Blastema cell-like formation by Msx1 and Msx2-transduced mBMSCs

Msh homeobox 1 (Msx1)- and Msx2-overexpressing bone marrow–derived mesenchymal stem cells resemble blastema cells and enhance regeneration in mice

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ABSTRACT
Amputation of the proximal region in mammals is not followed by regeneration because blastema cells (BCs) and expression of regenerative genes such as Msh homeobox (Msx) genes are absent in this animal group. The lack of BCs and positional information in other cells are therefore the main obstacle to therapeutic approaches for limb regeneration. Hence, this study aimed to create blastema-like cells (BlCs) by overexpressing Msx1 and Msx2 genes in mouse bone marrow–derived mesenchymal stem cells (mBMSCs) to regenerate a proximally amputated digit tip. We transduced mBMSCs with Msx1 and Msx2 genes and compared osteogenic activity and expression levels of several Msx-regulated genes (Bmp4, Fgf8, and keratin 14 (K14)) in BlCs groups including MSX1, MSX2, and MSX1/2 (in a 1:1 ratio) with those in mBMSCs and BCs in vitro and in vivo following injection into the amputation site. We found that Msx gene overexpression increased expression of specific blastemal markers and enhanced proliferation rate and osteogenesis of BCs compared with mBMSCs and BCs via activation of Fgf8 and Bmp4. Histological analyses indicated full regrowth of digit tips in the Msx-overexpressing groups, particularly in MSX1/2, through endochondral ossification 6 weeks post-injection. In contrast, mBMSCs and BCs formed abnormal bone and nail. Full digit tip was regenerated only in the MSX1/2 group and was related to boosted Bmp4, Fgf8, and K14 gene expression and to limb-patterning properties resulting from Msx1 and Msx2 overexpression. We propose that Msx-transduced cells that can regenerate epithelial and mesenchymal tissues may potentially be utilized in limb regeneration.

Digit tip regeneration is a well-documented example of organ/appendage regeneration in lower vertebrates such as salamanders as they exhibit robust regeneration potential in response to amputation anywhere along the proximal/distal limb axis (1). However, the level of amputation through the terminal phalanx causes various regeneration responses in mammals such as humans and mice. Amputation in the middle of the terminal phalanx involves complete skeletal restoration whereas only scar formation occurs
following amputation injury at the proximal level (2). Therefore, the main goal of clinical and experimental investigations is to restore lost parts of limbs damaged due to disease or injury. Despite numerous attempts, digit tip regrowth after amputation through terminal phalanges remains challenging. Naturally, blastema cells (BCs) are considered a key mediator of digit tip regeneration so that their removal following proximal amputation leads to scar formation in adult mammals (1).

BCs are believed to be a heterogeneous population of lineage-restricted progenitor cells derived from fibroblasts of connective tissue (3). There are several main surface biomarkers assigned to BCs - stem cell antigen-1 (Sca-1), endothelial marker (CD31), and vimentin (4). Msh homeobox (Msx) genes are members of the Hox gene family and include Msx1 (Hox7) and Msx2 (Hox8). They are highly conserved among vertebrates, and considered key genes in BCs (5,6). It has conclusively been shown that the absence of BCs as well as Msx1 and Msx2 genes resulted in regeneration failure in a proximal amputation of adult mice (7). Additionally, Msx genes are of crucial importance in ectomesodermal interactions that mediate cellular proliferation and differentiation during limb formation, AEC formation, and limb patterning (8). Bensoussan-Trigano et al. have shown that the Prx1-Cre Msx1null/null Msx2 null/Flox mutants display abnormal digit formation and pre-axial polydactyly in fetal mouse digit tip regeneration (9). Overexpressed Msx1 (Hox7) in hindlimb regeneration in a transgenic Xenopus model (M1) resulted in higher proliferation rate in both BCs and AEC, thickened wound epithelium, and more regenerated toes in M1 compared with WT animals in stage 54 (10).

More importantly, BCs have enabled the process of bone formation as a main process of limb regeneration by triggering cascade of cell signaling pathway such as bone morphogenetic proteins (BMPs) and fibroblast growth factors (FGFs) (11-13). Positional information is one of the key elements in successful regeneration. It has been proposed that the expression of region-specific Hox genes in early and late blastema tissues is more likely related to positional identity (14). Rao et al. have shown that fibroblastemas of xenopus limbs expressed PD axial patterning genes including hoxa9, hoxa11, and hoxa13 (15). Indeed, successful limb regeneration has been correlated to the formation of BCs and its related genes. Although transplantation of BCs at the amputation site could accelerate wound healing and digit regeneration, availability of BCs has always been a challenging issue in terms of digit regeneration. Hence, use of an alternative available cell source with high similarity to BCs would be a valuable strategy in limb regeneration.

