain a commitment to their differentiation programme	No conclusive protocols available for the isolation/ culture of these cells Limited studies on the stability of the acquired cartilage phenotype
Mesenchymal stromal/ stem cells	From adult tissues: – bone marrow, – adipose tissues – synovium – …	Large availability High proliferation capacity Immunomodulatory effects Trophic effects	Large donor-donor variability in the differentiation capacity Instability of the acquired cartilage phenotype
	From fetal tissues: – placenta – umbilical cord – umbilical cord blood	Large availability High proliferation capacity Cells with immuno-privileged status Immunomodulatory effects No ethical concerns	No conclusive protocols available for the isolation and culture of these cells Controversial evidence on the capacity of these cells to form hyaline-like cartilage
Pluripotent stem cells	Embryonic stem cells Inducible pluripotent stem cells	Potentially unlimited source of chondrocytes Possibility for personalised medicine	No conclusive protocols available Problems associated with tumour formation Use of the cells associated with ethical concerns

causes heterotopic joint-like structures [43, 44]. However, it has been demonstrated that Wnt is not a prerequisite for joint induction; rather, it is involved in the regulation of joint integrity by inhibiting chondrogenesis and by regulating Ihh expression [45]. Indian hedgehog (Ihh), in turn, is not only involved in controlling the phenotype of growth-plate chondrocytes, but also seems to be implicated in joint formation [46]. Tight regulation of BMP signalling is also crucial for joint formation [47]. Recently, it has been shown that proliferating cells in the distal part of the interzone contribute to both embryonic articular and growth plate cartilage formation, and that inhibition of BMP signalling by noggin next to the presumptive joint site allows preferential differentiation towards the embryonic articular cartilage phenotype [48]. However, the trigger for these cells to acquire a chondrogenic phenotype has not been elucidated yet. Formerly, it was demonstrated that articular cartilage initially shares a history with growth-plate chondrocytes (both are derived from cells expressing Sox9, collagen type II and doublecortin [49, 50]) until they eventually split their fates. Indeed, cells descended from GDF-5-positive cells do not contribute to growth plate development [51], but only to joint elements, including articular cartilage. On the other hand, cells of matrillin lineage give rise to growth plate chondrocytes [52].

Starting from the complexity of the described developmental processes (extensively reviewed elsewhere [17, 40-42]) and the intricate interplay between the occurrence of stable and transient cartilage, the developmental events leading to stable cartilage need to be extrapolated and validated for application on a model based on adult cells.

Step 2: Simplify and adapt – key pathways for adult MSC differentiation into stable cartilage

Recently, it has been demonstrated that the *in-vitro* activation/inhibition of pathways hypothesised to be active in development sequentially guides embryonic stem cell differentiation through mesoderm intermediates to a final chondrocyte population [53]. Interestingly, the differentiation of mouse chondrogenic mesodermal cells derived from embryonic stem cells (ESCs) could be directed *in vitro* either towards hypertrophy, under the influence of BMP4, or towards an articular cartilage phenotype by exposing them to GDF-5 and simultaneously inhibiting hedgehog and BMP pathways [54]. These results confirm the abovementioned postulated importance of a spatial restriction of BMP signalling for the *in-vivo* development of stable articular cartilage [48]. Similar outcomes have been achieved through human induced pluripotent stem cells (hiPSCs). In recent studies, hiPSC-derived chondrogenic progenitors, obtained *in vitro* through an intermediate state of mesoderm induction, were guided towards the acquisition of a stable cartilaginous phenotype (characterised by absence of BMP receptor type 2b expression) [55] by administration of transforming growth factor-beta (TGFβ) and leukaemia inhibitory factor (LIF) signals [56].

The direct application of developmental cues to embryonic or induced pluripotent cell sources thus confirms the potential of developmentally inspired approaches in generating cartilage templates. However, the translation of key developmental pathways to more clinically relevant adult stem cell sources, which are in different stages of commitment, requires further adaptation steps. Regarding the differentiation of MSCs to stable chondrocytes, several studies have focused on key signalling factors that are known to regulate hypertrophy during growth plate development (i.e., members of TGFβ, Wnt and fibroblast growth factor [FGF] protein families and the parathyroid hormone-related protein [PTHrP] / Ihh regulatory loop) [57].

