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TISSUE ENGINEERING AND REGENERATIVE MEDICINE

In Vivo Formation of Stable Hyaline Cartilage by Naïve Human Bone Marrow Stromal Cells with Modified Fibrin Microbeads

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ABSTRACT

Osteoarthritic and other types of articular cartilage defects never heal on their own. Medicinal and surgical approaches are often ineffective, and the supply of autologous chondrocytes for tissue engineering is very limited. Bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) have been suggested as an adequate cell source for cartilage reconstruction. However, the majority of studies employing BMSCs for cartilage tissue engineering have used BMSCs predifferentiated into cartilage prior to implantation. This strategy has failed to achieve formation of stable, hyaline-like cartilage, resistant to hypertrophy in vivo. We hypothesized that in vitro predifferentiation of BMSCs is not necessary when cells are combined with an adequate scaffold that supports the formation of stable cartilage in vivo. In this study, naïve (undifferentiated) human BMSCs were attached to dehydrothermally crosslinked stable fibrin microbeads (FMBs) without and with other scaffolds and implanted subcutaneously into immunocompromised mice. Optimal formation of abundant, hypertrophy-resistant, ectopic hyaline-like cartilage was achieved when BMSCs were attached to FMBs covalently coated with hyaluronic acid. The cartilage that was formed was of human origin and was stable for at least 28 weeks in vivo. STEM CELLS TRANSLATIONAL MEDICINE *2019;8:586–592*

SIGNIFICANCE STATEMENT

The present report is the first to demonstrate formation of stable cartilage in vivo by human bone marrow stromal cells (BMSCs). Using fibrin microbeads coated with hyaluronic acid as a scaffold combined with naïve BMSCs provides new possibilities for the restoration of damaged articular cartilage in regenerative medicine and for modeling of human cartilage diseases in vivo.

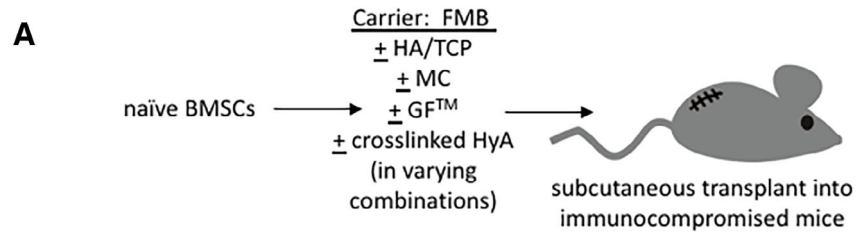
INTRODUCTION

Medicinal treatments for osteoarthritis are palliative, and surgical interventions have been less than optimal [1]. Populations of bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) contain skeletal stem cells, capable of differentiating into a spectrum of skeletal tissues, including cartilage (reviewed in [2]). Consequently, BMSCs have been proposed for use in cartilage regeneration. However, implantation of BMSCs predifferentiated into a chondrogenic phenotype, a common practice in current cartilage tissue engineering, has not resulted in formation of stable, hyaline-like cartilage, resistant to hypertrophic mineralization in vivo [3]. Proper handling of BMSCs ex vivo and choice of an appropriate scaffold appear to be critical.

Previous studies demonstrated that anchorage-dependent cells bind tightly to fibrin matrix, inspiring the development of dense fibrin microbeads (FMBs) [4, 5]. In the current study, naïve (undifferentiated) human BMSCs (hBMSCs) were attached to FMBs in combination with several secondary vehicles or to FMBs modified by covalent coating with hyaluronic acid (HyA-FMBs) and implanted subcutaneously into immunocompromised mice. We report for the first time that stable hyaline-like human cartilage can be formed in vivo by naïve human BMSCs in combination with HyA-FMBs.

MATERIALS AND METHODS

For detailed experimental procedures, see Supporting Information Materials and Methods section. Human bone surgical waste was obtained



B

Vehicles	Recipient mouse strain	Harvest times (weeks)	# recovered/ total #	Bone areas ave %	Cartilage scores (ave)	Mature cart areas ave %	Immature cart areas ave %	Vehicle areas ave %
FMBs	5 NSG	19	5/5 (panels C,E,G)	24.5±7.1	2,0,0,2,2 (1.2±1.1)	9.4±4.2	3.9±1.7	3.7+1.1
HyA-FMBs		19	5/5 (panels D,F,.H)	12.9±2.7 p = .2222	2,3,3,4,3 (3.0±0.7) p = .0317	38.5±6.7 p = .0159	6.7±0.9 p = .3095	9.7±0.6 p = .0079