Various cell sources have been isolated and characterized in terms of their potential in clinical settings. Fetal limb cells have a tremendous degree of similarity to BCs for limb regeneration, though their administration is hampered due to ethical concerns (16). Stem cells, in particular mesenchymal stem cells (MSCs), have tremendous potential in therapeutic approaches due to their unique characteristics of self-renewal and differentiation potential. MSCs are multipotent cells that exist in most adult tissues (17-19) and have the ability to give rise to multiple tissue-forming cell lineages (20-22). It has been postulated that MSCs affect cell migration, proliferation, and survival of the surrounding cells as well as provide anti-scarring properties through paracrine signaling (23,24). They may also regulate immune and inflammatory responses, making them an appropriate therapeutic candidate for treating inflammatory diseases (25,26). Thus far, numerous researches have been devoted to the application of MSCs in various diseased tissues due to their potent regenerative capacity and applied in over 350 clinical trials worldwide (27). However, there has been little investigation of MSCs in limb regeneration. Masaki et al. transplanted Bone marrow derived-MSCs (BMSCs) and limb bud transplantation into amputated limbs in neonatal mice and observed the generation of the segmented pattern of bone and cartilage (28). In another study, injection of the hematopoietic stem cells (HSCs) into an amputated digit did not lead to the formation of main structures of the digit but it contributed to the formation of blood cells and bone marrow tissue (29). However, lack of positional information in current efforts that use stem cells is more likely the cause of regeneration failure.

In our previous study, we isolated BCs from neonatal mice and compared their
characteristics with mouse BMSCs (mBMSCs) in vitro. Our study has shown that mBMSCs have similar characteristics to BCs in terms of growth and osteogenic activity, and can be considered as substitutes for BCs (30). Thus, the present study aims to create blastema-like cells (BlCs) through overexpression of the Hox gene family, including Msx1 and Msx2, that are involved in positional information in mBMSCs and regenerate a digit tip by transplantation of BlCs in vivo. Therefore, we transduced mBMSCs with Msx1 and Msx2 genes, after which their proliferation and differentiation potentials were examined by MTT and real-time PCR in vitro. In order to determine the regenerative potential of BlCs, we injected them into the amputated proximal digits of adult mice. The results demonstrated that BlCs could completely improve proximal digit tip amputation compared to mBMSCs and BCs.

RESULTS
Msx1 and Msx2- transduced mouse bone marrow-derived mesenchymal stem cells (mBMSCs)- In order to verify the accuracy of gene transduction, third generation self-inactivating (SIN) vectors with various fluorescent reporters' genes were constructed as diagrammed in Figure 1A. Msx1 and Msx2 genes were co-expressed by GFP and td tomato genes, respectively. Although the majority of cells expressed reporter genes, we needed a pure cell population which absolutely expressed Msx1 (GFP+) and Msx2 (tdTomato +). Therefore, the cells were sorted for GFP and tdTomato markers after 72 h of transduction as seen in Figure 1B (a, b). ICC analysis showed significantly lower protein expression levels of endogenous Msx1 and Msx2 in mBMSCs (less than 3%-5%) compared to BCs (30%-40%). Msx1 and Msx2 transduction led to drastic increase expression level of these exogenous genes in BlCs (100%), which was greater than BCs (Fig. 1C a, c).

We used RT-PCR to determine the quantitative expression levels of Msx1 and Msx2. Figure 1C shows that the expression levels of Msx1 and Msx2 in BCs was approximately 400-500-fold greater than mBMSCs (less than 3%-5%) compared to BCs (30%-40%). Msx1 and Msx2 transduction led to drastic increase expression level of these exogenous genes in BCs (100%), which was greater than BCs (Fig. 1C a, c).

Osteogenic activity- We assessed for osteogenic activity of BlCs, BCs and mBMSCs at various time points (culture days 7, 14, 21) by alizarin red S staining, ALP activity, and qRT-PCR. Alizarin red staining showed that nodules-like aggregates began to form during the first week and increased in abundance toward the end of the third week. There were significantly more mineralized nodules in the BlCs (MSX1, MSX2 and MSX1/2) plates compared to BCs and mBMSCs (Fig. 2A, E). ALP activity, as an early marker for osteogenic differentiation, was measured after 7, 14, and 21 days of incubation.
After 7 days, both MSX1 and MSX2-transduced cells as well as MSX1/2 group showed an almost equal increase in ALP activity. ALP activity significantly reduced in BlCs compared with BCs and mBMSCs. ALP activity significantly decreased in all the studied groups on days 14 and 21 (Fig. 2B). Figure 2C shows the calcium content of BlCs, BCs and mBMSCs cultures after 7, 14 and 21 days. The amount of calcium increased over time in all groups. After 14 days, we observed higher a calcium content in BlCs compared with those of BCs and mBMSCs. There was significantly increased calcium content observed between BCs (MSX1, MSX2 and MSX1/2), mBMSCs and BCs groups on day 21. Real-time PCR analysis revealed that the expression level of ColI as well as Runx2 and the OCN genes progressively up-regulated within 3 weeks (Fig. 2D a-c).

Analysis of Msx-related genes- We assessed the expression levels of several major Msx-related genes (Bmp4, Fgf8, ki67, and K14) by ICC and RT-PCR before and after gene transduction in mBMSCs as well as BCs. RT-PCR analysis showed that the expression level of Bmp4 gene increased by 350-fold in MSX1-transduced cells and by 300-fold in MSX2-transduced cells compared to mBMSCs (****p<0.0001; Fig. 3). The Fgf8 gene showed significant expression in both the MSX1 (170-fold) and MSX2 (150-fold) groups compared to mBMSCs (Fig. 3B a, b). ICC analysis indicated that mBMSCs did not express Bmp4 and Fgf8 endogenously, while these genes upregulated by 80%-90% in BlCs (Fig. 3B b, d). The expression level of the ectoderm-specific gene Keratin 14 (K14) and Ki-67 (also known as MKI67) was assessed using ICC. The former is important for nail formation in limb development and the latter is a cellular marker for proliferation. As shown in Figure 3Ca, we detected no endogenous expression of K14 in mBMSCs, whereas the protein expression level of K14 significantly up-regulated in MSX1 and MSX2-transduced cells (80%-90%; ****p<0.0001). In addition, BCs expressed K14 approximately 20-fold more than mBMSCs (Fig. 3D a). Likewise, ICC results indicated a low expression for Ki-67 in mBMSCs (Fig. 3Cb) compared to BCs. The expression level of Ki-67 protein increased by 90% in the MSX1 group and 80% in the MSX2 groups compared to the control group (****p<0.0001; Fig. 3D b).