Among the different signalling pathways, BMP is involved in many phases of limb development [58, 59]. BMP is indeed a key chondrogenic factor [60], but it is also involved in triggering endochondral ossification [48, 54]. Recently, it has, for example, been demonstrated how exogenous over-expression of Sox9 potentiates BMP2-induced chondrogenic differentiation while inhibiting BMP2-mediated hypertrophic maturation [61]. Temporally dynamic regulation of BMP pathways can thus be exploited to modulate MSC differentiation towards stable cartilage.

Temporal modulation of Wnt signalling is another currently investigated approach. Initial exposure to Wnt3a (either alone or in combination with FGF2) was indeed shown to enhance undifferentiated proliferation of MSCs, while priming cells towards more efficient chondrogenic differentiation [33, 62]. Endogenous Wnt signals, however, were also discovered to be the main driver of late hypertrophic maturation, suggesting the late inhibition of Wnt as a possible strategy for preventing calcification of cartilaginous templates [63]. Gremlin, a BMP inhibitor, and two inhibitors of Wnt signalling (frizzled-related protein [FRP] and dickkopf-related protein 1 [DKK1]) were also recently identified as distinctive markers of adult human articular cartilage. This further suggests that the inhibition of these pathways has to be considered for achieving stable chondrogenic differentiation of MSCs [64].

FGF family members have been shown to play a dynamic and time-dependent role during MSC chondrogenesis. Early exposure to FGF2 is well known to maximise the expansion potential of MSCs [65], but it has been associated with the early appearance of hypertrophy-related features as well [66]. Interestingly, Correa and colleagues recently demonstrated that this tendency can be modulated through late exposure of MSCs to the combination of endogenous FGF9 and FGF18 signals. FGF9 and FGF18, signalling mainly through FGF receptor 3, have been shown to induce both an anabolic effect on extracellular matrix production and a delay in the maturation of MSC-derived chondrocytes towards hypertrophy *in vitro* [66].

Finally, the PTHrP/Ihh regulatory loop is recognised as one of the main pathways involved in mediating chondrocyte hypertrophy [67]. Mueller and coworkers found that PTHrP treatment reduced alkaline phosphatase expression in MSC 3D pellet culture in a dose-dependent manner; however, when cultured under hypertrophy-enhancing conditions, PTHrP could not diminish the induced enhancement of hypertrophy in MSC pellets [68]. The intermittent supplementation of 3D pellet culture with PTHrP was also demonstrated to stimulate MSCs chondrogenesis (through an upregulation of collagen type II gene expression), while reducing endochondral ossification (through a reduction of Ihh and alkaline phosphatase activity) [69]. In contrast, Weiss and colleagues observed a concomitant down-reg-

ulation of chondrogenic and hypertrophic factors upon PTHrP treatment in MSC chondrogenic cultures that also resulted in unstable *in-vivo* cartilage formation [70].

These studies are examples of how complex developmental events may be approximated to discrete molecular pathways and highlight the importance of the temporal stage for delivery of instructive factors modulating MSC commitment. However, the stability of generated cartilaginous templates once implanted *in vivo* is the main limitation of all the above-mentioned approaches, suggesting the necessity for further refinements. The next required step will be to investigate interconnections among different pathways in MSC models in order to identify the most effective spatiotemporal sequence of instructive signals. Moreover, as mechanical factors are also involved in the development of articular cartilage, mechanotransduction can be considered as an alternative strategy to activate key articular chondrogenic pathways. Preliminary studies indeed showed that dynamic compressive loading suppressed a number of hypertrophic markers (collagen type X, matrix metalloproteinase-13 and ALP gene expression) in hMSC-derived constructs exposed to hypertrophic conditioning [71]. However, a thorough understanding of how individual mechanical factors influence hMSC is needed to utilise them predictably for mechanically-induced stable chondrogenesis [72].