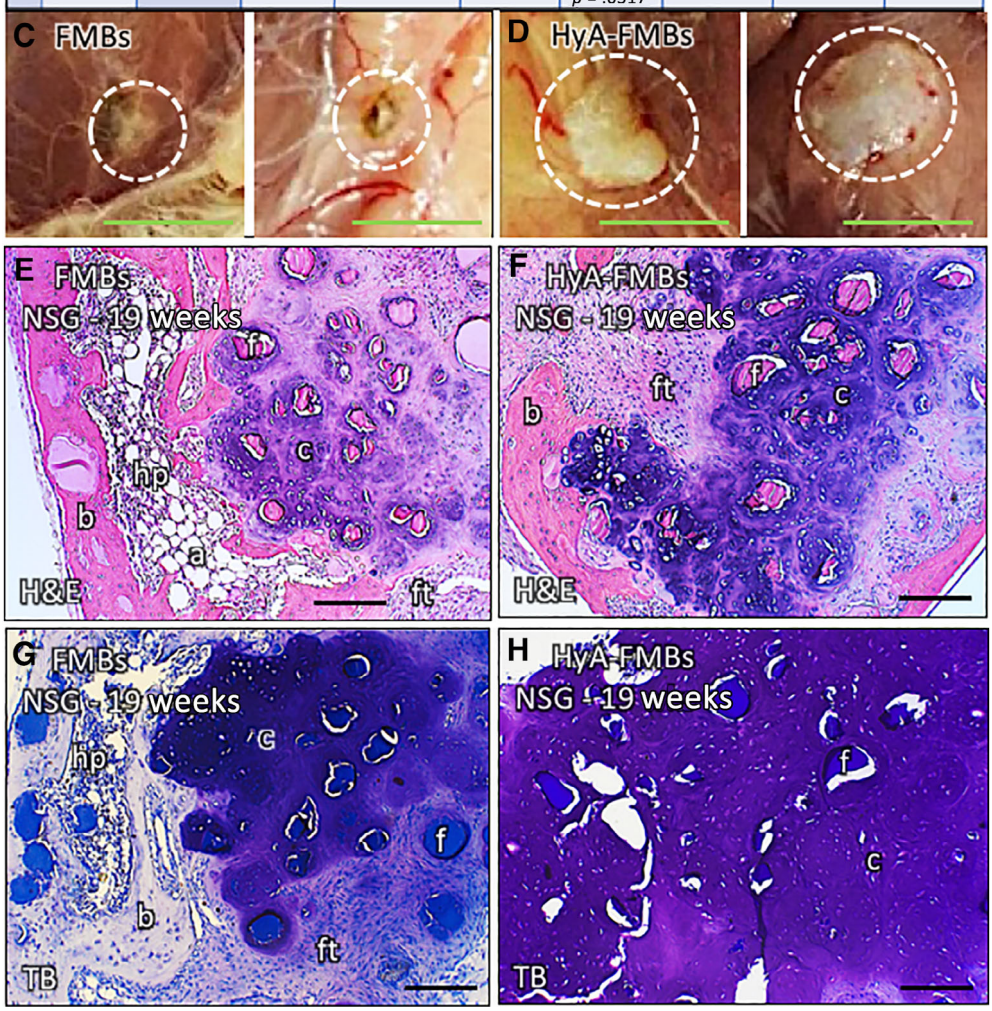


Figure 1. Cartilage formation in 19-week-old constructs of naïve human bone marrow stromal cells (hBMSCs) with FMBs, without and with covalently bound HyA-FMBs in NSG mice. **(A):** Study design. Scheme of subcutaneous placement of naïve hBMSCs with FMBs, without and with secondary carriers, into immunocompromised mice. **(B):** Details for the experiment. All constructs were recovered. More mature cartilage was formed by hBMSC/HyA-FMBs than by hBMSCs/FMBs (p = .0159). The bone and immature cartilage were also formed in both types of constructs, but with no statistical difference between the two types of constructs. Macroscopic views of cartilage formed **(C)** by hBMSC/FMB constructs and **(D)** by hBMSC/HyA-FMB constructs. **(E):** Staining of hBMSC/FMB constructs with H&E revealed strong basophilic staining of the extracellular matrix in areas with cartilage morphology. The bone, with some hematopoiesis-supporting foci, and fibrous tissue were found at the periphery of the constructs **(E, G)**. Cartilage-like areas in hBMSC/HyA-FMB constructs were **(F)** intensely basophilic in H&E-stained sections and **(H)** strongly metachromatic in TB-stained sections. Cartilaginous areas were more extensive in hBMSC/HyA-FMBs construct **(F, H)**. Residual FMBs were more abundant in hBMSC/HyA-FMB than in hBMSC/FMB constructs (p = .0079) **(E–H)**. Green scale bars = 5 mm; black scale bars = 200 μm. Abbreviations: a, adipocytes; b, bone; BMSC, bone marrow stromal cells; c, cartilage; f, residual fibrin microbeads; FMBs, fibrin microbeads; ft, fibrous tissue; GF, gelatin sponge; HA/TCP, hydroxyapatite/tricalcium phosphate; hp, hematopoiesis; HyA, hyaluronic acid; MC, micronized collagen; TB, toluidine blue (metachromatic staining of cartilage matrix).