Macroscopic evaluation of proximal regeneration- In our digit tip regeneration model, the adult mouse terminal phalanges were proximally amputated to ascertain that the model could not regenerate prior to injection of the cells (Supplementary Fig. 2A, B). The wound epidermis formed a scar wound healing response immediately after digit tip amputation at the proximal level of P3 in both the Nrm, Reg and Sham groups (Supplementary Fig. 2C-E). We observed closure in the epidermis completed in 4–5 day post amputation (DPA) without any new bone or nail formation. Therefore, we injected the cells 4 DPA when scar wound falls.

Whole mount digit tip analysis at 6 weeks after the injection (WPI) showed that the epidermal closure occurred in all experimental and control groups. We observed no trace of regeneration in the Nrm, Reg, and Sham groups, which was expected (Fig. 4B, C). In the BCs and mBMSCs groups, regeneration of the proximal regions occurred in less than 10% of the digit tips. Abnormal bone and nail organ formation with no elongation occurred in both groups (Fig. 4D, E; Supplementary Table 2). Surprisingly, more than 95% of the digit tips showed full digit tip regeneration, which included both formation and elongation of the bone and nail in the MSX1/2 group (Fig. 4 H; Supplementary Table 2).

In order to quantify the regenerated bone, we performed whole mount alizarin red staining to visualize the skeletal patterns to measure the proximal-distal length of each terminal phalanx (30 digits for each group). As illustrated in Figure 5 A, the Nrm, Reg and Sham groups formed no nails or new bone (Fig. 5A b, c). We observed newly-formed bone and nail in the mBMSCs and BCs groups (Fig. 5A d, e), even though they had abnormal morphologies with thin nails and short bones. On the other hand, morphological analysis revealed that bone and nail generated up to 50% of the intact group in both the MSX1 and MSX2 groups (Fig. 5A f g, B). There were no
remarkable differences in new bone length between the MSX1 and MSX2 groups. The thickness and length of the new nail remarkably increased in MSX1/2 as the length of the newly-formed bone was comparable to the intact group (Fig 5Ah, B).

**Histological analysis of digit tip regeneration** - In order to explore the presence of BICs in terminal phalangeal element regeneration, the labeled cells were first traced between the stump bone and wound epidermis position. Ferridex and GFP- MSX1 labeled cells comprised 15%-20% of total cells in digit tip regenerated regions (Supplementary Fig. 3). Similarly, BrdU labeling showed the presence of injected cells at the site of injury 6 WPI in all experimental groups (Fig. 6).

Histological analysis showed that epidermal closure occurred in all groups and the thickness of epidermis and dermis layer had no significant differences among the groups (Fig. 7). H&E and Masson’s trichrome staining results indicated that tissue atrophy occurred in the connective tissue (cartilage, nail, and bone stump) in the Nrm. Reg and Sham groups. However, we observed no apoptotic cells or tissue in these groups. (Fig. 7D, H). In the cell-injected groups, the distal end of the bone stump was elongated and formed a bony cap similar to bone callus (Fig. 6J, N). Specifically, the bone stump showed slight elongation in BCs and mBMSCs, whereas the bone callus was significantly larger in the BICs groups (Fig. 7Q-U, W, X). The formed bone callus was spongy in both BCs and mBMSCs. This structure was going to be replaced by a structure similar to compact bone in BICs. Interestingly, bone callus typically formed in the MSX1/2 groups which was greatly similar to the intact groups. As illustrated in Figure 7, we did not observe any distinct bone cavity in both BCs and mBMSCs; however, this cavity was generated in the MSX1 and MSX2 groups. In particular, the bone cavity structure in these groups was in similar to the intact group. We performed alcian blue and alizarin red S staining (Al& Al) to determine if the bone callus-like tissue was bone or cartilage. The results showed that the new bone callus stained a blue color, which indicated cartilage issue in all groups 6 WPI (Fig. 7). Long-term follow-up of the MSX1/2 groups after 10 WPI indicated that the cartilage tissue was replaced by mineralized bone tissue (Fig. 8A). IHC analysis for main osteogenic related genes including Col I and OCN also confirmed that newly formed osteoid tissue was matured bone (Fig. 8B).

Immunohistochemistry analysis showed that the BCs and mBMSCs groups expressed relatively low amounts of endogenous Msx1, Msx2, Bmp4 and Fgf8 proteins (Fig. 9A a-h, B). In contrast, the expression levels of the same markers dramatically increased in the BICs groups, particularly MSX1/2 (Fig. 9A i-t, B). These results agreed with the expression profiles of Msx1, Msx2, Fgf8, and Bmp4 under in vitro conditions.