To accomplish these aims, high-throughput *in-vitro* models [33, 34, 36] have been recently introduced as powerful tools for testing the effect of different combinations/concentrations of soluble factors, immobilised cues or mechanical stimuli [73] on MSC differentiation fate, in a fast and reliable fashion. They will thus be promising candidates for speeding up this preliminary screening step.

Step 3: Implement – developmental re-engineering strategies for cartilage repair

Once the network of signalling pathways necessary and sufficient for the generation of articular cartilage has been identified, strategies for transferring them to the site of the injury need to be implemented. In our vision, this can be achieved either by a direct approach, namely localised delivery of selected developmental signals, or indirectly through the exploitation of environmental features characterising the target adult/injured tissue.

Direct strategies

The direct delivery of key agonists and/or antagonists, defined in the previous section, at the site of the injury is a promising strategy to guide resident progenitor cells and/or implanted MSCs towards the generation of stable cartilage in the context of a traumatic joint environment. The envisioned implanted graft should thus ensure the timed delivery of factors for activating/inhibiting selected pathways on targeted cells. In this regard, possible strategies for the controlled and localised delivery of soluble signals are addressed in the next section.

Concerning the targeted cells for the repair process, a few studies have recently led to the identification of putative joint progenitor cells. They were defined within the joint site as slow-cycling proliferative cells showing numerous stem cell markers, as well as specific traits such as TGFβ receptor II [74] or proteoglycan 4 expression [75] in combination with *in vitro* assays of colony forming units and differentiation capacity. These cells localise to various joint tissues such as the superficial zone of articular cartilage, the infrapatellar fat pat, the synovium and the groove of Ranvier, and were shown to persist also in the (young) adult organism (reviewed in [76]). The relationship between these hypothetical progenitor cells and their role in articular cartilage regeneration and repair, however, remains largely unknown. If they could be activated to migrate to the site of injury and induced to differentiate, they would represent a promising cell source in addition to, or as a substitute for, implanted MSCs in cartilage TE approaches (reviewed in [25, 77]). In this regard, microfracture has traditionally been exploited as the main strategy for recruiting progenitor cells from the subchondral bone to the site of the injury [78, 79]. Moreover, a number of studies have recently addressed the use of acellular natural or synthetic scaffolds decorated with chemotactic factors as a promising approach to improve the homing of endogenous cells for cartilage regeneration. Generally, these scaffolds demonstrated in rabbit cartilage defect models a superior repair potential compared with control scaffolds. Zhang and colleagues used stromal derived factor-1 (SDF-1) in collagen type 1-based scaffolds for the repair of partial thickness defects. In this study, in contrast to other reports mentioned below, stem/progenitor cells from tissues other than bone marrow were recruited, since the subchondral bone plate was intact [80]. Huang and colleagues utilised a "MSC-affinity peptide" to functionalise a demineralised bone matrix filled with chitosan-based hydrogel in order to enhance full-thickness osteochondral defect repair by a microfracture procedure [81]. Luo et al. demonstrated the synergistic effect of mechanogrowth factor (MFG), an isoform of insulin-growth factor-1 with a chemokine-like function, with a TGFβ3-decorated spongy silk fibroin-based scaffold in osteochondral defect repair in terms of increased MSC recruitment and suppression of fibrocartilage formation [82].

Alternatively, the use of devitalised cartilaginous templates generated by genetically modified MSCs for the generation of the extracellular matrix template [83, 84] may presumably be applied for cartilage TE in order to attract resident stem/progenitor cells. As an example, MSCs overexpressing a potent, matrix-interacting antiangiogenic protein and undergoing early *in-vitro* chondrogenic differentiation could serve as a template for later decellularisation.

Indirect strategies

Alternative strategies consist of providing cues at the site of the injury to influence the recipient's environment, which in turn would induce differentiation by indirect activation/inhibition of key molecular pathways. The main cues of a traumatic joint environment have been extensively described in recent reviews [85, 86], and they are beyond the scope of this article. Briefly, key elements playing a role within a traumatic joint environment include (i) the trigger of inflammatory and immune responses and (ii) the increase of vascularisation. These events are not present during embryonic development of cartilage and they have to be considered while building up an efficient MSC-based

TE therapeutic approach for cartilage repair, eventually favouring final cartilage regeneration after the implantation of a tissue-engineered graft.