A	Vehicles	Recipient mouse strain	Harvest times (weeks)	# recovered/ total #	Bone areas %	Cartilage scores	Mature cart areas %	Immature cart areas %	Vehicle areas %
	HyA-FMBs	4 NSG	8	2/2 (B,C,F,G)	3.5;0.6	2,2	18.8;20.0	14.9;15.3	38.2;54.5
			16	1/1	1.1	3	40.8	13.2	25.4
			28	1/1	1.1	3	65.6	15.2	10.8
	Empty FMBs		8	0/1	0	0	0	0	0
			16	0/1	0	0	0	0	0
	Empty HyA-FMBs		8	1/1 (panel E)	0	0	0	0	79.2
			16	0/1	0	0	0	0	0
	HyA-FMBs	4 SHC	8	2/2 (panel D)	0, 0	1,1	1.9;5.9	11.5;12.9	72.3;68.0
			16	1/1	0.3	2	15.9	18.1	37.5
			28	1/1	0.2	4	74.3	4.6	18.3

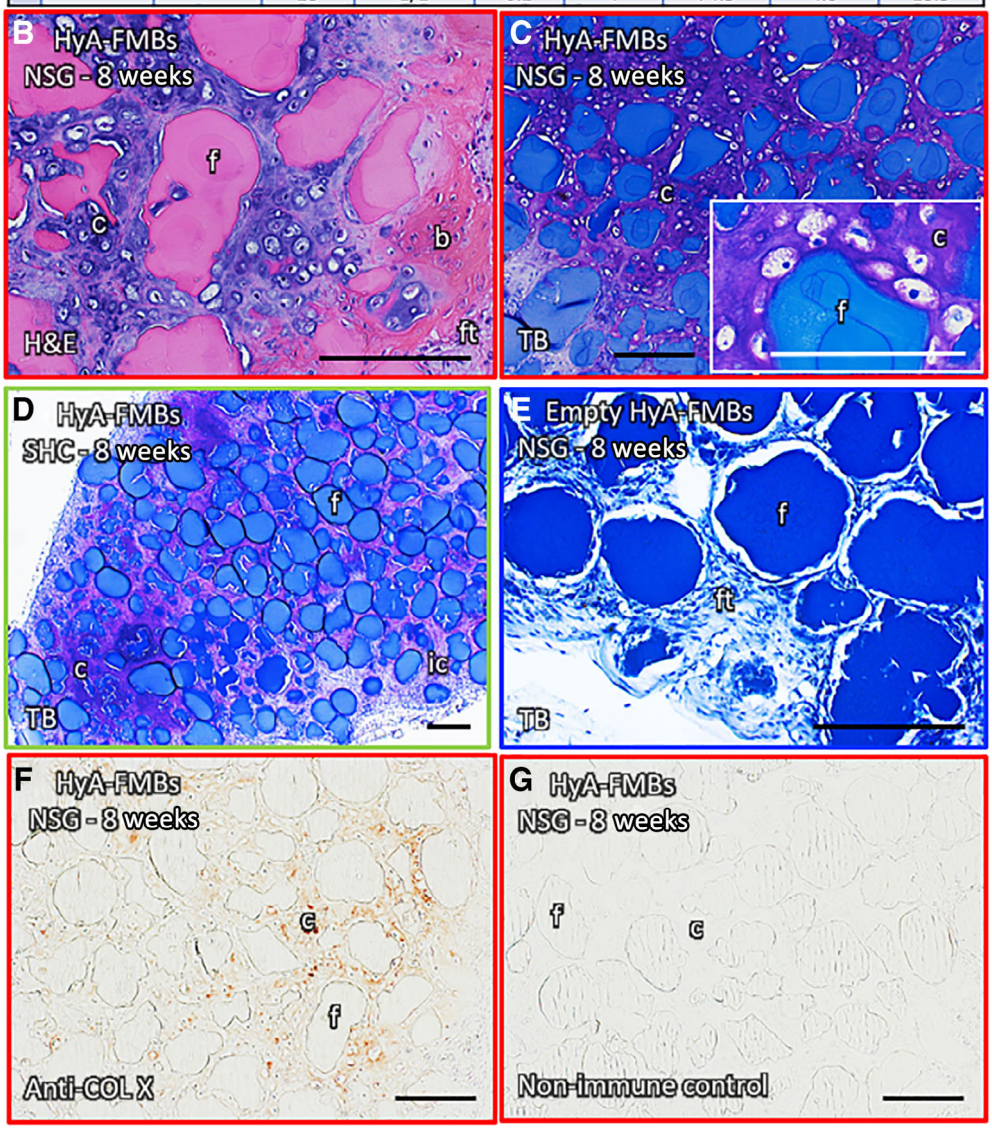


Figure 2. Cartilage formation in 8-week-old transplants of naïve human bone marrow stromal cells (hBMSCs)/HyA-FMBs and transplants of empty FMBs and HyA-FMBs. **(A):** Data collected using mice NSG, red box, and SHC, green box. All constructs containing naïve hBMSCs were recovered at all time points. At 8 weeks, substantial cartilage (scores 2, 2; mature cartilage: 18.8% and 20.0% of total transplant areas) and some bone (3.5% and 0.6%) were formed in hBMSC/HyA-FMBs in NSG mice and minimal cartilage (scores 1, 1; mature cartilage: 1.9% and 5.9%) but no bone in SHC mice. **(B):** In NSG mice, large fields of basophilic staining were noted that stained intensely with TB **(C)**. **(D):** Cartilage formed in SHC was slightly less mature than in NSG