**DISCUSSION**

Digit tip regeneration in adult mice is restricted to the distal half of the terminal phalangeal element (P3), while regeneration fails following amputation through the proximal third of P3 (7) due to lack of BCs and Msx genes. Msx genes regulate the cellular behavior like proliferation and differentiation as well as digit patterning during limb development and regeneration (31). The unavailability of BCs is a major challenge regarding BCs therapy. On the other hand, lack of positional information in other cell sources as an exclusive ability of BCs is hampered the cell-based therapeutic approaches. Therefore, in this study we have developed a new cell source by overexpression of the Msx1 and Msx2 genes in mBMSCs which created a BICs, after which we explored the regenerative potential these BICs in proximally amputated digit tips (Fig. 10).

Our ICC and qRT-PCR results showed that Msx1 and Msx2 genes accurately transduced and upregulated in mBMSCs. Additionally, transduction of exogenous Msx genes has resulted in upregulation of endogenous Msx2 and Msx1 in MSX1 and MSX2 cells, respectively. The Msx2 expression domain has been shown to expand into the Msx1 domain and they overlap (7). Therefore, upregulation of endogenous genes would be expected. Also, overexpression of one of the Msx may affect expression of the other related genes. A comparison of BICs and original BCs has confirmed the higher expression levels of the Bmp4, Fgf8 and Kit67 genes in BICs according to ICC results. To address that BICs
have the similar characteristics of BCs, we assessed the expression level of specific blastema markers, CD31, Vim and Sca1, in all groups. Surprisingly, the flow cytometry results confirmed that specific blastema cell surface markers significantly expressed in BlCs compared to mBMSCs. These significant differences among mBMSCs, BCs, and BlCs might be attributed to overexpression of Msx, which has led to a higher proliferation rate and osteogenic activity in the transduced cells. Bmp4 is considered to be a crucial osteogenic marker (32). Its upregulation in BlCs enhanced osteogenic differentiation capacity compared to mBMSCs and BCs. Likewise, the higher proliferation rate of BlCs occurred because of Fgf8 and Ki67 gene upregulation, as proliferation markers. The results of cell cycle analysis confirmed that S phase in BlCs was longer than mBMSCs and BCs. Of note, the decreased activity of BCs relative to BlCs was most likely related to cellular expansion following several passages in vitro.

We explored the regenerative capacity of BlCs following transplantation into non-regenerative digit tips of an adult mice model. In this model, the cartilage tissue of the joint, stump bone, and nail of P3 were preserved, whereas we removed the bone and nail within the bone marrow cavity. Our whole mount results combined with histological analysis showed a lack of new bone and nail formation as well as tissue atrophy in the non-cell injected groups (Nrm. Reg and Sham) after 6 WPI, which confirmed that the proximally amputated model was non-regenerative. We observed a significant regenerative response in BlCs, particularly MSX1/2 as indicated by complete nail and bone formation, which was like the intact group. We observed abnormal bone and thin nail formation in both the mBMSCs and BCs groups. These findings showed that the new cell fate of mBMSCs by overexpression of the MSX genes efficiently improved regrowth of the amputated digit.

In order to precisely address how the process of bone formation progresses, we performed Al & Al staining. According to the data, the cell-injected groups induced osteogenesis via an endochondral ossification manner as with a previous study that demonstrated endochondral bone formation in mouse digit tip regeneration (33). The transient cartilage was suggested to be replaced by newly mineralized bone. However, visible changes occurred in the generated bone tissue in terms of volume and histology among the BlCs, mBMSCs, and BCs groups. As expected, injection of BlCs led to regrowth of bone and nail organs, although full regeneration that contained normal bone cavity and compact bone-like was only observed in the MSX1/2 groups after 6 WPI. Long-term follow-up confirmed completion of ossification after 10 WPI in the MSX1/2 group. The presence of very large blood vessels containing blood cells was clearly detected within the regenerated region. Our results are consistent with previous studies that have shown that cells expressing the endothelial markers CD31 and stem cell antigen 1 (Sca1) in blastema region could differentiate into endothelial progenitor cells and formed blood vessels (4). Regenerated bone marrow cavity along with blood vessels formation in BlCs groups, particularly MSX1/2, compared to BCs and mBMSCs groups most probably related to the increased expression level of CD31 and Sca1 markers after MSXs transduction. In order to allocate the improved regeneration to our MSX-transduced cells, Ferridex and BrdU-labeled cells were tracked in the regenerated regions 6 WPI. The presence of labeled cells at injury site along with expression of Msx genes as evidenced by GFP + cells demonstrated the contribution of BlCs to digit regeneration.

The expression of Msx and its related genes was also shown by IHC results. Interestingly, excellent regeneration occurred in the MSX1, MSX2, and particularly MSX1/2 groups that expressed the highest levels of Msx1 and Msx2. Msx1 and Msx2 are greatly expressed in mesenchymal and epithelial cells, respectively (9). Thus, their combination in MSX1/2 caused regeneration of both mesenchymal tissue such as bone and epithelial one like nail.

It should be taken into consideration that we could not distinguish the expression level of endogenous and exogenous genes due to the lack of a distinct marker in vivo. However, the discrepancy of expressed genes between various groups is related to the exogenous gene
transduction which contributes to acceleration of
digit regeneration.

The Msx genes directly regulate Bmp4 expression as an essential signaling molecule for
bone formation and digit development (13,34). Runx2 is also controlled indirectly by Msx genes
through upregulation of Bmp4. Therefore, enhanced expression level of the Bmp4 gene in
the BCs group compared to mBMSCs and BCs resulted in remarkable bone formation.

According to the literature, although essential for
bone formation, Bmp4 expression is not sufficient
for full digit tip regeneration. Yu et al. have
reported improved regeneration, but not complete
restoration of an injury within the terminal phalangeal element after BMP4 treatment (34).