As a first example, inflammatory cues could be exploited and/or modulated in order to improve cartilage repair and the integration of a restored chondral surface to the subchondral bone. It is well known that inflammation is the first phase of tissue repair and that inflammatory cells are crucially involved in the initiation of chondrogenic differentiation and repair processes [87]. Inflammatory cells (i.e., macrophages) have indeed been demonstrated to be key players in healing processes by orchestrating the early regenerative response to injuries [88]. Recent studies have shown that monocytes polarised towards tissue repair – namely anti-inflammatory macrophages (M2) [89] – consistently had a synergistic effect on the cartilage-forming capacity of MSCs in *in-vitro* co-culture models [90]. Inflammatory cells infiltrating the damaged cartilage area could thus be exploited as a strategy to improve osteochondral repair at injured joint sites by directly enhancing the chondrogenic capacity of implanted/recruited MSCs. This can be accomplished by the development of scaffolds capable of promoting the recruitment/polarisation of tissue-repair macrophages [91] and concomitantly stimulating MSC chondrogenesis through the controlled release of instructive factors.

It is well known that continuous inflammation at the defect site leads to aberrant angiogenesis [92]. This may affect the fate of both cartilage generated through an implanted graft and the repaired tissue itself. Indeed, chondrocytes are exposed to a hypoxic environment from development throughout adulthood and a beneficial effect of low-oxygen conditions on chondrogenic phenotype has been observed in cultured human articular chondrocytes [93, 94]. In contrast, vascular invasion through vascular endothelial growth factor (VEGF) signalling is essential for progression from cartilaginous towards bone tissue during endochondral ossification [95]. Based on this, several studies have addressed the influence of blocking angiogenesis and of low oxygen tension on the stability of engineered cartilage. When muscle-derived stem cells were genetically modified to overexpress a soluble VEGF inhibitor and BMP-4, cartilage formation by these cells, in comparison with cells modified for BMP-4 only, was improved in a rat articular cartilage defect model in both healthy [96] and osteoarthritic conditions [97]. Hyaluronic acid- / fibrin-based scaffold functionalised with bevacizumab (an anti-VEGF drug currently in use) and seeded with nasal chondrocytes reliably developed into cartilaginous tissue stable upon subcutaneous implantation, whereas nonfunctionalised scaffolds were mostly resorbed as a result of vessel-mediated ingrowth of matrix-digesting monocytes [98]. Moreover, in a comparison of the effect of hypoxia and normoxia on MSCs cultured under standard 3D chondrogenic conditions in the presence of TGFβ, suppression of hypertrophic markers and a phenotype resembling articular cartilage was observed [99]. Such hypoxic conditions were necessary for the whole *in-vitro* culture period and the hypoxia-primed cartilage templates showed a reduced extent of calcification upon ectopic implantation [100]. Collectively, these findings indicate that the inhibition of angiogenesis and the maintenance of a hypoxic environment might be key requisites for functional performance of engineered cartilage repair tissue.

Biomaterials for the controlled delivery of bioactive signals for developmental re-engineering

Once the effective stimuli for stable cartilage repair have been identified (see previous section), a strategy for their efficient delivery to the injury site has to be defined. To this end, we envision immobilising them within grafts and controlling their spatiotemporal release (fig. 3). This requires the exploitation of innovative technologies in the field of biomaterials.

There are currently numerous techniques available for simple localised delivery of growth factors, in particular by hydrogels based either on biomolecules such as extracellular matrix proteins or polysaccharides, or on synthetic polymers such as poly(ethylene)glycol. Growth factors can be physically incorporated into and immobilised in the gel network by covalent (reviewed in [101]) or affinity binding (reviewed in [102, 103]). Specific tethering of the growth factors allows their controlled release. In the case of covalent attachment, the release can be tuned by additionally inserting cell-responsive linker sequences which are, for example, recognition-sites for metalloproteases. With affinity binding, the affinity of the specific interaction partners governs release kinetics. Furthermore, affinity binding offers the possibility of manipulating the activity of the growth factor. There are hydrogel systems using naturally derived growth factor binding segments from heparin, fib-