mice. Empty (without cells) FMBs were not found at 8 or 16 weeks, and empty HyA-FMBs were only found at 8 weeks (blue box) and consisted of large, unresorbed particles (79.2% of the transplant area) surrounded with a loose fibrous tissue, with no signs of cartilage formation **(E)**. **(F):** Small cartilaginous areas stained positively for type X collagen. A nonimmune immunoglobulin of the same isotype elicited no staining **(G)**. Black scale bars = 100 µm; white scale bar = 50 µm. Abbreviations: b, bone; c, cartilage; f, residual hyaluronic acid-fibrin microbeads; FMBs, fibrin microbeads; ft, fibrous tissue; HyA, hyaluronic acid; ic, immature cartilage; TB, toluidine blue; anti-COL X, immunostaining with an antibody against type X collagen; Non-immune control, immunostaining with a nonimmune immunoglobulin.

A

Vehicles	Recipient mouse strain	Harvest times (weeks)	# recovered/ total #	Bone areas %	Cartilage scores	Mature cart areas %	Immature cart areas %	Vehicle areas %
HyA-FMBs	4 NSG	8	2/2	3.5;0.6	2,2	18.8;20.0	14.9;15.3	38.2;54.5
		16	1/1 (panels B,C,E-G)	1.1	3	40.8	13.2	25.4
		28	1/1	1.1	3	65.6	15.2	10.8
Empty FMBs		8	0/1	0	0	0	0	
		16	0/1	0	0	0	0	
Empty HyA-FMBs		8	1/1	0	0	0	0	79.2
		16	0/1	0	0	0	0	
HyA-FMBs	4 SHC	8	2/2	0, 0	1,1	1.9;5.9	11.5;12.9	72.3;68.0
		16	1/1 (panel D)	0.3	2	15.9	18.1	37.5
		28	1/1	0.2	4	74.3	4.6	18.3