Exogenous BMP4 partially rescued a digit defect
in an Msx1 mutant in cultured explanted autopods
of E14.5 neonatal mice. This effect was dose-
dependent (7). Therefore, full digit tip
regeneration in our study might be related to an
enhanced expression level of FGF-8 which
recovered the ecto-mesodermal interactions as
vital events in limb regeneration and
development. Msx genes control the Fgf8 expression indirectly through either BMP or
sonic hedgehog (SHH) signaling pathway (35).

The crucial role of Fgf8 in cell proliferation as
assessed by BrdU labeling showed significant
outgrowth of the regenerating digit in the
MSX1/2 group compared to the mBMSCs and
BCs groups. Recently, Satoh et al. observed the
cooperative inputs of Fgf- and Bmp-signaling for
full-competence limb regeneration in axolotl
(36). Therefore, normal digit regeneration in
MSX1/2 might be attributed to direct contribution
of BCs as well as paracrine activity of BCs
through co-overexpression of Fgf8 and Bmp4
genes caused by Msx1 and Msx2 gene
transduction.

More importantly, Msx genes exert their
role through the positional information properties
on limb patterning [38]. Therefore, lack of HOX
genes (i.e. Msx1 & Msx2) in mBMSCs led to bone
formation yet without patterning.

Our results demonstrated that over-
expression of Msx1 and Msx2 in mBMSCs fully
regenerated proximally amputated digit tips in
adult mice. This study showed that MSX
transduced cells exhibited upregulation of Bmp4
and Fgf8, as well as K14 compared to BCs and
mBMSCs. This finding has suggested that
ectopic MSCs, which contain exogenous
essential genes activate endogenous signaling
pathways and effectively accelerate the
regeneration process (Fig. 10B). Further
experiments are necessary to clearly elucidate the
molecular mechanism implicated in upregulation
of the K14 and Fgf8 genes related to Msx.

**EXPERIMENTAL PROCEDURES**

**Isolation and expansion of mouse bone
marrow-derived mesenchymal stem cells**
(mBMSCs) and blastema cells (BCs)- MSCs were
isolated from bone marrow of inbred C57BL/6
strain as described previously (39). Isolated cells
were cultured in Dulbecco's Modified Eagle
Medium (DMEM; Gibco) supplemented with
15% fetal bovine serum (FBS; Gibco), 2 mM L-
glutamine (Sigma-Aldrich) and 1%
penicillin/streptomycin (Gibco) with media
changes every 3 days. Non-adherent cells were
removed by changing the media, allowing MSCs
to proliferate. mBMSCs at passage 3 were used
for further experiments.

As we previously reported, BCs were
isolated from digit tips of 3-day-old newborn
C57BL/6 mice, followed by digestion with 0.2%
collagenase type I (Gibco) and 0.5% dispase
(Sigma-Aldrich). Collected cells were cultured in
DMEM medium supplemented with 15% FBS, 2
mM L-glutamine and 1%
penicillin/streptomycin (Gibco) with media
changes every 3 days. Non-adherent cells were
removed by changing the media, allowing MSCs
to proliferate. mBMSCs at passage 3 were used
for further experiments.

**Plasmid constructions, virus production,**
and Msx1 and Msx2 gene transduction-
Lentiviral plasmids and virus production
performed according to a previously described
protocol (40,41). Briefly, the cDNAs of Msx1 and
Msx2 were amplified by PCR and cloned into third generation lentiviral expression vectors. Msx1 and Msx2 were cloned into IRES-GFP and IRES-tdTomato viral plasmids, respectively, to form Msx1-IRES-GFP and Msx2-IRES-tdTomato vectors. The plasmids had similar constitutive promoters but they differed in co-overexpression marker genes.

In order to produce lentiviral particles, the HEK293 cells were cultured in fibroblast media that included G418 (200 µg/ml; Sigma) and NEAAs (10 µl/ml; Life Technologies). Next, 5×10^6 cells were transfected by using Lipofectamine 3000 (Life Technologies, Inc.) along with the vectors and lentiviral packaging vectors: pMDL, pRev and pVSVG. Transfection medium was renewed after several hours. The filtered viral supernatant was used for mBMSCs transduction along with polybrene (6 µg/ml; Sigma). Thereafter, transduced cells were expanded for several passages in culture media. The GFP+ and tdTomato+ cells were sorted using a FACS sorting (BD FACS Aria™ II Cell Sorter, USA). Sorted cells were expanded for several passages and used for subsequent experiments.

**Flow cytometry**- To analyze the expression of cell surface markers, cell surface antigens were detected using flow cytometric technique. Passaged-3 BCs, and BlCs (mBMSCs as a control group) were first trypsinized, washed and suspended in PBS. Next, they were incubated with phycoerythrin (PE)-conjugated anti-mouse sca1, CD31 (abcam) and fluorescein isothiocyanate (FITC)-conjugated anti mouse Vim (sigma). As isotype controls, murine FITC-conjugated IgG1 and PE-conjugated IgG2b (eBioscience) were substituted for primary antibodies. Data from all samples were collected using FACScan flow cytometer (BD FACS Caliber, BD Biosciences, San Jose CA, USA) and analyzed by flowing software version 2.5.