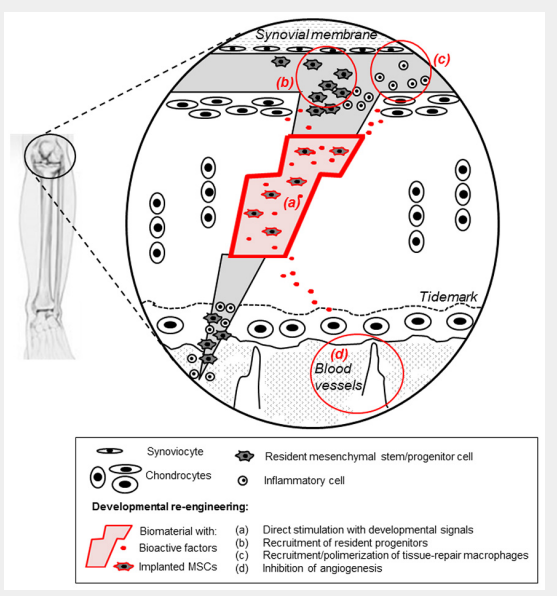

Figure 3

Developmental re-engineering strategy for cartilage repair. We envision the implantation of a graft fitting the site of the injury and able to integrate and release in a spatiotemporally controlled manner either one or several bioactive factors among: (a) developmentally inspired signals to guide either implanted or (b) resident progenitor cells towards a chondrogenic phenotype, (c) factors for the recruitment and polarisation of tissue-repair macrophages or (d) anti-angiogenic factors.
MSC = mesenchymal stromal/stem cell

rin and akin that either do not interfere or enhance the activity of the growth factor. In contrast, bound factors are inactive in hydrogels in which binding happens to the active site of the morphogen as, for example, when peptides derived from receptors are included in the gel backbone. Thus, these hydrogel systems can act to sequester and eventually to release growth factors, possibly generating gradients [102].

For the dynamic adjustability of biomaterials targeting temporal control over growth factor release, light-mediated chemistries have been recently introduced as an emerging tool (reviewed in [104]). Methods such as light-activated enzymatic patterning of synthetic hydrogels, in which the light-reversible protection group blocks the recognition site for the enzyme in the gel network, allow the functionalisation of the hydrogel with signalling factors with high spatial resolution while maintaining their bioactivity [105]. However, these strategies allow the material to be tuneable only *in vitro* prior to implantation. Cartilage is naturally exposed to mechanical loading; therefore, systems in which growth factor release is governed mechanically might lead to the possibility to adjust growth factor delivery after implantation. Moghadam et al. showed that cyclic loading of a hydrogel consisting of thermosensitive nanoparticles and a physically entrapped drug triggers, within a few minutes, a temperature-mediated shrinkage of the nanoparticles that results in higher permeability of the hydrogel and thereby facilitates release of the drug [106]. Moreover, the field of drug release from photosensitive beads has been growing [107] and may also be adapted for TE applications. Lee and colleagues demonstrated that photo-caged adhesion ligand RGD (the tripeptide Arg-Gly-Asp) in subcutaneously implanted hydrogels can be activated and host cell colonisation can be controlled spatiotemporally upon exposure to transdermal light [108].

Moreover, there is increasing evidence that physical properties of the scaffold can steer chondrogenic differentiation. It was shown that glycosaminoglycan-based scaffolds of lower crosslinking density and lower stiffness support cartilaginous matrix accumulation by human MSCs in a chondrogenic medium [109] or induce increased SOX9 expression in rat MSCs in absence of any other differentiation supplements [110]. Therefore, in addition to biochemical factors, the physical parameters of scaffolds also should be taken into account.

Conclusions

In this review, we discuss the possibility to apply the paradigm of "developmental re-engineering" to cartilage repair and regeneration, by following three main steps: learn, simplify/adapt and implement.

According to this vision, the regeneration of functional and stable cartilaginous tissues should start with an understanding of the pathways involved in cartilage embryonic development. A simplification/adaptation step is then proposed to extrapolate from the *in-vivo* complexity key signals that are specifically efficient on clinically relevant adult stem cells models. Developmentally inspired protocols obtained as outcomes of this second step have finally to be implemented in the native tissue, while considering and possibly exploiting the peculiar features of an adult environment. This would lead to the design of artificial developmental pathways in the attempt to either recapitulate directly developmental events or to alter the adult environment to become compatible with developmental processes. The latter case is exemplified by an anti-angiogenic strategy. During development and throughout life articular cartilage is an avascular tissue, but upon injury vascular ingrowth can occur. Therefore, re-establishing the physiological low oxygen tension by blocking angiogenesis may restore conditions that steer resident or injected mesenchymal progenitor cells towards chondrogenic differentiation [111]. Ultimately, the proposed design of artificial developmental pathways may offer a chance for increasing the robustness of regeneration strategies, especially for those tissues, like cartilage, where after development the self-healing capacity remains limited.

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Figures (large format)

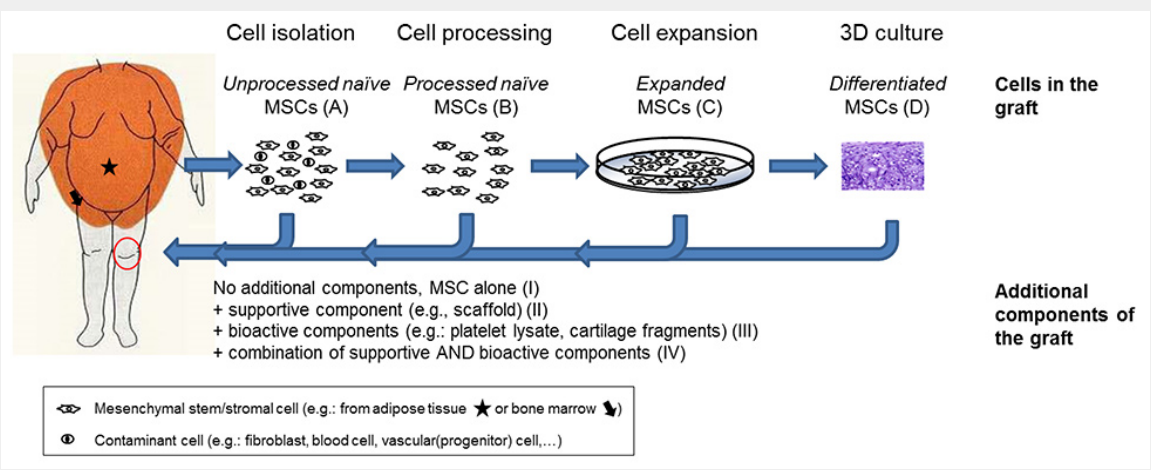

Figure 1

Classification of mesenchymal stromal/stem cell (MSC)-based strategies based on the type and differentiation stage of cells (capital letters in parenthesis) and additional components (Roman numbers in parenthesis) present in the graft. Below are listed examples of MSC-based graft material used in selected clinical trials registered at http://clinicaltrials.gov (keywords used for the research: stem cells AND cartilage repair).
A–II: bone marrow MSC aspirate in a scaffold (identifier NCT00885729).
B–III: MSCs isolated from adipose tissue specimens (liposuction) combined with platelet-rich plasma (identifier NCT01739504).
B–IV: concentrated bone marrow-derived cells on a collagen scaffold, covered with a platelet gel (identifier NCT02005861); MSCs separated from redundant joint tissues (bone marrow or synovium) combined with cartilage fragments in a fibrin gel (identifier NCT01301664).
C–I: expanded adipose tissue-derived MSCs alone (identifier NCT01399749).
C–III: expanded bone marrow MSCs combined with platelet lysate (identifier NCT02118519).
C–IV: expanded bone marrow MSCs combined with chondrocytes in a fibrin gel (identifier NCT02037204).
D–II: chondrogenic pellets derived by bone marrow MSCs embedded in a scaffold (identifier NCT00891501).