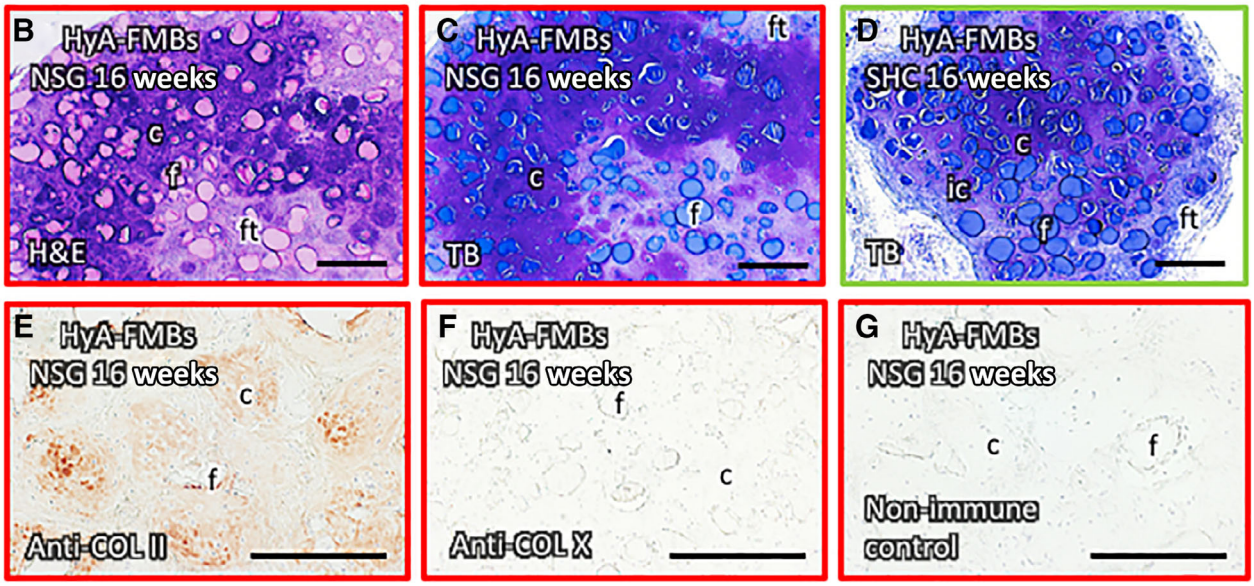


Figure 3. Cartilage formation in 16-week-old transplants of naïve human bone marrow stromal cells (hBMSCs)/HyA-FMBs. **(A):** Data collected using NSG mice, red box, and SHC mice, green box. At 16 weeks, extensive cartilage (score 3, 40.8% of mature cartilage) and some bone (1.1%) were formed using hBMSC/HyA-FMBs in NSG mouse; less cartilage (score 2, 15.9% of mature cartilage) was formed in SHC mouse. Large fields of basophilic staining were noted **(B)**, that stained intensely with TB **(C)**, with some fibrous tissue; cartilage formed in SHC mouse was slightly less mature than in NSG **(D)**. Cartilaginous areas stained positively fort type II collagen **(E)**, but not for type X collagen **(F)**. A nonimmune immunoglobulin of the same isotype as both antibodies elicited no staining **(G)**. Scale bars = 200 μm. Abbreviations: c, cartilage; f, residual fibrin microbeads; FMBs, fibrin microbeads; ft, fibrous tissue; HyA, hyaluronic acid; ic, immature cartilage; TB, toluidine blue; anti-COL II, immunostaining with an antibody against type II collagen; anti-COL X, immunostaining with an antibody against type X collagen; Non-immune control, immunostaining with a nonimmune immunoglobulin.

in accordance with the National Institute of Health (NIH) regulations, governing the use of human subjects under Office of Human Subjects Research Protection (OHSRP) assurance NIDCR-00183. In vivo implantation was performed in accordance to an institutionally approved animal protocol and the NIH Guide for the Care and Use of Laboratory Animals. A semiquantitative scoring system (range 0–4) as well as histomorphometry using Adobe Photoshop CS6 were used to evaluate cartilage and bone formation.

Results

In preliminary experiments, hBMSCs attached to FMBs formed small amounts of cartilage in beige (bg)/nude mice (Supporting Information Fig. S1). To improve the cartilage formation, FMBs were combined with several secondary scaffolds (Fig. 1A) or precultivated with hBMSCs and other vehicles in vitro. These combinations failed to increase cartilage formation (Supporting Information Figs. S2, S3A, S3B). Furthermore, based on inconsistent results using noncongenic bg/nude mice, two strains of congenic, highly immunocompromised mice were used in subsequent experiments.

FMBs were then modified with HyA covalently linked to their surfaces and compared with unmodified FMBs in Non-obese Diabetic Severe Combined Immunodeficiency Gamma (NSG) mice (Fig. 1B). At 19 weeks, hBMSC/FMB constructs were small and grayish-pinkish (Fig. 1C); minimal cartilage formation was observed in three of five constructs (Fig. 1B, 1E, 1G). hBMSC/HyA-FMB constructs were large, white, glossy (Fig. 1D),