**Cell proliferation assay**- The expression levels of osteogenic-related markers (Col1, Runx2, and OCN) were examined by qRT-PCR. Briefly, total RNA from cells was extracted using TRI Reagent® (Sigma-Aldrich, T9424). cDNA was produced by the RevertAid First Strand cDNA Synthesis Kit (Fermentas, K1632) according to the manufacturer’s instructions. The expression levels of the target genes were normalized to GAPDH as a reference gene. Analysis was performed by the comparative ∆∆CT method. Supplementary Data Table 1 lists the primers used in this study.

**Real-time PCR analysis**- The osteogenic differentiation of BICs, BCs and mBMSCs was examined as a function of alkaline phosphatase (ALP) activity at days 7, 14, and 21. ALP activity was determined with respect to the release of p-nitrophenol from p-nitrophenyl phosphate substrate using an Alkaline Phosphatase Assay Kit (Colorimetric; Abcam, USA, ab83369) according to the manufacturer's protocol. Briefly, cells were cultured on 24-well plates at a density of 5×10^4 cells per well. The medium was replaced after 48 h with osteogenic media that contained 0.2 mM ascorbic acid, 10 mM β–glycerophosphate, and 1 nM dexamethasone. For each of the time points, we used lysis buffer to scrape the cell layer from the
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surface, followed by sonication and centrifugation to collect the cell lysis solution. We added p-nitrophenyl phosphate to the cell lysis solution to begin the reaction, which was halted after 60 min by the addition of a stop solution. Optical density was analyzed at 405 nm with a Thermo Scientific Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, USA). ALP activity values were normalized with respect to the total protein content obtained from the same cell lysate and expressed as units per microgram of total protein. Total protein content was measured using the BCA protein assay kit (EMD Millipore Company, Darmstadt, Germany). The absorbance of the reaction product was measured at 562 nm. The protein concentration was calculated from a standard curve.

**Calcium content**- Calcium content was measured to evaluate the matrix mineralization ability of BlCs at days 7, 14 and 21 post-induction. The calcium concentration was determined using a Calcium Colorimetric Assay Kit (Biovision, Inc., USA) based on the formation of stable purple colored complexes with free calcium. The color intensity was detected at 575 nm by a Thermo Scientific Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, USA). Color intensity is directly proportional to the calcium concentration of the samples.

**Immunocytochemistry (ICC) and protein expression analysis**- We used the immunofluorescent technique to assess for the presence of Msx1, Msx2, as main markers of blastema, as well as Bmp4, Fgf8, Ki67 and K14 as bone differentiation, proliferation, and nail formation markers. Cells were fixed in 4% paraformaldehyde for 20 min and permeabilized with 1% Triton X-100. The fixed cells were blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature, then incubated overnight at 4°C with primary antibodies that included rat polyclonal anti-mouse BMP4, FGF8, MSX1, MSX2, (Invitrogen), Ki67 (Santa Cruz), and K14 (Biorbyt). Cells were finally incubated with goat anti-rat Alexa Fluor® 488 secondary antibody (1:500, Invitrogen) and goat anti-rat Alexa Fluor® 568 secondary antibody (1:500, Invitrogen) for 60 min at room temperature. Nuclei were counterstained with DAPI (Invitrogen), and analyzed using a fluorescence microscope (Olympus BX51, Japan).

**Digit tip amputation**- C57B/L6 female mice were used in all experiments. Adult mice were anesthetized by ketamine (80 mg/kg body weight) and xylazine (8 mg/kg body weight). Amputations were performed out on digits 2, 3, and 4 of the forelimb, at the proximal non-regenerating level. Procedures for care and use of mice were performed in accordance with the Standard Operating Procedures approved by the Institution’s Animal Care & Ethics Committee of Royan Institute.

**Cell labeling by Feridex and BrdU**- We used two cell labeling methods to track cells following transplantation. In the first method, cells were labeled with super-paramagnetic iron oxide (SPIO) nanoparticles known as Feridex IV (Sigma) prior to transplantation. A mixture of Feridex IV (100 µg/mL) and protamine sulfate (45 µg/mL) prepared in serum-free culture medium was directly applied to the attached cells after which they were incubated for 2 h at 37°C. Subsequently, 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin were added to the cells and incubated for 48 h. Cell labeling was performed by incorporation of 5-bromo-2-deoxyuridine (BrdU) using a BrdU Detection Kit II (Roche) according to the manufacturer’s instructions. The labelled cells in the histological sections were tracked using an IX70 inverted microscope (Olympus, Japan).

**Cell preparation and transplantation**- We injected a solution of fibrin glue and media that contained labeled-cells (at a 1:1 ratio) with a final cell concentration of 1×10^5 cells/μl, (approximately 5-7×10^5 cells per digit) into the amputation wound 4 days post-amputation (4 DPA). Injection was performed using a Hamilton needle under a Stereo Loop (Olympus, Japan) between the wound epidermis and the stump of the amputated bone and allowed to hydrate in situ before needle removal. The experimental groups consisted of cell-injected groups that included mBMSCs, BCs, MSX1, MSX2, and the combination of MSX1 and MSX2 (MSX1/2, 1:1 ratio). Non-cell-injected groups included an intact digit group (control), normal regeneration (Nrm. Reg), and Sham (fibrin glue+media, 1:1 ratio) groups.
Digit harvest and bone length analysis- Mice were euthanized by CO2 euthanasia chambers and their digit tips were harvested from both the left and right forelimbs at 6 and 10 weeks post-injection (WPI). The right digit tip was used for the intact digit group (control). In order to observe the entire mount digit regeneration, we fixed a random number of digits in 4% PFA overnight at 4°C. After washing in 1% KOH in H2O, the digits were incubated serially in 20% glycerol/1% KOH for 3–6 h, 50% glycerol/1% KOH for 4–16 h, and 100% glycerol for 2-3 h at room temperature.