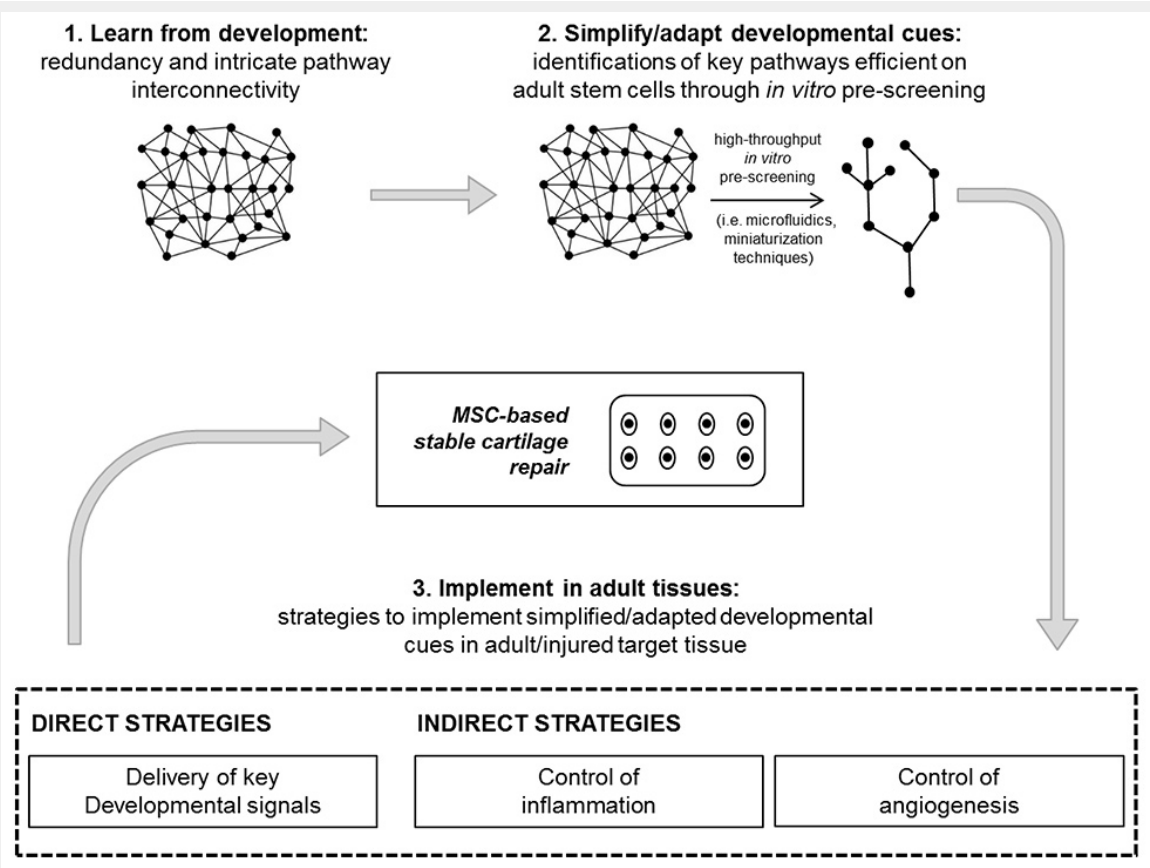

Figure 2

Developmental re-engineering concept: learn, simplify/adapt, implement. After the understanding of pathways involved in the embryonic development of the targeted tissue to repair (1), a simplification/adaptation step is required to extrapolate from the *in-vivo* complexity the key signals that are effective in adult stem cells models. This step (2) could be accelerated by using recently introduced high-throughput *in-vitro* screening tools. The most effective developmentally inspired protocol defined *in vitro* for the acquisition of functional cartilage is then implemented in the adult/injured target tissue. For this aim, different strategies, either direct or indirect, can be identified (3).

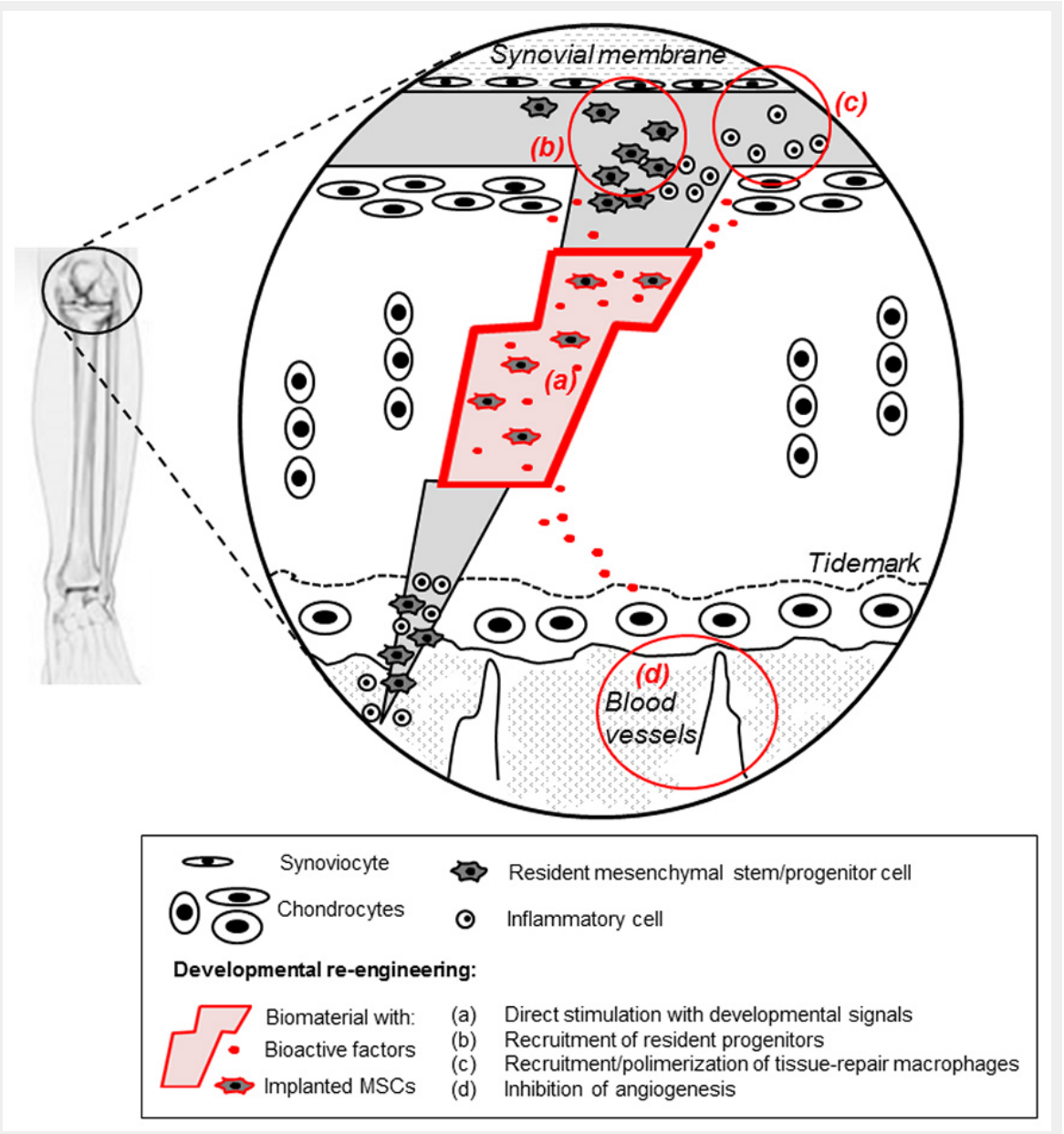

Figure 3

Developmental re-engineering strategy for cartilage repair. We envision the implantation of a graft fitting the site of the injury and able to integrate and release in a spatiotemporally controlled manner either one or several bioactive factors among: (a) developmentally inspired signals to guide either implanted or (b) resident progenitor cells towards a chondrogenic phenotype, (c) factors for the recruitment and polarisation of tissue-repair macrophages or (d) anti-angiogenic factors.
MSC = mesenchymal stromal/stem cell