A	Vehicles	Recipient mouse strain	Harvest times (weeks)	# recovered/ total #	Bone areas %	Cartilage scores	Mature cart areas %	Immature cart areas %	Vehicle areas %
	HyA-FMBs	4 NSG	8	2/2	3.5;0.6	2,2	18.8;20.0	14.9;15.3	38.2;54.5
			16	1/1	1.1	3	40.8	13.2	25.4
			28	1/1 (panels B,D,F,H-J,L,M)	1.1	4	65.6	15.2	10.8
	Empty FMBs		8	0/1	0	0	0	0	
			16	0/1	0	0	0	0	
	Empty HyA-FMBs		8	1/1	0	0	0	0	79.2
			16	0/1	0	0	0	0	
	HyA-FMBs	4 SHC	8	2/2	0, 0	1,1	1.9;5.9	11.5;12.9	72.3;68.0
			16	1/1	0.3	2	15.9	18.1	37.5
			28	1/1 (panels C,E,G)	0.2	4	74.3	4.6	18.3

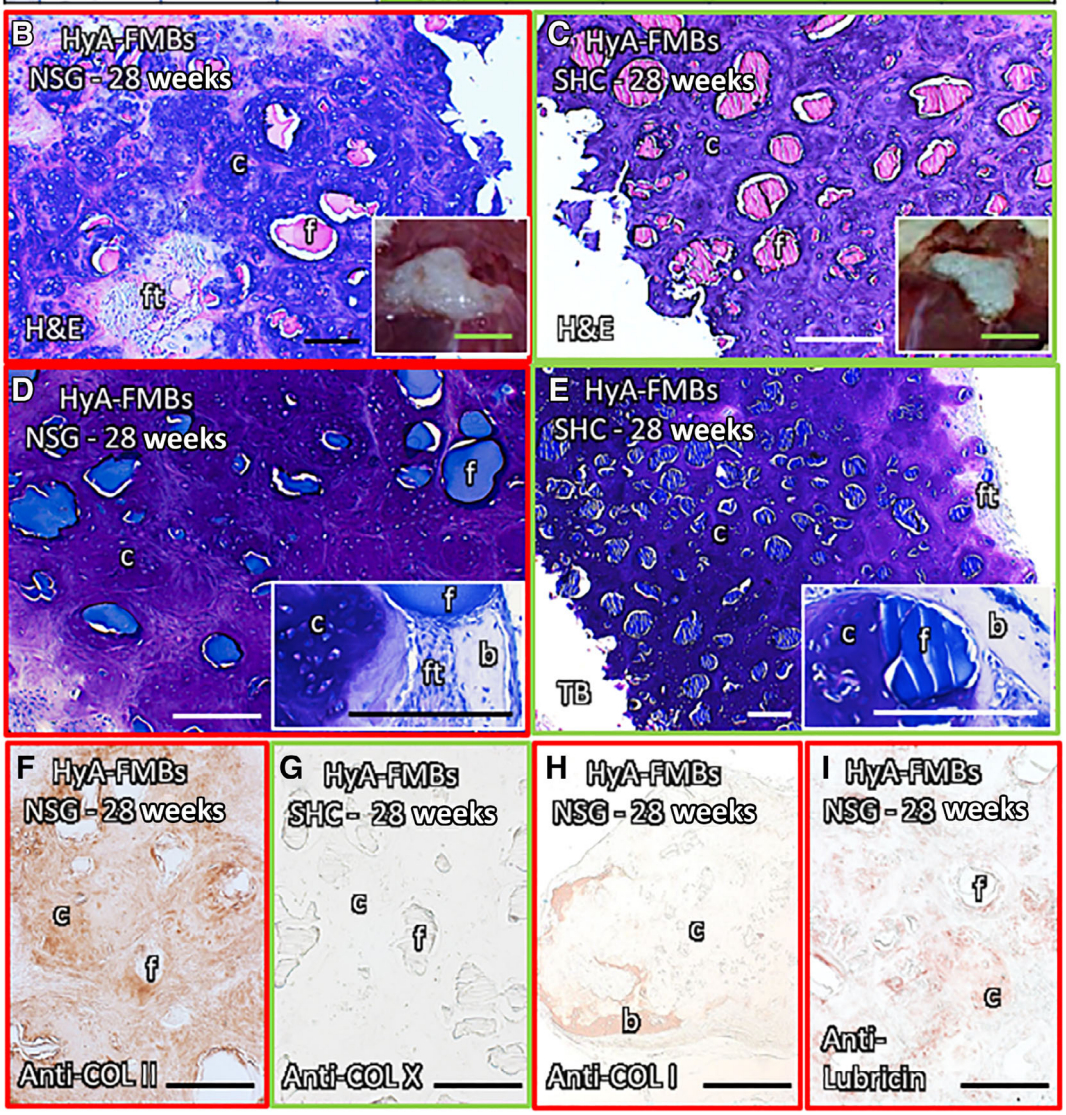


Figure 4. Cartilage formation in 28-week-old transplants of naïve human bone marrow stromal cells (hBMSCs)/HyA-FMBs. **(A):** Data collected using NSG mice, red box, and SHC mice, green box. **(B, C):** The white, glossy appearance of the transplants persisted (insets), and vast fields of cartilage were formed in both NSG and SHC constructs. **(D, E):** The cartilage matrix stained intensely purple with TB; small islands of bone were noted on the periphery of constructs in both strains of mice (insets). **(F):** Cartilage matrix stained intensely positive for type II collagen in NSG transplant. **(G):** No staining for type X collagen was noted in SHC transplant. **(H):** Bone matrix stained positive for type I collagen, whereas cartilage matrix stained negative in an NSG transplant. **(I):** Cartilage matrix stained positive for Lubricin in an NSG transplant. The non-immune control for all four of these antibodies is shown in Figure S4C. Green scale bars = 5 mm; black and white scale bars = 100 μm. Abbreviations: b, bone; c, cartilage; f, residual hyaluronic acid-fibrin microbeads; ft, fibrous tissue; FMBs, fibrin microbeads; HyA, hyaluronic acid; TB, toluidine blue; anti-COL II, immunostaining with an antibody against type II collagen; anti-COL X, immunostaining with an antibody against type X collagen; anti-COL I, immunostaining with an antibody against type I collagen. Anti-Lubricin, immunostaining with antibody against Lubricin.

and hard. Cartilage was found in all five constructs at a statistically higher amount compared with hBMSC/FMB constructs (scores: 3.0 ± 0.7 vs. 1.2 ± 1.1, p = .0317; areas of mature cartilage: 38.5% ± 6.7% vs. 9.4% ± 4.2%, p = .0159). Vast fields of cartilage contained chondrocytes in lacunae surrounded by extracellular matrix (ECM) staining strongly basophilic with H&E (Fig. 1F) and intensely metachromatic with toluidine blue (Fig. 1H). In all constructs, sizable bone trabeculae were located at the periphery (Fig. 1E, 1F), surrounding areas of hematopoiesis (Fig. 1E, 1G), but not at statistically different levels between constructs (p = .2222). By 19 weeks, nonresorbed HyA-FMBs were more abundant than FMBs (p = .0079).

Cartilage formation in hBMSC/HyA-FMB constructs was evaluated at 8, 16, or 28 weeks in NSG and Severe Combined Immunodeficiency Hairless Congenic (SHC) mice (Fig. 2A). At 8 weeks, NSG constructs revealed good cartilage (score 2; areas of mature cartilage, 18.8% and 20.0%; Fig. 2A–2C) and little bone; SHC constructs demonstrated minimal mature cartilage (score 1; areas 1.9% and 5.9%) and no bone (Fig. 2A, 2D). NSG constructs displayed limited positive staining for type X collagen (Fig. 2F, 2G). In positive areas, some cells resembled hypertrophic chondrocytes (increased size, pyknotic nuclei, and fragmented cytoplasm; not shown).

At 16 weeks, large areas of mature cartilage (score 3; 40.8%) were formed in NSG construct (Fig. 3A–3C). ECM surrounding chondrocytes demonstrated moderate to strong staining for type II collagen (Fig. 3E, 3G) but no immunoreactivity for type X collagen (Fig. 3F). In SHC constructs, less mature cartilage (score 2; 15.9%) was formed (Fig. 3A, 3D).

At 28 weeks, both NSG and SHC constructs preserved their white, glossy, pearl-like appearance (insets, Fig. 4B, 4C) and rigid consistency. Mature cartilage was the most prevalent tissue in the sections (score 4; areas 65.6% and 74.3%, respectively; Fig. 4A–4E). Narrow strips of fibrous tissue and thin, isolated bone trabeculae were observed at the periphery (insets, Fig. 4D, 4E). The ECM displayed moderate to strong staining for type II collagen (Fig. 4F). Staining for type X collagen was negative throughout the entire constructs (Fig. 4G). Although the new bone stained positive for type I collagen, cartilage was negative (Fig. 4H) and displayed moderate staining for Lubricin (Fig. 4I). The lack of vascularization in the new cartilage was emphasized by negative staining for von Willebrand factor (Supporting Information Fig. S4A, S4B). Residual HyA-FMBs persisted in both types of constructs; at all time points, more nonresorbed HyA-FMBs remained in SHC than in NSG mice (Fig. 4A–4E). Thus, stable, abundant, hyaline-like cartilage persisted, or expanded, up to the latest time point of 28 weeks (Fig. 4A).

The human origin of the cartilage was demonstrated by positive staining with an antibody specific for human mitochondria (Supporting Information Fig. S4D, S4E) in NSG 28-week constructs. FMB constructs without BMSCs (empty) were not found at 8 or 16 weeks, whereas empty HyA-FMBs were found at 8 weeks only (Fig. 2A), consisting of large, nonresorbed HyA-FMB particles (occupying 79.2% of the areas of the sections), sparsely populated with fibrous tissue without cartilage (Fig. 2A, 2E).

Discussion

Pellet cultures, the current gold standard for hBMSC chondrogenic differentiation [6], undergo endochondral bone formation in vitro [7]. Consequently, it is not surprising that preformed cartilage constructs do not generate cartilage in vivo and instead undergo either hypertrophy and mineralization [8] or remodeling into a bone/marrow organ [9]. No protocols currently exist for generation of functional hyaline cartilage by hBMSCs in vivo [10]. We hypothesized that naïve hBMSCs could form hyaline cartilage in vivo if a scaffold promoting chondrogenic differentiation is identified. Fibrin-based matrices and fibrin/HyA hydrogels supported early chondrogenesis in vitro but degraded too quickly in vivo [11]. More stable dense FMBs that retained cell binding properties of fibrin were then developed for cell implantation [12].

In our study, HyA-FMBs with naïve hBMSC generated stable, glossy-white, resilient tissues in vivo, which were histologically similar to, and demonstrated the important markers of, hyaline cartilage—type II collagen and Lubricin [13]—but lacking both type I collagen and vascularization. At the earliest time point (8 weeks), small regions of newly formed cartilage demonstrated hypertrophic chondrocyte morphology and stained positively for type X collagen. Importantly, these type X collagen-positive areas totally disappeared by 28 weeks, suggesting that the vast majority of the cartilage formed had no tendency to become hypertrophic or to undergo endochondral ossification. Immunostaining for human mitochondria verified the human origin of the chondrocytes, even at 28 weeks (~0.5 years). The mature-looking human cartilage not only persisted but also significantly expanded, occupying almost the entire areas of the constructs.

The subcutaneous location is considered to be nonpermissive for development of hyaline cartilage [14]. However, fresh or low passage human articular chondrocytes do form stable cartilage even in the hostile subcutaneous environment (e.g., [3]), the very same cells that have been most successful clinically. It is likely that in more permissive orthotopic conditions, cells that have passed rigorous subcutaneous tests will have higher chances of forming permanent, articular-type cartilage. Indeed, human induced pluripotent stem cell-derived chondrocytes formed better cartilage in joint defects than subcutaneously [15].

Of note, constructs supporting stable cartilage formation in vivo were formed following a simple 90-minute incubation of HyA-FMBs with naïve BMSCs. Neither creation of complex, tissue-like constructs in vitro nor the use of biphasic or triphasic scaffolds improved cartilage formation by hBMSCs in vivo (see Supporting Information Results section). However, the timing and amount of cartilage (and peripheral bone) formed by hBMSC using HyA-FMBs and other scaffolds differed among bg/nude, NSG, and SHC mice. Additional studies are needed to identify critical elements in both the recipient and donor that promote cartilage versus bone formation. It will be necessary to determine if hBMSCs from older, clinically relevant individuals will generate abundant cartilage with HyA-FMBs, as pediatric BMSCs do. Interestingly, in our previous studies, comparable amounts of bone were formed in vivo by hBMSCs from pediatric and elderly donors (S. A. Kuznetsov and P. G. Robey, unpublished observations). Preclinical studies on repair of intra-articular defects by hBMSCs in immunocompromised mice and rats are underway, moving toward the development of a technique for human cartilage defect restoration. Lastly, this in vivo model system can be developed to study pathological mechanisms of human genetic and acquired diseases of cartilage.

Conclusion

We have demonstrated that chondrogenic pre-differentiation of BMSCs is not required in order to form cartilage in vivo. Naïve, undifferentiated human BMSCs, combined with HyA-FMBs and implanted subcutaneously into immunocompromised mice, generated abundant, hypertrophy-resistant, hyaline-like cartilage of human origin, stable for at least 28 weeks in vivo. This approach is promising for restoration of joint cartilage defects in humans.

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Author Contributions

S.A.K., R.G.: conception/design, collection and/or assembly of data, data analysis and interpretation, final approval of manuscript; A.H.-L., N.C., L.F.d.C.: collection and/or assembly of data, final approval of manuscript; P.G.R.: conception/design, collection and/or assembly of data, data analysis and interpretation, statistical analysis, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicated no potential conflicts of interest.

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