In order to quantify newly formed bone, we measured the proximal-distal length of each terminal phalanx (about 30 random digits for each group). Prior to staining, the skin was thoroughly removed and the limbs were fixed in 100% ethanol for 4–6 h. Fixed limbs were then stained with 60% ethanol/5% acetic acid/0.015% alcian blue/0.005% alizarin red at room temperature for 24 h, followed by washing in 1% KOH, then transferred into 50% glycerol, and stored in 100% glycerol. Histological analysis- Digits from all experimental groups as well as cell tracking samples were fixed in 4% paraformaldehyde for 24 h at 4°C, followed by decalcification in Morse’s solution (20% formic acid and 10% sodium citrate, pH 7.2) with constant agitation at room temperature for 4 days. Tissues were then embedded in paraffin, and cut into 6 µm thickness sections for histological staining.

Prussian blue staining- Ferridex labeled cells were tracked using Prussian blue staining. Tissue sections from the cell tracking groups were re-hydrated and incubated in 2% potassium ferrocyanide (Ajax Chemical) in 6% hydrochloric acid for 30 min. After washing, nuclear staining was carried out with hematoxylin.

Hematoxylin and eosin (H&E), Masson’s trichrome, Alizarin red S and Alcian blue (Al & Al) staining- Samples were first deparaffinized and dehydrated according to standard protocols. Subsequently, sections were stained with Masson’s trichrome according to the manufacturer’s instructions (Gomori, procedure HT10, Sigma-Aldrich). Hematoxylin and eosin (H&E) stains used Gill’s hematoxylin to stain nuclei and acidified eosin to counterstain the cytoplasm. In order to observe the skeletal elements, we stained the samples with alizarin red S and alcian blue (Al & Al) to detect bone and cartilage, respectively.

Immunohistochemistry- Immunostaining was performed using the following primary antibodies: MSX1 (Abcam), MSX2 (Abcam), BMP4 (eBioscience), and FGF8 (eBioscience). Briefly, the slides were blocked for 30 min in 10% BSA with 2% goat serum, followed by an overnight incubation with primary antibodies at 4°C. Horseradish peroxidase (HRP) was the secondary antibody at 1:5000 concentration for 1 h (Invitrogen). The results were visualized by a light microscope (Olympus, Japan).

Statistical analysis- Statistical analyses were carried out on datasets that consisted of at least three independent experiments, using an unpaired Student’s t-test that compared two groups; one-way ANOVA with Tukey’s multiple comparison test which compared more than two groups; or two-way ANOVA with Tukey’s multiple comparison test for nonparametric results with Graph Pad Prism software (GraphPad, San Diego, CA, USA). All data are expressed as the mean ± SD. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: LT designed, performed and analyzed the experiments shown in all figures and wrote the paper. MH designed and constructed vectors for transduction and expression of Msx genes. FAS provided qPCR technical assay and contributed to the data analysis of qPCR. LS provided technical assistance and cell injection. SH edited the paper and contributed to the preparation of the figures. NA
coordinated the study. MBE conceived, coordinated the study, provided technical assistance and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

REFERENCE
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FOOTNOTES:
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The abbreviations are used are: mBMSCs, mouse bone marrow-derived mesenchymal stem cells; BCs, blastema cells; BICs, Blastema like Cells; HSCs, hematopoietic stem cells; MSCs, mesenchymal stem cells; Msx1, Msh homebox 1; Msx2, Msh homebox 2; CFU-F, Colony-forming unit fibroblast; Coll, collagen type I; OCN, osteocalcin; ALP, alkaline phosphatase; DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; NEAAs, Nonessential amino acids; Bmp4, bone morphogenic protein 4; Fgf8, fibroblast growth factor 8; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; ICC, Immunocytochemistry; BSA, bovine serum albumin; FBS, Fetal bovine serum; PBS, phosphate buffer solution; BrdU, 5-bromo-2-deoxyuridine; Nrm. Reg, normal regeneration; Al& AL st, Alizarin red & Alcian blue staining; H&E, hematoxylin and eosin; WPI, weeks post-injection; DPA, days post-amputation; K14, keratin 14; mM, millimolar; µl, microliter; µm, micrometer; ml, milliliter; µg, microgram; mg, milligram; nm, nanometer; kg, kilogram,

FIGURE LEGENDS:
Figure 1. Msx1 and Msx2 gene transduction in mouse bone marrow-derived mesenchymal stem cells (mBMSCs). A, Vector map. Msx1 and Msx2 co-expressed by GFP and tdTomato,
respectively, in order to follow MSX-positive cells in the same vectors. B, The immunocytochemistry (ICC) images represent Msx1 and Msx2 genes endogenously expressed in mBMSCs and BCs, as well as exogenous Msx1 (GFP) and Msx2 (tdTomato) genes expression in BlCs cells (a, b). C, Real-time PCR analysis shows the gene expression level of Msx1 (b) and Msx2 (d) in BCs, mBMSCs and BlCs groups. The histograms represent the percentage of Msx1 and Msx2 protein expression levels in BCs, mBMSCs and BlCs groups (a, c). D, Flow cytometry analysis of CD31, Sca1 and Vim cell surface markers for BlCs, BCs and mBMSCs. Scale bar: 50 μm. Data are presented as means ± SD. ****p<0.0001.

