The Secretome of Hypoxia Conditioned hMSC Loaded in a Central Depot Induces Chemotaxis and Angiogenesis in a Biomimetic Mineralized Collagen Bone Replacement Material

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The development of biomaterials with intrinsic potential to stimulate endogenous tissue regeneration at the site of injury is a main demand on future implants in regenerative medicine. For critical-sized bone defects, an in situ tissue engineering concept is devised based on biomimetic mineralized collagen scaffolds. These scaffolds are functionalized with a central depot loaded with a signaling factor cocktail, obtained from secretome of hypoxia-conditioned human mesenchymal stem cells (MSC). Therefore, hypoxia-conditioned medium (HCM)-production is standardized and adapted to achieve high signaling factor-yields; a concentration protocol based on dialysis and freeze-drying is established to enable the integration of sufficient and defined amounts into the depot. In humid milieu—as after implantation—signaling factors are released by forming a chemotactic gradient, inducing a directed migration of human bone marrow stroma cells (hBMSC) into the scaffold. Angiogenic potential, determined by coculturing human umbilical vein endothelial cells (HUVEC) with osteogenically induced hBMSC shows prevascular structures, which sprout throughout the interconnected pores in a HCM-concentration-dependent manner. Retarded release by alginate-based (1 vol%) depots, significantly improves sprouting-depth and morphology of tubular structures. With the intrinsic potential to supply attracted cells with oxygen and nutrients, this bioactive material system has great potential for clinical translation.

1. Introduction

The challenging treatment of critical-sized bone defects, which arise from systemically diseased bone, trauma, or surgical resection of infectious or tumorous tissue, is still predominantly treated via autologous bone grafting. This current gold standard is limited by the amount of harvestable spongiosa and other drawbacks of the operative procedure like donor site morbidity.[1] Tissue engineering is suggested to provide a potent solution; however, the classical approach, which involves a colonization of a biomaterial scaffold in vitro, suffers from an insufficient supply of the cells after implantation due to the lack of vascularization.[2] Therefore, in situ tissue engineering approaches have emerged as a promising alternative. For this purpose, biomimetic and bioresorbable scaffolds are required with the intrinsic ability to recruit cells with regenerative potential in situ on the one hand but also constitute a favorable environment for proliferation and differentiation in order to stimulate neo-tissue formation and remodeling without the need of previous extra corporeal cell seeding and cultivation. Moreover, as for successful bone fracture defect healing a functional vascular network is mandatory to sufficiently supply cells in deeper scaffold regions with oxygen and nutrients, the scaffolds should support and stimulate ingrowth of blood vessels. Mineralized collagen scaffolds provide such a favorable environment with an interconnected-porous microarchitecture.
suitable for cell ingrowth (~90% porosity, pore size 180 ± 12 µm).[3,4] This nanocomposite is produced in a biomimetic process of synchronous collagen fibril reassembly and mineralization,[5] whereby nanocrystalline hydroxyapatite (65–67 wt%) is tightly bound to the collagen type I matrix (28–30 wt%), which closely resembles natural extracellular bone matrix on the microscale.[3] After freeze drying scaffolds are chemically crosslinked with EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) leading to highly elastic scaffolds in wet state with a compressive modulus ≈ 28 kPa at 50% uniaxial compression.[3] Several in vitro studies verified that this artificial bone matrix exhibits an excellent suitability to support attachment, proliferation, and osteogenic differentiation of human bone marrow stroma cells (hBMSC).[6,7] Investigations in bone defect models in vivo (rat and sheep) showed the active remodeling of the scaffold material and new bone formation within 6–12 weeks.[8–12] In order to upscale scaffolds to clinically relevant dimensions, strategies to efficiently colonize and vascularize these scaffolds are urgently required. Our concept to achieve this is to functionalize these scaffolds with a central depot loaded with a comprehensive signaling factor cocktail. In humid milieu—the gradient formation of released factors simultaneously induces chemo-attraction of cells with regenerative potential and vascularization, which will promote accelerated bone defect healing.

![Figure 1](image)

**Figure 1.** Bone defect healing concept based on biomimetic mineralized collagen scaffolds functionalized with a central depot loaded with a signaling factor-enriched cocktail. In humid milieu—like after implantation—the gradient formation of released factors simultaneously induces chemo-attraction of cells with regenerative potential and vascularization, which will promote accelerated bone defect healing.}

half-lives of only minutes and the lack of clinically approved release systems to enable a sustained release, exogenous therapeutic rhBMP-2 is administered in the range of milligrams.[17] Since under normal bone repair conditions BMP’s are secreted in nanograms the clinically applied doses exceed those concentrations one million times.[18] Those supraphysiological doses are afflicted with a concerning number of adverse effects including ectopic bone formation with spinal cord impingement,[19] increased bone resorption by increased osteoclast activity[20] or cyst-like bone void formation,[21] In order to reduce the applied doses, the controlled release from scaffolds or the combination with other synergistically acting factors rather than the single-agent treatment has been widely reported.[10,22] A cost-effective and potent alternative to recombinant growth factors are autologous or allogenic growth factor mixtures. Recently, a growing number of literature is rising awareness that the secretome of mesenchymal stem cells (MSC) is a key regulator for tissue regeneration – rather than the cells themselves.[23] The entirety of secreted cytokines, chemokines, angiogenic and growth factors and their auto- and paracrine roles comprise a great potential in a variety of clinical applications. Furthermore, the secretome can be manipulated, e.g., by hypoxic culture conditions.[24] Hypoxia-conditioned hBMSC has been shown to be highly potent to attract hBMSC and exhibit a wide range of angiogenic factors and cytokines.[25–27] Among many others also stroma cell-derived factor 1 alpha (SDF1-α, also CXCL12) is secreted, which is the most prominent homing factor for MSC, specifically binds to plasma membrane receptor C-X-C chemokine receptor type 4 (CXCR4) and initiates signaling pathways that lead to their directional migration and homing.[28] Since hypoxia-conditioned medium (HCM) from hBMSC has been shown to have chemotactic and angiogenic potential,
the aim of the present study was to exploit this potential for scaffold functionalization to create a bone replacement material with intrinsic cell-attractive and angiogenic ability to promote and accelerate bone regeneration.

2. Results

2.1. Optimization of Cultivation Settings for HCM Generation

The process of HCM production was intensely optimized aiming to maximize the yield of secreted factors. Since it is known that MSC secrete VEGF under hypoxic conditions, VEGF was selected as reference protein and benchmark to monitor the amount of secreted factors. Since the application of secreted signaling factors is challenged by the variation between donor cells, we compared HCM generated from primary hBMSC (three different donors; one representatively shown in Figure 2A) and cells of the immortalized human mesenchymal stem cell line expressing human telomerase reverse transcriptase (hTERT-MSC) regarding the secreted VEGF content after 2 d of cultivation in hypoxia (Figure 2A). Normalization to the cell number revealed that both exhibited similar VEGF secretion. However, due to a significantly higher proliferation rate (doubling time: hTERT-MSC 2–3 d; primary hBMSC 5–6 d), VEGF concentrations in HCM derived from hTERT-MSC was around twofold higher.

The duration of hypoxia treatment had a significant impact on the VEGF concentration in HCM, for instance prolongation from 2 to 5 d led to an increase in the range of 2.5-fold (Figure 2B). Reducing the content of fetal calf serum (FCS) led to decreased VEGF secretion. However, due to a significantly higher proliferation rate (doubling time: hTERT-MSC 2–3 d; primary hBMSC 5–6 d), VEGF concentrations in HCM derived from hTERT-MSC was around twofold higher.

In order to determine the impact of different sera on the secretion of selected proteins, HCM was generated in serum-free medium or in the presence of 2 vol% serum (w/ and w/o heat inactivation; FCS, hiFCS, HS, hiHS). Via antibody array the amount of 55 angiogenesis-related proteins was determined semiquantitatively for the different HCM-variants shown in Table 1 and the normoxic control (Figure 3). Figure 3B shows selected upregulated angiogenic and downregulated antiangiogenic factors (complete relative luminescence intensity of all represented antibodies on the array is shown in Figure S1 in the Supporting Information). No clear trend in favor of any particular serum-supplementation is visible. VEGF as the most potent inducer of angiogenesis was analyzed in detail via ELISA (Figure 3C). The results confirmed that the cultivation under hypoxic conditions led to a strong increase of VEGF secretion. Compared to normoxic cultivation (≈29 ng mL⁻¹), VEGF-concentration in w/o_HCM, FCS_HCM and hiFCS_HCM was significantly increased up to fivefold (≈140 ng mL⁻¹) and even to sevenfold in HCM supplemented with HS and hiHS (≈200 ng mL⁻¹).

2.2. Influence of Serum on Composition and Chemotactic Potential of HCM

Table 1. Tested HCM-Variants.

| Name                  | Serum     | Culture conditions |
|-----------------------|-----------|--------------------|
| w/o_NormCM            | w/o       | normoxic           |
| w/o_HCM               | w/o       | hypoxic            |
| FCS_HCM               | 2% FCS    | hypoxic            |
| hiFCS_HCM             | 2% hiFCS  | hypoxic            |
| HS_HCM                | 2% HS     | hypoxic            |
| hiHS_HCM              | 2% hiHS   | hypoxic            |
The chemotactic potential of the HCM groups was determined using a transwell assay. Compared to the positive control (expansion medium +20 vol% FCS), all tested HCM variants attracted hBMSC significantly stronger (Figure 3D). The unconditioned blank medium (Expansion Medium +2% of the respective serum or without serum) did not lead to enhanced

**Figure 3.** Influence of serum on secretion of selected angiogenesis-related proteins and chemotactic potential of HCM. A) Antibody array for 55 angiogenesis-related proteins and B) analysis of relative luminescence intensity (RLI) (Ang: Angiogenin; GM-CSF: granulocyte macrophage colony-stimulating factor; IGFBP-1: Insulin-like growth factor-binding protein 1; MCP-1: monocyte chemotactic protein 1; uPA: urokinase-type plasminogen activator; VEGF: vascular endothelial cell growth factor). C) VEGF-concentration via ELISA (n = 3). D) Chemotactic potential of HCM-variants and unconditioned blank media via transwell-migration assay (n = 6). Mean ± standard deviation, *p < 0.05, ***p < 0.001.
migration, demonstrating that the chemotactic potential is directly caused by the secreted factors from hypoxia-treated hiTERT-MSC. All serum-containing HCM-variants showed similar chemotactic potential towards hBSMC, with a slightly increased migration rate towards HS_HCM. Serum-free HCM (HCM_w/o), on the contrary, exhibited significantly lower chemo-attractive potential towards hBMSC.

2.3. Angiogenic Potential of HCM

The ability of HCM to induce angiogenesis, which is expected from its composition, was investigated for all HCM-variants (Table 1) in a HUVEC/hBMSC coculture angiogenesis assay.[31] Immunohistological staining of CD31-positive structures allowed the visualization of endothelial tube formation after 7 d of incubation and revealed that all tested HCM-variants induced angiogenesis significantly stronger than the positive control (20 ng recombinant human (rh)VEGF mL⁻¹) (Figure 4). While serum-containing HCM-variants showed similar results, the addition of w/o_HCM to the cocultures induced tube formation to a reduced extent regarding total tubule length, number of tubules, and number of junctions (Figure 4B).

Since VEGF concentration in HCM is much higher than in the positive control (Figure 3C) and in order to determine whether HCM also with lower VEGF concentrations shows angiogenic potential, the HCM variants were diluted to contain 1, 2.5, 5, 10, 20 ng VEGF per mL and tested in the coculture angiogenesis assay. Staining CD31-positive structures after 7 d revealed that all HCM-variants diluted to 20 ng VEGF mL⁻¹ induced tube formation significantly stronger than 20 ng mL⁻¹ of the single growth factor rhVEGF₁₆₅ (Figure 5). Interestingly, even HCM with very low concentrations of VEGF—starting from 2.5 ng mL⁻¹—induced angiogenesis significantly, while on the contrary at least 10 ng mL⁻¹ of rhVEGF₁₆₅ alone were necessary to achieve significant effects in comparison to the negative control (Figure 5B). For all tested concentrations tube formation—meaning the number of junctions, tubules, and the total tube length—induced by HCM-derived cocktails was significantly higher compared to the respective concentration of recombinant VEGF demonstrating HCM’s impressive angiogenic potential. Comparing the tested HCM variants among one another, no significant difference regarding their angiogenic potential was measurable at these dilutions.

2.4. Concentration of HCM-Derived Cocktails for Central Depot Integration

To integrate HCM-derived cocktails into the small volume of the central scaffold-depot, increase of the signaling factor concentration was necessary. After collection, HCM was dialyzed for several hours, frozen in aliquots at −80 °C, freeze dried and successfully resuspended in up to 1/50 of its previous volume generating 50x HCM. With a molecular weight cutoff of 3.5 kDa the used dialysis tubes enabled desalting of the samples and simultaneously holding back the secreted functional factors. The control of VEGF-concentration after dialysis and freeze-drying proved that around 90% of the growth factors remained in the reconstituted 50x concentrated growth factor mixtures (Figure S2, Supporting Information). To determine the impact of different concentration grades on chemotaxis, transwell assays were carried out exemplarily for hiFCS_HCM. Figure 6A shows a qualitative overview of hBMSC migration towards a broad range of HCM concentrations (0.1x–50x HCM) which was obtained via NeuroProbe transwell assay. The results revealed a direct correlation between the number of migrated hBMSC and increasing HCM-concentration. However, starting from tenfold concentration the amount of migrated cells decreased. For further quantitative investigation, HCM-concentrations until fivefold were examined via Corning transwell-migration assay (0.01x–5x HCM; Figure 6B). Even diluted HCM led to
significantly higher migration compared to the negative control. While cells migrated around sixfold stronger towards 0.01x HCM, migration strongly increased with rising HCM-concentration until 22-fold for 5x HCM.

2.5. In Vitro Characterization of 3D Scaffolds Loaded with a HCM-Derived Cocktail Depot

Considering the final aim of clinical translation, HCM generated from hTERT-MSC cultivated in medium with 2 vol% of HS instead of FCS was used for scaffold functionalization (HS_HCM containing 97.55 ng VEGF mL\(^{-1}\)). Freeze-dried HCM was diluted in either serum-free medium or 1 wt% alginate and injected as central depot in mineralized collagen scaffolds (VEGF-amounts in 15 \(\mu\)L injected as central depot: 10x – 14.6 ng; 20x – 29.3 ng; and 50x – 73.2 ng). The influence of the central depot containing the HCM-derived cocktail on chemotactic cell migration and angiogenic sprouting into mineralized collagen scaffolds was investigated in vitro.

2.6. Migration of hBMSC into 3D Scaffolds

Figure 7 shows the migration of hBMSC into the scaffolds within 3 d after seeding onto the surface. As observed in 2D, also in porous scaffolds the cell migration was stimulated by the HCM-derived signaling factor mixtures in a concentration-dependent manner (Figure 7B,C). While in scaffolds without depot the majority of cells remained close to the scaffold surface, in scaffolds with HCM-derived cocktail depot an increasing cell number migrated to deeper scaffold regions. For 50x HCM in medium-based depots, the cells were evenly distributed until a depth of 1.5 mm (Figure 7B). In deeper scaffold regions, the detected cell number decreased. Significant differences between the scaffolds with increasing HCM-concentrations became visible in depths from 0.75 to 2.0 mm. The results shown in Figure 7C revealed that hBMSC migrated significantly deeper in scaffolds with medium-based depots compared to alginate-based depots (Figure 7D,E).

2.7. Angiogenesis in 3D Scaffolds

For 3D angiogenesis assays in mineralized collagen scaffolds, depots based on medium or alginate were loaded with HCM-derived cocktails as described above or rhVEGF (100 ng) which was used as positive control. HUVEC/hBMSC cocultures were seeded onto the scaffold surface and immunohistochemically stained after 10 d (Figure 8). VEGF release from the depot-containing scaffolds was determined: the amount of VEGF in the supernatant at day 1, 4, 7, and 10 was determined at the depot (Figure 8B). Furthermore, alginate-based depots significantly retarded the VEGF-release as significantly lower values were measured in comparison to medium-based depots (Figure 8B). The formation of well-developed tubular networks on the scaffold surface revealed the strong angiogenic potential of all functionalized scaffold.
variants (Figure 8F; upper rows). Interestingly, the prevascular structures appeared to be more pronounced on scaffolds modified with an alginate-based depot. Quantitative analysis via imaging software Angiosys 2.0 substantiated these observations by showing significantly increased total tube lengths for the alginate group compared to the corresponding medium-based samples (Figure 8C). Higher HCM-concentrations led to enhanced angiogenesis by increasing the branching and total tube length of the formed prevascular networks. Control scaffolds functionalized with 100 ng VEGF per depot showed strong tube formation at the scaffold surface to an extent comparable to 10x HCM. Scaffold functionalization with higher HCM concentrations led to even stronger tube formation. Analysis of the scaffold sections revealed that depending on the functionalization prevascular structures sprouted through the interconnected pores to different extents. Control scaffolds with VEGF-loaded depots showed only weak sprouting, while HCM-functionalization clearly enhanced angiogenesis and sprouting depths in a concentration-dependent manner (Figure 8F; lower row). Alginate-based depots significantly increased tube morphology and sprouting depths. Deepest sprouting until 1500 µm and most pronounced prevascular structures were detected in scaffolds functionalized with 50x HCM (Figure 8D,F).

3. Discussion

In order to develop an effective in situ tissue engineering approach for the treatment of critical-sized bone defects, we focused on the application of highly potent angiogenic and chemotactic signaling factor cocktails secreted by hypoxia-treated mesenchymal stem cells as central signaling factor depot within biomimetic 3D scaffolds of mineralized collagen. Accordingly, the intention of the present study was to optimize the procedure of HCM generation, increasing the concentration of secreted signaling factors in order to integrate the generated cocktail as depot into mineralized collagen scaffolds and to characterize these functionalized scaffolds in vitro.

For clinical application, a standardized cocktail of signaling factors is desirable; therefore, we utilized the immortalized hiTERT-MSC line for HCM generation to overcome the drawback of donor variation. By monitoring VEGF as benchmark for protein secretion, we found that both—hiTERT-MSC and primary hBMSC—secreted similar amounts of VEGF per cell after 2 d of hypoxia but due to higher proliferation rates of hiTERT-MSC the yield of secreted factors was significantly increased (Figure 2). Also, by optimizing the cultivation settings a strong increase of the growth factor yield was achieved: (i) hypoxia
duration was prolonged from 2 to 5 d, (ii) medium to surface ratio was halved. By establishing a concentration protocol based on dialysis, freeze-drying and resuspension, we were able to generate a highly potent signaling factor-enriched cocktail. To minimize the required amount of resuspension medium (DMEM w/o phenol red) and to maximize the signaling factor concentration, the amount of serum was reduced from 10 vol% to 0–2 vol%. The protocol optimization and concentration procedure led to a tremendous increase of the signaling factor amount proven by means of the benchmark protein VEGF. While Gabrielyan et al. measured \( \approx 1.99 \text{ ng VEGF per mL} \),\(^{[25]} \) we were able to achieve \( \approx 150 \text{ ng mL}^{-1} \) (75-fold) from HCM generated in serum-free or 2 vol% FCS-containing medium and \( \approx 200 \text{ ng mL}^{-1} \) (100-fold) from HCM supplemented with 2 vol% HS (Figure 3C).

Antibody arrays revealed the hypoxia-induced upregulation of several factors involved in angiogenesis (Figure 3A,B), among them VEGF, angiogenin (ANG), and transforming growth factor-\( \beta \)1 (TGF-\( \beta \)1). These three factors have been shown to have crucial impact on angiogenesis. By binding to the VEGF-receptors multiple downstream signaling pathways are activated by VEGF leading to the regulation of vascular permeability, promotion of endothelial cell migration, proliferation and survival and finally formation of new vessels.\(^{[32]} \) Like VEGF, also ANG is involved in various physiological processes. In the course of neovascularization, ANG stimulates degradation of the basement membrane and the extracellular matrix, enabling cell migration and tissue invasion.\(^{[33]} \) Especially in wound healing ANG is known to facilitate rapid blood vessel growth and tissue repair.\(^{[34]} \) By exerting a dual effect on angiogenesis—meaning the induction of angiogenesis and simultaneous inhibition of endothelial cell proliferation—TGF-\( \beta \)1 serves as control-mechanism for angiogenesis and regulates the capillary morphogenesis and lumen formation.\(^{[35,36]} \) In turn, the angiogenic effect of monocyte chemoattractant protein-1 (MCP-1) mediates TGF-\( \beta \)-stimulated angiogenesis by recruiting vascular smooth muscle cells toward endothelial cells, which promotes the maturation of new blood vessels.\(^{[37]} \) Another synergistic interplay between the expressed factors involves the upregulation of the insulin growth factor binding protein-2 (IGFBP-2), which enhances the VEGF gene promoter activity and induces endothelial cells to produce and secrete VEGF-A.\(^{[38]} \) Other factors like granulocyte macrophage colony-stimulating factor (GM-CSF)\(^{[39]} \) and urokinase-type plasminogen activator (uPA)\(^{[40]} \) are predominantly involved in proteolysis and degradation of the basement membrane and extracellular matrix components in order to facilitate sprouting and new vessel formation. While many angiogenic factors are upregulated, antiangiogenic factors like tissue inhibitor of metalloproteinase-1 (TIMP-1)\(^{[41]} \) and plasminogen activator inhibitor-1 (PAI-1)\(^{[42]} \) are downregulated, which demonstrates the comprehensive angiogenic effect of HCM. The remarkable angiogenic potential has been demonstrated via angiogenesis assays based on hBMSC/HUVEC cocultures. HCM generated either without or with 2% serum induced the formation of prevascular structures significantly better than the positive control containing the single recombinant VEGF (20 ng mL\(^{-1} \)) (Figure 4). However, angiogenic potential of HCM generated in serum-free medium stays behind the other variants, which could be caused by a diverged factor secretion caused by the sudden serum deprivation—even though no indication for this effect can be seen from the array results of 55 angiogenic factors (Section S1, Supporting Information).
Also, an additional synergistic interaction between secreted factors and serum-originating factors is possible as well as a stabilizing effect during freeze-drying procedure. By diluting the HCM-variants to VEGF-concentration below the positive control (20 ng mL$^{-1}$), the synergistic effect of the entirety of all soluble factors in HCM has been impressively demonstrated (Figure 5). Here, even high dilutions with only 2.5 ng mL$^{-1}$ partial VEGF concentration induced tube formation, while the same concentration of rhVEGF showed no effect. That outstanding angiogenic potential can be explained by synergistic interactions of diverse factors expressed under hypoxic conditions. Compared to single factors, this physiologically concerted factor mix resembles the natural situation much better. For injured tissue in vivo, hypoxia serves as major stimulus for the upregulation of proangiogenic factor production, which leads to the secretion of an indeterminable endogenous proangiogenic cocktail by various invading cells.[43] Future investigations will further characterize the functionality of HCM by either combining or blocking selected factors and analyzing their impact on the angiogenic potential.

Similar to the angiogenic potential all HCM-variants showed significantly higher chemotactic potential compared to
the positive control (20 vol% FCS), while HCM from serum-free medium stays behind the other groups (Figure 3D). Since hBMSC did not migrate towards blank media, the chemo-attractive capacity was directly caused by the factors secreted by hTERT-MSC during hypoxic-treatment. Transwell arrays conducted with various HCM-concentrations showed that even diluted HCM (starting from 0.01-fold; 0.579 ng VEGF mL$^{-1}$; Figure 6) exhibit a significant chemo-attractive potential, which correlated with the increase of HCM concentration until fivefold (289.5 ng VEGF mL$^{-1}$). Higher HCM-concentrations in 2D migration assays inhibited hBMSC migration, leading to a bell-shaped concentration-response curve, which is typical for most chemotactic factors in chemotaxis assays due to the impeded maintenance of the concentration gradient between both chambers.[44] Investigating the hBMSC migration in the porous 3D scaffolds bypasses this limitation of transwell assays. For 3D experiments, concentrated HCM was injected 10-, 20-, and 50-fold into the scaffolds depot. Despite the concentration procedure, applied signaling factors are still $10^{5}$−$10^{6}$ times lower concentrated (ng) compared to the clinically applied rhBMP (mg).[17] Released in a retarded manner, cells at the scaffolds surface are exposed to reduced concentrations in the physiological range, which gives more insights into migration properties and resembles the in vivo situation most closely.

Considering the final aim of clinical translation, all 3D-experiments were carried out with HCM generated from DMEM with 2 vol% of human serum (HS; HCM; allogen) which could also be derived autologous from the respective patient if personalized therapies are desired. HBMSC were seeded on the surface of mineralized collagen scaffolds modified with depots loaded with 10x, 20x, or 50x HCM, dissolved in either serum-free medium or 1 vol% alginate (Figure 7). The growth factor release from the central depot leads to the formation of a gradient within the scaffold, which in turn is expected to induce the directed migration along the gradient. As proof of concept, we recently demonstrated this effect by utilizing VEGF as chemoattractant for endothelial cells,[14] which migrated deepest in scaffolds with a depot based on alginate and heparin—hence scaffolds with the most retarded VEGF-release. We concluded from these findings that the migration distance correlates with the gradient’s steepness.[3] Astonishingly, in this study hBMSC migrated deepest in scaffolds with medium-based depots loaded with 50x HCM (Figure 7C). Without alginate-caused retardation, signaling factors are quickly released from the depot. For VEGF, around 75% was released within the first 3 d.[14] However, the screening for suitable hBMSC donors for migration assays showed, that even though all tested cells migrated actively towards HCM, all donors exhibited different migration characteristics, which makes it difficult to determine a defined threshold for required HCM concentrations. Beside the general donor diversity, also factors like age, passage number, or culture conditions affect the MSC’s ability of migration and homing, which complicate migration studies in vitro.[55, 56]

Beside the chemo-attractive potential, also the angiogenic capacity of HCM has been demonstrated in 3D-scaffolds. Figure 8 shows that with increasing HCM concentrations in the depots the tubular structures sprouted deeper into the scaffolds and formed longer and more branched networks. On the surface, scaffolds functionalized with 10x HCM per depot showed tube formation in the range of the positive control with 100 ng VEGF/depot. Since depots with 10x HCM contained only a proportional amount of $\approx$14.6 ng VEGF this result clearly demonstrates the benefit of the entirety of factors and their complex synergistic interplay.

The addition of alginate to the depot showed an overall positive effect with significantly increased sprouting depths and, furthermore, clearly enhanced morphology of the formed prevascular structures. Both effects can be explained with the depot-stabilizing effect of alginate and the sustained factor release, which have previously been shown for VEGF over 28 d.[14] The cross-linked alginate forms a dense network, which acts as physical barrier that prolongs the maintenance of the growth factor gradient, shields the proteins from denaturation and bypasses their— in most cases—short half-lives.[47] Since endothelial cells have been shown to migrate significantly deeper in scaffolds with retarded growth factor release,[14] an enhanced migration depth of HUVEC could have contributed to the formation of tubular structures in deeper scaffold regions, which in turn persisted longer due to a sustained release of proangiogenic factors.

Beside the physical barrier, the release kinetics of molecules depend on their interaction with alginate. Without chemical interactions, the release depends mainly on the charge polarization of the molecule, i.e., hydrophilic molecules may diffuse very quickly while hydrophobic drugs diffuse slowly through the gel pores.[48] The ionic interaction between polyanionic alginate and positively charged factors like VEGF, Ang, and basic fibroblast growth factor (bFGF) has also been shown to increase their biological activity.[49–51] which could have contributed to the stabilization of formed prevascular structures and their enhanced morphology.

Interestingly, the prevascular structures in scaffolds functionalized with VEGF showed significantly reduced ingrowth compared to all HCM-functionalized scaffolds. Preliminary tests revealed only weak chemotactic response of hBMSC towards VEGF alone (data not shown). Since the composition of secreted factors by hTERT-MSC during hypoxic treatment closely resembles the cellular response on hypoxia in vivo (e.g., in hematoma after trauma), the synergistic effects of these signaling factors are most decisive for the chemotactic and angiogenic potential of HCM. The strong migration capacity of hBMSC towards HCM-modified depots led to significantly increased sprouting depths with deepest sprouting (1500 µm) on scaffolds modified with 50x HCM released from alginate-based depots (proportional amount of $\approx$73.2 ng VEGF). The physical proximity of hBMSC and HUVEC in deeper scaffold regions facilitate their cellular crossstalk, by which both cell types are positively affected.[52] Beside the cellular communication, the secretion of extra cellular matrix proteins by stroma cells have been shown to promote angiogenesis[53] and significantly enhance the life span of endothelial cells,[54] which also could have positively affected the deeper sprouting. Therefore, the attraction of both endothelial cells and stroma or stem cells is of great importance to augment and accelerated bone defect healing. This complex augmentation can only be achieved by a synergistic interplay of bioactive factors and the
Secretome of hypoxia-conditioned hBMSC particularly exhibits this potential. It contains a diverse array of bioactive molecules including growth- and angiogenic factors, hormones, cytokines, chemokines, extracellular matrix proteins, and also extracellular vesicles and exosomes with genetic material. Exosomes itself have been emerged as promising therapeutic agent. Due to that remarkable spectrum of secreted factors, many studies state that the effects of MSCs are predominantly mediated by their secretome, which makes it the key regulator of various physiological processes like directing endogenous and progenitor cells to the sites of injury, initiating multipotent differentiation, promoting wound healing, or mediating tissue revascularization. Preliminary in vitro experiments concerning the osteogenic potential of HCM showed that calcium deposition as marker for mineralization was significantly increased in the presence of HCM even without the addition of osteogenic supplements. This experiments also revealed a proregenerative effect of HCM for hBMSC (Figure S3, Supporting Information). Recent studies suggest that hTERT expression influences the expression of genes involved in cell cycle regulation, proliferation and differentiation. However, due to their short half-lives in vivo the proproliferative effect of the released factors would only be short termed, which could be even advantageous especially for older or diseased patients with reduced regenerative potential.

Recently, Bari et al. published a standardized GMP-compliant process—based on ultrafiltration and freeze-drying—to generate freeze-dried soluble powder from adipose MSC-secretome for clinical applications. Standardizing the generation of signaling factor-enriched cocktails from hypoxia conditioned hTERT-MSC bears a great potential for the treatment of a wide variety of diseases. Even though the complexity of secretome makes it difficult to study, the entirety of factors and their complex interplay account for its significant therapeutic potential. The application of HCM-derived signaling factor-enriched cocktails as central depot in the herewith described in situ tissue engineering concept would bypass present interfering concerns afflicted with cell-based therapies in regenerative medicine like immune compatibility, tumorigenicity, infections, as well as saving on time and cost required for ex vivo expansion of autologous cell preparations. Furthermore, this bioactive material system could supersede the application of supraphysiological dosed rhBMP-2 in the clinical practice and avoid the associated risk of severe side effects. Our future investigations envisage the in vivo validation and standardization of protocols for cell isolation, characterization, expansion, and secretome generation with regard to clinical translation.

4. Conclusion

The secretome of hypoxia-conditioned hTERT-MSC (HCM) has a tremendous potential to induce the directed migration of hBMSC and simultaneously promotes the formation of vascular structures, which sprouted deeply into mineralized collagen scaffolds. Based on HCM, signaling factor-enriched cocktails were generated by optimizing the culture conditions to increase the yield of secreted factors and by establishing a concentration protocol including a dialysis and freeze-drying procedure. The integration of the HCM-derived cocktails as central depot in mineralized collagen scaffolds resulted in the generation of a bioactive material system with great potential for the treatment of critical-sized bone defects as demonstrated in a set of in vitro experiments. While cells with regenerative potential will be recruited from the surrounding tissue to the site of injury, the newly formed vessels will ensure their supply with essential oxygen and nutrients. This approach has the potential to be a cell-free and ready-to-use therapeutic solution for accelerated bone defect healing that can be produced at affordable costs and with better quality control and consistency compared to classical cell-based tissue engineering concepts.

5. Experimental Section

Preparation of Porous Scaffolds of Mineralized Collagen: Scaffolds (diameter: 6 mm; height: 8 mm) consisting of fibrillated, mineralized collagen were prepared as described previously using collagen type I from bovine tendon (Syntacoll, Germany). In brief, collagen dissolved in 10 × 10^−3 m hydrochloric acid was mixed with a 0.1 m calcium chloride solution; the pH value was adjusted to 7.0 by addition of 0.5 m TRIS and 0.5 m Sørensen phosphate buffer. The neutralized mixture was incubated at 37 °C for 12 h to run the process of synchronous collagen fibril reassembly and nanocrystalline hydroxyapatite precipitation. Via centrifugation, the mineralized collagen fibrils were collected. After resuspending the collagen pellet in distilled water (1.5 g mL^−1), the suspension was filled in wells of a 96-well tissue culture polystyrene plate, frozen at −20 °C and freeze-dried. Crosslinking of the porous scaffolds was carried out by incubation in 1 wt% 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC; Sigma-Aldrich, USA) dissolved in 80% ethanol for 1 h. After thorough rinsing of the scaffolds in water, 1 wt% glycine solution and again in water, final freeze-drying was conducted. Scaffolds were sterilized by γ-radiation at 25 KGY.

Cell Cultivation: For cell culture experiments, human bone marrow stroma cells (hBMSC) were isolated from bone marrow aspirates of healthy donors at the age of 29–33 (male, Caucasian) after informed consent. The use of hBMSC for the experiments was approved by the ethics commission of the Technische Universität Dresden (EK261092009). Immortalized hTERT-MSC were used for the generation of HCM. Both, hBMSC and hTERT-MSC, were cultivated under standard cell culture conditions (humidified atmosphere, 21% O_2, 37 °C, 5% CO_2) in expansion medium consisting of α-MEM (Biochrom, Germany) with 10% FCS; Corning, USA), 100 U mL^−1 penicillin and 100 μg mL^−1 streptomycin and 2 × 10^−3 m L-glutamine (−Glut). For in vitro angiogenesis experiments, hBMSC were cocultured with human umbilical vein endothelial cells (HUVEC; passage 4–6; Promocell, Germany), which were expanded in Endothelial Cell Growth Medium (Promocell).

Cell Number Quantification via LDH-Activity: Cells were lysed in 1% Triton X-100/PBS for 30 min at 37 °C. Lactate dehydrogenase (LDH) activity was determined using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, USA) according to the manufacturer’s instructions. The absorbance was measured at 492 nm (infinite M200 PRO, Tecan, Switzerland) and correlated to a calibration line, generated from defined numbers of cells from the respective experiment.

Generation of Hypoxia Conditioned Medium and Production of Signaling Factor-Enriched Cocktails—Optimization of HCM Generation: (i) Standardized cell line: HCM was generated from hBMSC of three donors and hTERT-MSC. 2 × 10^6 cells were seeded in 6-well plates, incubated in expansion medium for 3 d under standard conditions, followed by 2 d of incubation at hypoxic conditions (1% O_2, 37 °C, 5% CO_2) as described by Gabrielyan et al. medium volume to cell
growth area: 0.1 mL cm$^{-2}$. After collection, the VEGF-content of HCM was measured via ELISA and cell number was determined via LDH-activity. (ii) Increase of yield of secreted factors: The hypoxia duration was prolonged until 5 d and the ratio of medium volume to cell growth area was reduced to 0.057 mL cm$^{-2}$. VEGF served as reference protein and was determined to monitor the increase of yielded factors.

**Generation of Hypoxia Conditioned Medium and Production of Signaling Factor-Enriched Cocktails**—Production of Concentrated Signaling Factor Cocktails: For HCM-generation, hTERT-MSC were cultured with expansion medium in a T175 cell culture flask until 95% confluency. Then the medium was removed, the cell layer was washed twice with phosphate buffered saline (PBS; Gibco, USA) and 10 mL phenolred-free DMEM (Gibco) was added (0.057 mL cm$^{-2}$). According to the experimental design, the medium was supplemented with 2 vol% native or heat inactivated FCS (hiFCS) or human serum (HS/hiHS). Table 1 shows the HCM-variants used for further characterization. Under gentle shaking (Biosan Rocker-Shaker, Latvia), the cells were cultured for 5 d under hypoxic conditions (1% O$_2$, 37 °C, 5% CO$_2$). Then the supernatant was centrifuged at 1500 rpm to separate the medium from cell debris and dialyzed against deionized water to remove ions and molecules below 3.5 kDa (GEBAflex dialysis tubes, cut off 3.5 kDa; Scienova GmbH, Germany); 4 mL aliquots were frozen at −80 °C. To obtain concentrated cocktails, aliquots were freeze-dried and resuspended in 400, 200, or 80 µL (10x, 20x, 50x HCM) (Figure 9).[14]

**Antibody Array** For HCM characterization, an antibody array for human angiogenesis-related proteins was performed according to the manufacturer’s instructions (Proteome Profiler Human Angiogenesis Array Kit, R&D, USA). Chemiluminescence signals were detected with a gel documentation system (G:BOX, Syngene, UK) and analyzed qualitatively and quantitatively using ImageJ V1.44p freeware (http://rsbweb.nih.gov/ij; National Institutes of Health, Bethesda, Maryland, USA). ELISA for VEGF Quantification: VEGF concentration was determined via enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Human VEGF Standard TMB ELISA Development Kit, Peprotech, France). Color development was measured with an ELISA plate reader (infinite M200 PRO, Tecan, Switzerland) at 450 nm with wavelength correction at 620 nm.

**Integration of HCM-Derived Signaling Factor Cocktails into Scaffolds as Central Depot:** For the functionalization of mineralized collagen scaffolds with a central depot a signaling factor-enriched cocktail derived from one batch HS_HCM was applied (97.55 ng VEGF mL$^{-1}$). Freeze-dried aliquots were resuspended in α-MEM (w/o supplements) or 1 wt% alginate (autoclaved alginate dissolved in deionized water; ISP Capitel Inc., USA) under sterile conditions to obtain concentrated cocktails (10x, 20x, 50x HCM).[14] A gel loading pipet tip (GELoader, Eppendorf, Germany) was used to inject 15 µL of the concentrated cocktail into dry mineralized collagen scaffolds. Scaffolds with alginate-based depots were incubated for 10 min in 0.1 M CaCl$_2$ solution to crosslink the alginate.

**Biological Characterization of Hypoxia Conditioned Medium**—2D Chemotaxis Assay: To determine the chemotactic potential of the generated HCM-variants towards hBMSC two transwell-based chemotaxis-assays were performed. For both assays hBMSC were starved for 12 h in serum-free expansion medium to enhance their migration potential. Due to the small required sample volume of 30 µL the transwell-migration assay from NeuroProbe (ChemoTx System 96 well polycarbonate track-etch membrane (PCTE); pore size 8 µm, NeuroProbe, USA) was used to obtain a first qualitative overview (0.1x–50x HCM). The cells were prestained with Cell Tracker CM-Dil according to the manufacturer’s instructions (1 µg mL$^{-1}$ in PBS; Thermo Fisher Scientific, USA), resuspended in serum-free expansion medium and seeded as droplet with 1 × 10$^5$ hBMSC in 30 µL onto the membrane. After 24 h incubation under standard cell culture conditions the cells on the upper side of the membrane were manually removed with a cotton swab and the lower membrane side was fluorescence-microscopically examined (Keyence BZ9000E; Keyence, Japan).

Quantitative analysis of the most relevant HCM-concentrations was conducted via Corning transwell-migration assay (Corning HTS Transwell-96 permeable supports; pore size 8 µm, Sigma Aldrich, Germany). $3 \times 10^6$ hBMSC in 80 µL of serum-free expansion medium were seeded into each upper chamber. 150 µL of HCM-variants and controls were added into the lower chamber. After 24 h incubation under standard cell culture conditions, the cells on the upper side of the membrane were lysed and cell number was correlated to the measured LDH-activity. For both assays, expansion medium without serum was used as negative and with 20 vol% FCS as positive control.

**Biological Characterization of Hypoxia Conditioned Medium**—3D Migration Assay into Scaffolds: Prior to seeding, the scaffolds containing a depot loaded with an HS_HCM-derived cocktail were equilibrated for 1 h in serum-free expansion medium. The fully soaked scaffolds were placed upright (injection canal on the bottom) into a bovine serum albumin (BSA)-blocked 48-well plate (1 wt% BSA in PBS, 30 min). For seeding, 10 µL containing 1.5 × 10$^5$ hBMSC were dropped on top of each scaffold. After 30 min incubation at 37 °C, 490 µL serum-free expansion medium was added carefully. After 72 h incubation under standard conditions, the scaffolds were washed with PBS, fixed for 30 min with 4 vol% formaldehyde in PBS (FA; SAV Liquid Production GmbH; Germany), cut in longitudinal direction with a razor blade and fixed again for 5 min with 4 vol% FA. To investigate the cell migration, the nuclei were stained with Hoechst 33 342 (2 µg mL$^{-1}$ in PBS, Invitrogen, USA). The stained scaffold halves were transferred to cryo-molds (Sakura Finetek, VWR, USA) and frozen at −80 °C in section medium (Richard-Allain Scientific Neg-50, Thermo Scientific, USA). 50 µm thin slices were prepared by cryo-sectioning with a Leica CM 1900 cryotom (Leica, Germany), mounted with Roti-Mount FluorCare (Roth, Germany) and imaged with a fluorescence-microscope (Keyence). Cell migration was analyzed via Arivis Vision 4D (Arivis AG, Germany): starting from the surface, each slice was divided in
frames with 250 µm steps up to a depth of 3000 µm. By counting the cell number in each frame the cell distribution was determined.

Biological Characterization of Hypoxia Conditioned Medium—2D Angiogenesis Assay: Coculture of hBMSC and HUVEC. The angiogenic potential of HCM-derived cocktails was characterized with an angiogenesis assay consisting of a HUVEC/hBMSC coculture.[11] 6.8 × 10^4 hBMSC were seeded into each well of a 96-well plate and cultured in expansion medium under standard conditions. After 3 d, 1.7 × 10^3 HUVEC were seeded on top of the hBMSC monolayer and coculture medium (α-MEM and endothelial basal medium (Promocell) [1:1; 15 vol% hiFCS, 100 U mL⁻¹ penicillin, and 100 µg mL⁻¹ streptomycin, osteogenic supplements 10^−8 M dexamethasone, 0.05 × 10⁻³ M ascorbic acid 2-phosphate, 5 × 10⁻³ M β-glycerophosphate; all from Sigma-Aldrich]) was added. To determine the angiogenic potential of HCM variants, 50x HCM was serially diluted in coculture medium; as positive control, 20 ng VEGF mL⁻¹ (rh VEGF165; Biomol, Germany) was added instead of HCM. The coculture run for 7 d with a medium change at day 4. The cells were then fixed in 4% FA for immunohistochemical staining of prevascular structures.

Biological Characterization of Hypoxia Conditioned Medium—3D Angiogenesis Assay: Coculture of hBMSC and HUVEC in Scaffolds. Scaffolds containing a depot loaded with an HS-HCM-derived cocktail were equilibrated in coculture medium for 30 min, shortly dipped on a filter paper to remove the excess medium from the pores and placed into a BSA-coated 48-well plate (1% BSA in PBS, 30 min) with the injection site facing towards the well-bottom. The scaffolds were seeded by dropping 100 µL of coculture medium containing 1.25 × 10^5 HUVEC and 5 × 10^4 hBMSC on top of each scaffold followed by incubation for 30 min at 37 °C and addition of 400 µL coculture medium to each well. With medium changes at day 4 and 8, the scaffolds were incubated for 10 d. For analysis, the scaffolds were washed with PBS, fixed with 4% FA and immunohistochemically stained for prevascular structures. To visualize the ingrowth of tubular structures, the scaffolds were longitudinally cut with a razor blade and microscopically analyzed. Scaffolds with 100 ng VEGF-loaded depot served as positive control, and 8, the scaffolds were incubated for 10 d. For analysis, the scaffolds were longitudinally cut with a razor blade and microscopically analyzed. Scaffolds with 100 ng VEGF-loaded depot served as positive control, and 8, the scaffolds were incubated for 10 d. For analysis, the scaffolds were longitudinally cut with a razor blade and microscopically analyzed. Scaffolds with 100 ng VEGF-loaded depot served as positive control, and

Statistical Analysis: The cell culture experiments were performed using at least triplicates; the results are given as mean value ± standard deviation. GraphPad Prism 8.1.1 (GraphPad Software, USA) was used to perform a one-way analysis of variance (ANOVA) to evaluate statistical significance. Significance levels were set as *p < 0.05, **p < 0.01 and ***p < 0.001. Tukey’s multiple comparison post-hoc test was used to determine differences within the groups.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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