Figure 2. Osteogenic activity. The data represent osteogenic differentiation of BlCs, BCs and mBMSCs after 7, 14, and 21 days. A, Nodule-like aggregations increased over time during 3 weeks and showed strong mineralization in all groups. B, Histogram shows alkaline phosphatase (ALP) activity in the BCs, mBMSCs, MSX1, MSX2 and MSX1/2 groups. C, Histogram represents the calcium content in BCs, mBMSCs, MSX1, MSX2 and MSX1/2 groups. D, Real-time PCR analysis for Col I (a), Runx2 (b), and OCN (c) genes showed enhanced expression levels in the MSX groups compared to mBMSc and BCs. E, Histogram shows quantitative analysis of mineralized nodules in the BCs, mBMSCs, MSX1, MSX2 and MSX1/2 groups. Data are presented as means ± SD (n=3). ****p<0.0001.

Figure 3. Gene and protein expression analysis of Bmp4, Fgf8, K14, and Ki 67. A, Immunostaining of Bmp4 (a), Fgf8 (b), and nuclei (DAPI; blue). Left panel shows merged image with DAPI. B, Real-time PCR analysis shows the expression level of the Bmp4 (a) and Fgf8 (c) genes in all groups. Histogram shows the amount of BMP4 (b) and FGF8 (d) protein expressions analyzed by Image-J software on immune-stained slides. C, Immunostaining of k14+ (a), ki67+ (b). The left panel shows a merged image with DAPI. D, Histogram shows k14 (a) and ki67+ (b) protein expression level analyzed by Image-J software. Scale bar: 50 μm. Data are presented as means ± SD. ****p<0.0001.

Figure 4. Whole mount digit tip evaluation at 6 WPI. The images show the macroscopic evaluation of regenerated digit tip of intact (A), Nrm. Reg (B), sham (C), BCs (D), mBMSCs (E), MSX1 (F), MSX2 (G) and MSX1/2 (H) groups.

Figure 5. Measurement of bone and nail elongation. A, Whole mount alizarin red staining shows bone and nail formation (a-h). B, Histogram shows bone length measurements for all experimental groups.

Figure 6. Tracking of BrdU labeled cells after 6 WPI. A) Immunohistochemistry illustrates BrdU labeled cells in mBMSCs, BCs, MSX1, MSX2 and MSX1/2 groups, B) Histogram shows quantitative analysis of BrdU+ cells percentage in bone regenerated regions by image-J software in all groups. n=3, Arrows show the position of labeled cells.

Figure 7. Histological analysis. Hematoxylin and eosin (H&E), Masson's trichrome, alcian blue, and alizarin red S staining in the intact (A-C), Nrm. Reg (D-F), Sham (G-I), BCs (J-L), mBMSCs (M-O), MSX1 (P-R), MSX2 (S-U), and MSX1/2 (V-Y) groups. The H&E results showed that no apoptotic cells or tissues were found in any of the groups. Alcian blue-alizarin red S staining
indicated that the region containing new bone callus was a blue color; we assumed the presence of cartilage tissues that stained by Alcian blue.

Figure 8. **Bone regeneration analysis in MSX1/2 after 10 WPI.** A, Al & Al staining of MSX1/2 group 10 WPI that shows the endochondral bone was replaced by mineralized bone. B, IHC analysis shows the expression of Col I and OCN in mature bone generated in MSX1/2 group after 10 weeks.

Figure 9. **Immunohistochemistry analysis of the MSX1, MSX2, BMP4, and FGF8.** A. IHC staining shows that MSX1, MSX2, BMP4, and FGF8 expressed in the BCs, mBMSCs, MSX1, MSX2, and MSX1/2 groups. B. Histograms show the percentage of MSX1, MSX2, BMP4, and FGF8 protein expressions in the BCs, mBMSCs, MSX1, MSX2, and MSX1/2 groups (a-d).

Figure 10. **Schematic representation of study design and related mechanism.** A) mBMSCs are transduced by *Msx1* and *Msx2* genes (a). Proximal none-regenerative digit tip model is created (b), followed by BlCs injection 4DPA into the models (c). Digit tip regeneration is occurred 6WPI (d). B) Schematic representation of the proposed mechanism by MSX transduced cells on limb regeneration. In this model, MSXs indirectly regulate Runx2 through up-regulation of Bmp4, and Runx2 in turn triggers osteogenic pathway via OCN and Col I expression. MSXs also control the expression of Fgf8 indirectly either by Bmp4 or SHH, which has caused cell proliferation and bone elongation. The up-regulation of K14 as a consequence of MSX genes led to nail formation.
Figure 1:
Figure 2:

A. Blastema cell-like formation by Msx1 and Msx2-transduced mBMSCs

B. ALP activity

C. Calcium content

D. COL I

E. OCN

F. RUNX2

G. Alogarithmic fold change over 7 days

H. Alogarithmic fold change over 2 days
Figure 3:
Figure 4:
Blastema cell-like formation by Msx1 and Msx2-transduced mBMSCs

Figure 5:
Figure 6:

Blastema cell-like formation by Msx1 and Msx2-transduced mBMSCs
Figure 7:
Blastema cell-like formation by Msx1 and Msx2-transduced mBMSCs

Figure 8:
Figure 9:
Blastema cell-like formation by Msx1 and Msx2-transduced mBMSCs

Figure 10: