Endochondral bone tissue engineering using embryonic stem cells

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Embryonic stem cells can provide an unlimited supply of pluripotent cells for tissue engineering applications. Bone tissue engineering by directly differentiating ES cells (ESCs) into osteoblasts has been unsuccessful so far. Therefore, we investigated an alternative approach, based on the process of endochondral ossification. A cartilage matrix was formed *in vitro* by mouse ESCs seeded on a scaffold. When these cartilage tissue-engineered constructs (CTECs) were implanted s.c., the cartilage matured, became hypertrophic, calcified, and was ultimately replaced by bone tissue in the course of 21 days. Bone aligning hypertrophic cartilage was observed frequently. Using various chondrogenic differentiation periods *in vitro*, we demonstrated that a cartilage matrix is required for bone formation by ESCs. Chondrogenic differentiation of mesenchymal stem cells and articular chondrocytes showed that a cartilage matrix alone was not sufficient to drive endochondral bone formation. Moreover, when CTECs were implanted orthotopically into critical-size cranial defects in rats, efficient bone formation was observed. We report previously undescribed ESC-based bone tissue engineering under controlled reproducible conditions. Furthermore, our data indicate that ESCs can also be used as a model system to study endochondral bone formation.

osteoblast | cartilage | endochondral ossification | scaffold | *in vivo*

Bone tissue engineering is generally approached by combining osteogenic cells with a porous biodegradable ceramic scaffold. Human bone marrow-derived mesenchymal stem cells (MSCs) can be differentiated into the osteogenic lineage by culturing the cells in the presence of the osteogenic differentiation supplements dexamethasone, ascorbic acid, and β-glycerophosphate (1, 2). Mineralized bone matrix is deposited *in vitro* as a result of the expression of osteogenic genes (3), and *de novo* bone formation is observed when human MSCs are implanted into an ectopic or orthotopic site (2). Even though MSCs from most human donors show osteogenic potential, there is large variation in bone-forming capacity by human MSCs, and multipotency is gradually lost upon expansion (4). Most importantly, bone formation by MSCs is currently insufficient for successful tissue engineering (5). Besides efforts to increase bone formation by MSCs *in vivo*, we also explore ES cells (ESCs) (6–8) as a potential source for bone tissue engineering. ESCs are capable of indefinite undifferentiated proliferation *in vitro* and can provide an unlimited supply of cells, which can be differentiated into various cell types (9).

Before ESCs can be used in clinical applications, some technical issues have to be addressed, such as the labor-intensive procedure and the use of animal-derived reagents to expand human ESCs, the immunogenicity of allogeneic ESCs and the potential risk of tumorigenicity. Moreover, a differentiation scheme has to be designed to obtain the desired cell or tissue type. Osteogenic differentiation of mouse and human ESCs has been established *in vitro* by culturing the cells in medium supplemented with ascorbic acid, β-glycerophosphate, dexamethasone (10–12), BMP2 (13), compactin (13), or vitamin D3 (14). Mineralization was observed, and qPCR analysis showed up-regulation of osteogenic markers such as Cbfa-1/Runx2, osteopontin, bone sialoprotein, and osteocalcin. We observed similar results when mouse and human ESCs were differentiated into the osteogenic lineage *in vitro* (unpublished work). To assess bone tissue engineering using ESCs, we seeded human or mouse ESCs onto ceramic scaffolds and cultured them in osteogenic media for 7 or 21 days. Six weeks after implantation into immunodeficient mice, no bone tissue was observed in samples of mouse ESCs (unpublished work). For human ESCs, we observed some *in vivo* mineralized tissue, but no bone tissue, as reported (11). So far, *in vivo* bone formation by ESCs has been observed only in teratomas. Strikingly, it occurred to us that bone tissue in teratomas frequently aligns hypertrophic cartilage, which resembles the process of endochondral ossification. Most bones in the body are formed via endochondral ossification, which involves the formation of cartilage tissue from condensed mesenchymal cells and the subsequent replacement of the cartilage template by bone. In contrast, direct conversion of mesenchymal tissue into bone is called intramembranous ossification, which occurs primarily in the craniofacial skeleton. Here, we describe an alternative approach to *in vivo* bone formation using ESCs, based on the process of endochondral ossification.

Results

Chondrogenic Differentiation of Mouse ESCs *in Vitro* and Bone Formation *in Vivo*. It is well established that osteogenic human MSC mineralize *in vitro* (Fig. 1*A*) and form bone *in vivo* (Fig. 1*B*) (2). This process of ossification occurs through intramembranous ossification, without the intermediate production of cartilage (data not shown). Moreover, it is known that mouse ESCs, like MSCs, can be induced into the osteogenic lineage *in vitro* as indicated by mineralization (Fig. 1*C*) and the up-regulation of osteogenic genes. However, in contrast to human MSCs, these osteogenic mouse ESCs did not form bone upon implantation (Fig. 1*D*). Therefore, we assessed an alternative approach: endochondral ossification, in which ESCs first deposit cartilage, which may serve as a template for ossification. In previous studies, we have shown cartilage formation by mouse ESCs in pellets, on polymeric scaffolds, and in hydrogels (15). We now assessed the chondrogenic potential of mouse ESCs on ceramic scaffolds. ESC-derived embryoid body (EB) cells were seeded on ceramic particles and cultured in serum-free chondrogenic differentiation medium containing TGFβ3 for 21 days. Regions with typical cartilage morphology, that is round cells with

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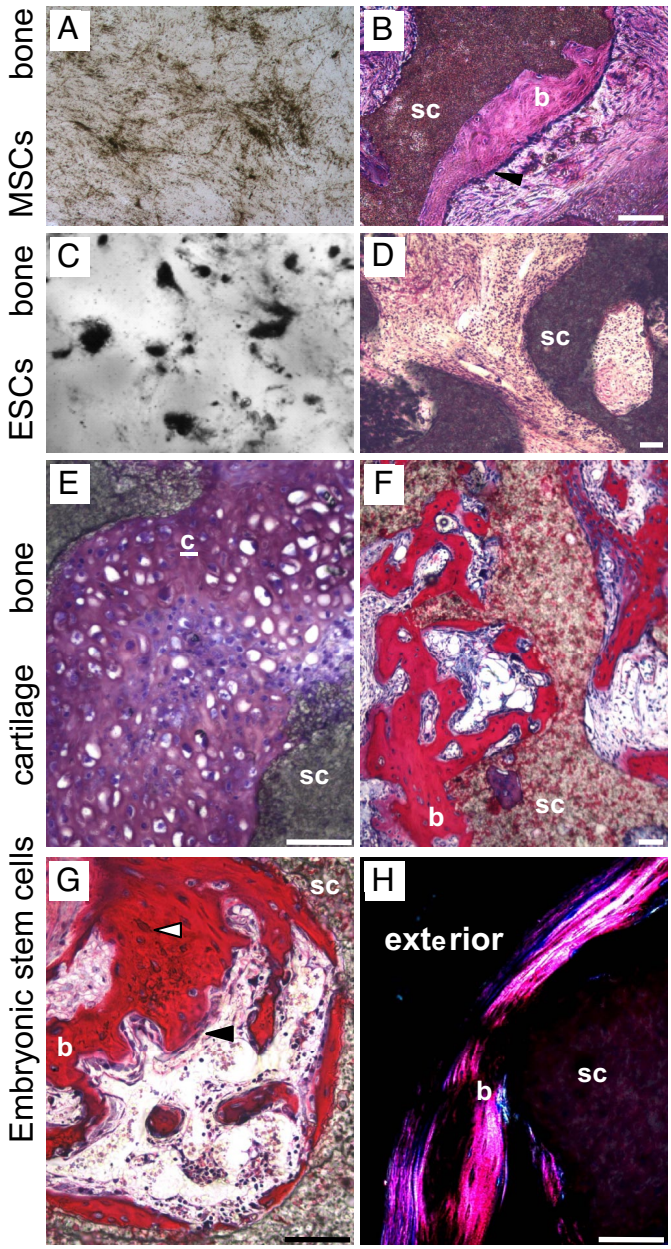


Fig. 1. Two approaches for *in vivo* bone formation by MSCs and ESCs. (*A*) *In vitro* osteogenic differentiation of human MSCs cultured on tissue culture plastic for 21 days, indicated by von Kossa staining, which stains mineralized matrix black. (*B*) *In vivo*, bone is formed by human MSCs, as shown by the methylene blue and basic fuchsin stained sections of cells grown on ceramic particles for 7 days and implanted s.c. into immunodeficient mice for 6 weeks. Bone tissue stains pink, and bone-lining cells are indicated by a black arrowhead. (*C*) *In vitro* osteogenic differentiation of mouse ESCs cultured on tissue culture plastic for 21 days, indicated by black von Kossa staining of the mineralized matrix. (*D*) After 21 days, no bone is formed *in vivo* by mouse ESCs, which were precultured *in vitro* for 21 days on ceramic particles in osteogenic medium. (*E*) In the process of endochondral ossification, bone is formed on a cartilage template. Mouse ESCs were cultured in chondrogenic medium on ceramic particles for 21 days. Cells displayed a chondrocyte phenotype, as indicated by round cells in lacunae surrounded by extracellular matrix, which stained positive for glycosaminoglycans (indicated by pink thionin staining). (*F*) CTECs were implanted s.c. for another 21 days to demonstrate bone formation. Bone tissue is stained dark pink by basic fuchsin. (*G*) Higher magnification of bone tissue observed on implanted CTECs. Bone-lining cells are indicated by a black arrowhead and osteocytes by an open arrowhead. (*H*) The bone tissue that was formed consisted of lamellar bone, as indicated by polarized light. sc, ceramic scaffolds; b, bone; c, cartilage. (Scale bar, 100 μm.)

lacunae surrounded by extracellular matrix, were found on the outside of the particles and inside the pores (Fig. 1*E*). Cartilage was formed in each sample cultured in chondrogenic medium and approximately one-tenth to one-third of the cells on the particles differentiated into chondrocytes. Collagen type II expression was substantially up-regulated compared to control cultures (data not shown). Particles cultured in control or osteogenic medium did not show formation of cartilage (data not shown).

After creating a cartilage template on ceramic particles by differentiating mouse ESCs into the chondrogenic lineage for 21 days *in vitro* [hereafter referred to as cartilage tissue-engineered constructs (CTECs)], the next step was to demonstrate *in vivo* bone formation. Therefore, CTECs were implanted s.c. in the back of immunodeficient mice for 21 days. Bone-like tissue was formed in all samples, which were differentiated into the chondrogenic lineage (Fig. 1*F*), in contrast to the samples, which were differentiated into the osteogenic lineage *in vitro* (Fig. 1*D*), in which bone tissue was never observed. The newly formed bone, also known as osteoid, was aligned with osteoblasts, which were visible in the mature and mineralized bone tissue (Fig. 1*G*). Bone was formed both on the outside of the particles, as within the pores, and the tissue consisted of lamellar bone as demonstrated by polarized light (Fig. 1*H*). We demonstrate directed, reproducible bone formation using mouse ESCs *in vivo*.

A Time Course of Endochondral Bone Formation by Mouse ESCs. Next, we investigated the fate of *in vitro* formed cartilage after implantation and the process of bone formation *in vivo*. Therefore, we analyzed the CTECs 2, 7, 14, or 21 days after implantation (Fig. 2*A*). At the time of implantation, cartilage tissue was present on the CTECs, and hardly any cellular stroma was observed. After 2 days *in vivo*, cartilaginous tissue was still present on the implanted CTECs, indicating the survival of the implanted tissue. The CTECs showed different amounts of fibrous immature scar-like tissue that showed a resemblance to mesenchymal cells in tissue cultures. After 7 days *in vivo*, the cartilage showed the beginning of maturation indicated by larger lacunae with smaller uniform nuclei. The onset of endochondral calcification was indicated by slight basic fuchsin staining within the mature cartilage. Furthermore, the fibrous stroma became more cellular and dense and contained more vessels. Bone formation was first seen after 14 days *in vivo*. Bone surrounded hypertrophic chondrocytes and the mineralized cartilage matrix (Fig. 2*B*). In some regions, cartilage seemed to be totally replaced by bone, and in other regions, mature cartilage was still present. After 21 days *in vivo*, hardly any cartilage remained, and more bone tissue was observed than after 14 days. We even observed tissue resembling bone marrow in some bone lacunae (Fig. 2*A*).

The gradual decrease in the amount of cartilage and a gradual increase in the amount of bone tissue in time were confirmed by histomorphometric analysis. After 21 days, there was significantly less cartilage and significantly more bone per scaffold area than at earlier time points. There was also significantly more bone than cartilage after 21 days (Fig. 2*C*). Thus, in the course of 21 days *in vivo*, almost all cartilage matured and was replaced by bone tissue.

A Cartilage Template from ESCs Is Necessary for Endochondral Bone Formation. We investigated whether either chondrogenic stimulation or cartilaginous tissue was required for *in vivo* bone formation. Therefore, we differentiated cells *in vitro* for 3, 7, 14, and 21 days and subsequently implanted these samples for another 21 days into immunodeficient mice. *In vitro* chondrogenic differentiation for 3 and 7 days did not result in tissue with typical cartilage morphology. After 14 days, the first, mainly small, cartilaginous regions were observed, and more and larger

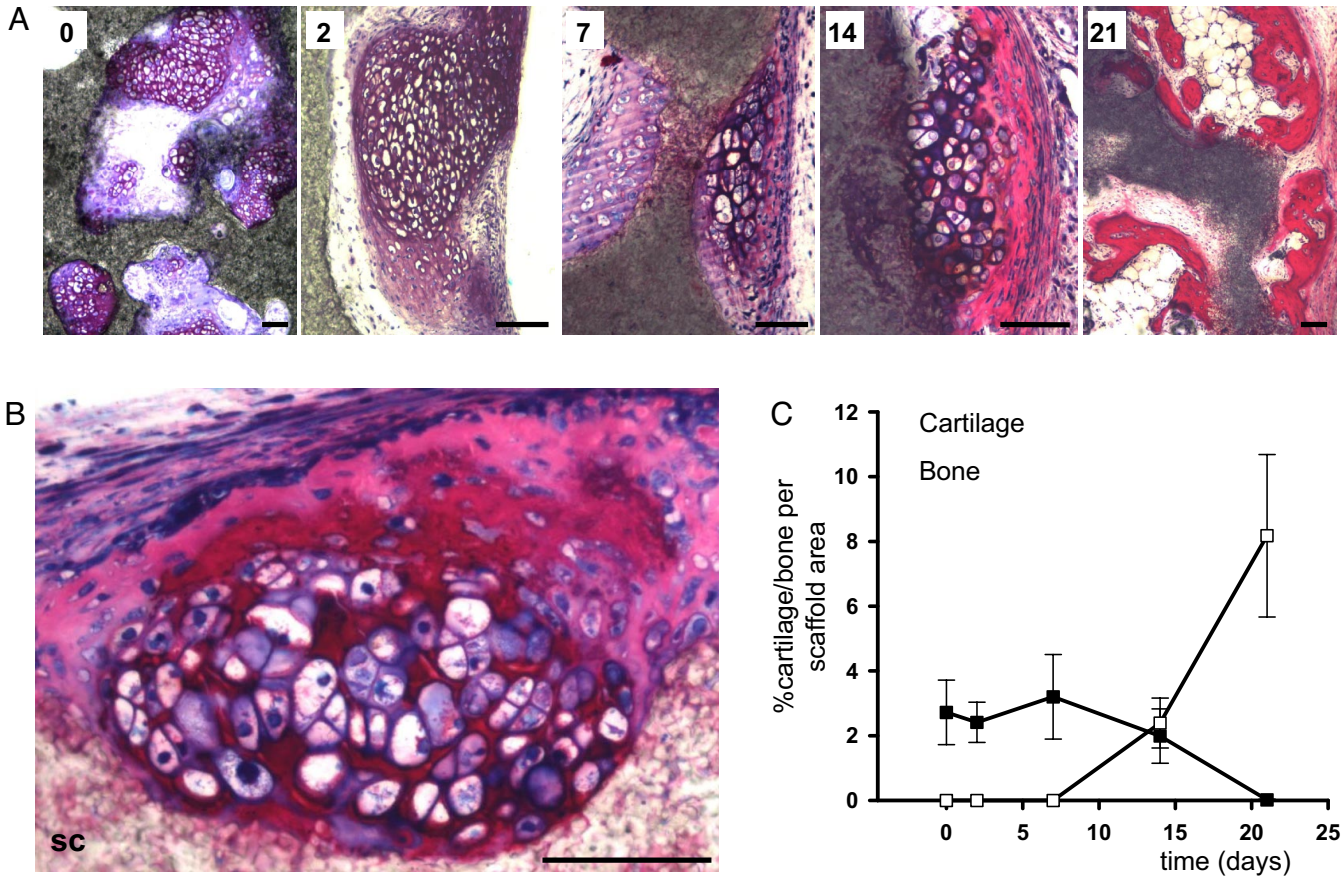


Fig. 2. *In vivo* bone formation throughout time. (*A*) Representative images of histological sections of CTECs 0, 2, 7, 14, and 21 days after implantation. At 0 and 2 days *in vivo*, cartilage matrix is visualized by pink thionin staining of glycosaminoglycans. At 7, 14, and 21 days *in vivo*, bone tissue is stained by methylene blue and basic fuchsin staining, which stains cells blue and bone tissue dark pink. Cartilage can still be recognized by morphology, that is extracellular matrix in which single cells in a lacuna can be distinguished. (*B*) Higher-magnification image of a CTEC after 14 days *in vivo*, showing the process of endochondral ossification. Hypertrophic chondrocytes in mineralized cartilage were surrounded by bone tissue. (Scale bars, 100 μm.) (*C*) Histomorphometric analysis of the amount of cartilage and bone per available scaffold area in time.

regions of cartilage tissue were formed after 21 days of *in vitro* culture (Fig. 3*A*). After 14 days in *vitro*, we scored 0–5 cartilage nodules, with an average of 2.4 (12 sections), and for the 21-day CTECs, we observed 11–34 nodules, with an average of 21.7 nodules/section (Fig. 3*C*). After subsequent implantation for 21 days, we scored the amount of bone nodules aligning the ceramic particle in all histological sections (five to seven sections per sample, six mice per time point). No bone nodules were observed in the 3-day samples. For the samples that had been differentiated *in vitro* for 7 days, we observed one bone nodule in a few sections, with an average of 0.2 bone nodules per section. For the 14-day CTECs, we observed 0–13 bone nodules in the sections with an average of 4.6, and for the 21-day CTECs, we observed 2–20 bone nodules with an average of 9.2 bone nodules/section (Fig. 3*C*). Thus, bone was mainly observed in the samples that had been differentiated into the chondrogenic lineage for 14 days and 21 days (Fig. 3*B*). In consistency with the higher amount of cartilage *in vitro*, the highest amount of bone was found in the 21 days samples. We conclude that a cartilage template is required for bone formation.

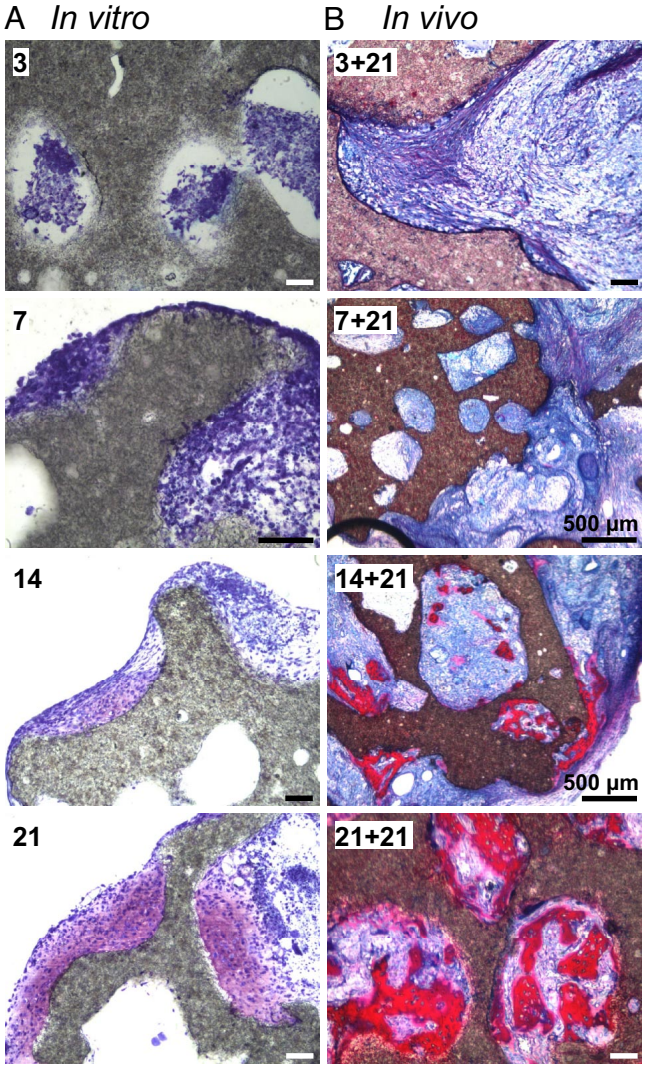
A Cartilage Template Is Not Sufficient for Endochondral Bone Formation. To investigate whether any cartilage template will mature, calcify and will be replaced by bone, we implanted cartilage derived from articular chondrocytes and adult stem cells.

Freshly isolated calf chondrocytes were cultured on ceramic particles in chondrocyte proliferation medium. After 21 days *in vitro*, cartilage and some fibrous tissue was formed on the ceramic particles (Fig. 4*A*). Subsequently, these constructs were implanted into immunodeficient mice. The cartilage phenotype was stable *in vivo* and more cartilage matrix was deposited (Fig. 4*B*). Hypertrophy and calcification of the cartilage matrix was not observed. No signs of endochondral ossification were observed when articular chondrocyte-derived cartilage was implanted.

We also investigated the fate of a cartilage template derived from adult stem cells. Given that even nonstimulated MSCs can form bone *in vivo*, we had to look for signs of endochondral ossification other than sheer bone formation, such as hypertrophic chondrocytes, calcified matrix, and regions where bone aligns and replaces cartilage. We seeded goat and human MSCs on ceramic particles and differentiated these cells into the chondrogenic lineage as described for ESCs. Cartilaginous tissue was formed by goat MSCs, which was almost homogeneous in some samples (Fig. 4*A*). Human MSCs proliferated on the ceramic particles, and a small amount of GAG-positive tissue was observed (Fig. 4*A*), limited to one to three small regions per sample.

Next, these CTECs were implanted into immunodeficient mice for an additional 21 days. Cartilaginous tissue was still observed, and bone was formed *in vivo* (Fig. 4*B*).

Bone formation in human MSC samples remained limited to an average of 2.6 small bone nodules per sample, similar to the rather inefficient chondrogenic differentiation *in vitro*. No signs



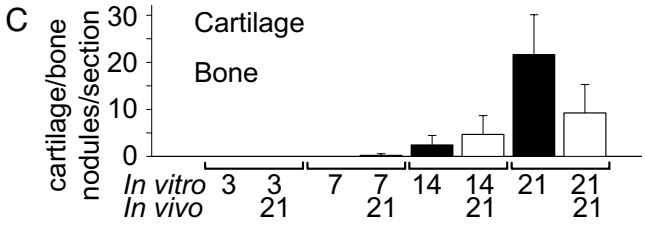


Fig. 3. The necessity of a cartilage template for *in vivo* bone formation using mouse ESCs. (*A*) Chondrogenic differentiation of mouse ESCs for 3, 7, 14, and 21 days *in vitro*, as indicated by thionin staining. Cartilage matrix was first observed after 14 days of chondrogenic differentiation and the amount increased in time as seen after 21 days. (*B*) Subsequent implantation for 21 days after *in vitro* differentiation for 3, 7, 14, and 21 days. Bone tissue was observed in the 14 + 21 and 21 + 21 days samples, as indicated by basic fuchsin staining. (Scale bars, 100 μm.) (*C*) Average amount of cartilage nodules *in vitro* and bone nodules *in vivo* in time, scored per section.

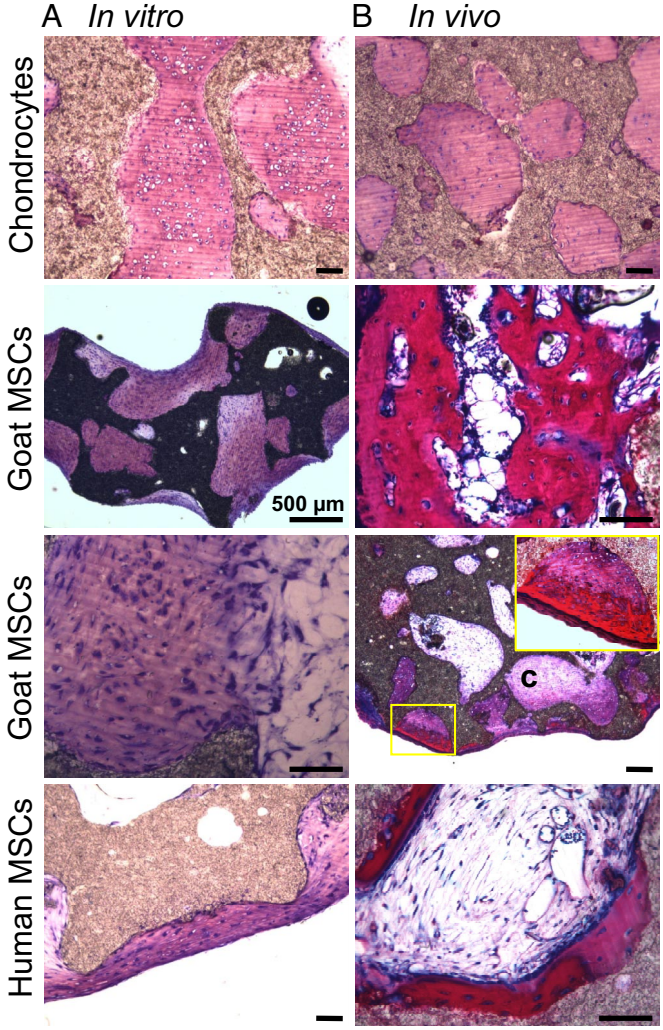


Fig. 4. A cartilage template is not sufficient to induce endochondral bone formation. (*A*) *In vitro* cartilage formation by bovine chondrocytes, goat MSCs, and human MSCS, stained by thionin. (*B*) Subsequent implantation of the *in vitro* samples as displayed in *A*. No bone was formed in the bovine cartilage samples. Bone was observed in both goat MSCs and human MSC samples, as indicated by basic fuchsin staining. (*Inset*) In some regions, bone aligned cartilage. Cartilaginous tissue (c) was still present in goat MSC samples. (Scale bars, 100 μm.)

of endochondral ossification were observed in these few bone nodules.

Extensive bone formation was observed in the implanted goat MSC CTECs, up to 40 bone nodules per section. Most bone appeared to be formed by the process of intramembranous ossification, as also observed when nonstimulated cells were implanted. However, in 14% of the bone regions, bone-aligned cartilage or calcified areas were observed in the cartilaginous tissue. Even though we did not observe the typical replacement of hypertrophic cartilage by bone, as observed with mouse ESCs (Fig. 2*B*), these areas are indications of the onset of endochondral bone formation by goat MSCs. In studies with mouse ESCs, hardly any cartilage was observed after 21 days *in vivo* (Fig. 2*C*). However, large regions of cartilage were still present in the implanted goat MSCs samples (Fig. 4*B*). Of the implanted large cartilaginous regions, ≈80% did not show signs of endochondral ossification after implantation.

Based on these experiments, we conclude that a cartilaginous matrix does not automatically lead to endochondral bone formation, but rather that stem-cell- and especially ESC-derived cartilage has a tendency to mature and enter the process of endochondral ossification.

Bone Tissue Engineering in an Orthotopic Defect. The above-mentioned *in vivo* studies were restricted to ectopic implantation sites. To study bone formation in an orthotopic defect,

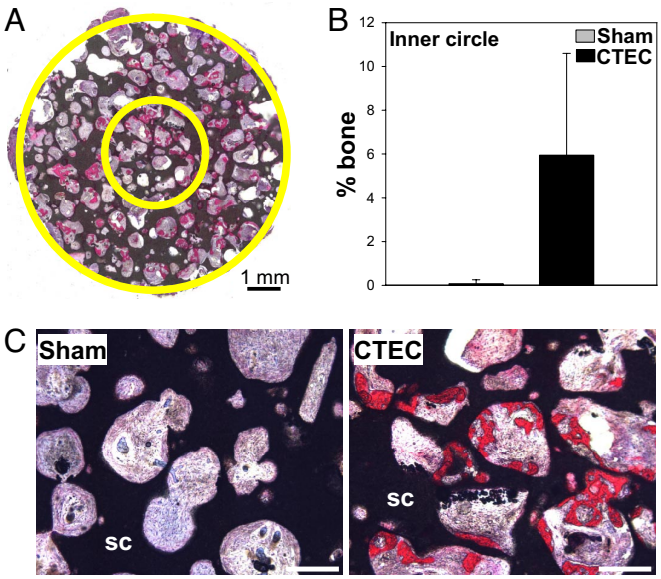


Fig. 5. Orthotopic bone formation using ESCs in a rat cranial defect. (*A*) Bone formation in an 8-mm CTEC implanted for 21 days in a critical-size cranial defect in a rat, visualized by methylene blue and basic fuchsin staining. For subsequent analysis, the sample was divided in an outer and inner ring, indicated by yellow circles. (*B*) Histomorphometric analysis of bone tissue formed in the inner circle of sham implants and CTECs. (*C*) Higher-magnification view of the inner circle (2.7 mm) of a sham implant and a CTEC. sc, scaffold. (Scale bar, 500 μm.)

we implanted CTECs or empty scaffolds in an 8-mm critical-size cranial defect in an immunodeficient rat. After 21 days, cartilage was observed in all *in vitro* discs (CTECs; data not shown).

The cranial defect proved critical, because bone in-growth did not bridge the sham implant. To distinguish between bone in-growth from the cranium and bone formation using ESCs, all samples were divided in an outer ring, containing the regions of bone in-growth, and an inner ring, in which bone in-growth was not observed in the sham implants (Fig. 5*A*). The percentage of bone in the inner circle of sham implants and CTECs (Fig. 5*C*) was determined by histomorphometry (Fig. 5*B*). Significantly more bone was observed in the inner circle of CTECs, as compared with sham implants. Hereby, we show efficient bone formation using ESCs in an orthotopic defect.

Discussion

In this article, we describe *in vivo* bone formation using mouse ESCs. We first attempted to directly differentiate ESCs into the osteogenic lineage, based on differentiation protocols and media established for adult stem cells (3) and osteoblasts. Although *in vitro* results were satisfying, *in vivo* experiments did not result in bone formation, by either mouse or human ESCs. We opted for an alternative approach by first forming a cartilage template and subsequently allowing the cartilage to be replaced by bone. Using this approach, we demonstrated previously undescribed directed and reproducible *in vivo* bone formation using ESCs in ectopic and orthotopic sites. The process is very robust, because we observed bone in all experiments where an ESC-derived cartilage template was formed *in vitro*. The amount of bone formed in these experiments was comparable to the amounts formed by rat and goat MSCs (data not shown). We also observed endochondral bone formation on polymeric scaffolds, indicating that *in vivo* bone formation was not induced by and is not exclusive to ceramic scaffolds (data not shown).

Most, if not all, differentiation protocols result in a heterogeneous population of ESCs differentiated into various lineages but enriched for the desired cell type. Cartilage and bone were formed in all experiments, but ESCs also differentiated into other less or more advanced tissue types, like squamous and cylindrical epithelial cells lining cyst-like spaces and tubules, endothelial cells lining (blood) vessels, fat, and stroma. In some experiments, we even observed teratoma formation. Cartilage, hypertrophic and calcified cartilage, and some bone were observed in the teratomas, but in contrast to our directed differentiation, these tissues did not align the scaffold material. For future application, the constructs should be purified from residual undifferentiated ESCs to avoid teratoma formation *in vivo*. In addition, purification could result in more homogeneous cartilage formation, which might result in improved bone formation.

Currently, we are investigating which cell population is responsible for bone deposition. A subpopulation of mesodermal or osteoprogenitor cells might be present in the implanted heterogeneous ESC population, which differentiate into osteoblasts and deposit bone matrix. Alternatively, as during bone growth, blood vessels infiltrate the cartilage matrix, and host cells might form bone tissue stimulated by factors secreted by the hypertrophic chondrocytes. Blood vessels close to the cartilage matrix were observed frequently in the samples.

The next step will be to show *in vivo* bone formation using human ESCs. When human ESCs were differentiated into the osteogenic lineage, small fragments of mineralized tissue were observed *in vivo* (11), but no osteocytes and osteoblasts were observed. The first step in endochondral bone formation by human ESCs would be the formation of a cartilage template. The chondrogenic potential of human ESCs has been demonstrated in indirect coculture experiments with primary chondrocytes (16). Whereas mouse ESCs show consistent cartilage formation, we did not observe cartilage formation when the chondrogenic protocols were transferred to human ESCs (data not shown). Not only did the serum-free chondrogenic medium containing TGFβ3 result in heterogeneous cartilage formation by mouse ESCs, but also the cartilaginous tissue that was formed by human MSCs in our studies was not homogeneous (data not shown), even when supplemented with BMP6. Further optimization of the growth-factor regime for both adult and ESCs is necessary (17, 18).

Mouse ESC-derived cartilage displayed maturation, calcification, and subsequent replacement by bone tissue. Indications of maturation were also observed for human ESC-derived cartilaginous tissue (16, 18) Apparently, ESCs followed the route of embryonic development. This implicates that ESCs can be used as a model system to study endochondral bone formation. Where most current models use chicken eggs, *in vitro* limb cultures, or transgenic mice, our results show that endochondral bone formation can now be studied with ESCs. Transgenic ESCs, rather than transgenic mice, can be used to investigate the influence of several genes in the process of endochondral bone formation. Besides *in vitro* assays, *in vivo* bone formation can be studied in ectopic and orthotopic models. The orthotopic defect used in our studies might not be the most logical model for endochondral bone formation, because it is well known that the bones of the craniofacium form through intramembranous ossification. However, this study does demonstrate that the implanted mouse ESCs result in bone formation in an orthotopic defect in a rat.

In conclusion, our data show that mouse ESCs readily undergo endochondral ossification after deposition of a cartilage matrix, which can benefit both bone tissue engineering and the genetic dissemination of endochondral bone formation.

Materials and Methods

Cell Culture. Mouse ESC line IB10 was cultured, and embryoid bodies were formed as described in ref. 15. ESC-derived embryoid body cells were used for differentiation experiments. Human MSCs were isolated from bone marrow aspirates from donors who had given written informed consent (2). Goat MSCs and calf articular chondrocytes were isolated as described (19, 20).

Differentiation. Aliquots of 1.5 million cells were seeded onto three ceramic particles of 2–3 mm, prepared as described by Yuan *et al.* (21). For cranial implants, one million cells were seeded statically on both sides of the 8 × 1.5-mm disk. Further differentiation of stem cells into the chondrogenic lineage was performed in serum-free chondrogenic medium containing TGFβ3 (22). For differentiation of human MSCs, the chondrogenic medium was supplemented with 250 ng/ml human BMP6 (Biovision) (23). For differentiation into the osteogenic lineage, EB cells were cultured for another 3 days in medium supplemented with 10^{-7} M retinoic acid (Sigma) and subsequently in medium supplemented with 0.2 mM ascorbic acid, 2.5 μM compactin (Sigma) and 0.01 M β-glycerophosphate (Sigma) (adapted from ref. 13). Osteogenic differentiation of human MSC was described by Both *et al.* (2).

***In Vivo* Studies.** Samples were precultured in chondrogenic or osteogenic medium for 21 days and subsequently implanted into immunodeficient mice (HsdCpb:NMRI-nu Harlan, $n = 6$) for 21 days, unless indicated otherwise (15). For cranial implantation, immunodeficient rats (Crl:NIH-*Foxn*^{rnu}; Charles River) were injected s.c. with 0.02 mg/kg buprenorphine (Temgesic) for pain relief. The rats were induced with 4–5% isoflurane, and during the operation, they were maintained with a mixture of isoflurane (1.5–3%), O_2 (200–300 ml/min) and N_2O (50–200 ml/min). An incision was made in the skin over the cranium from the middle of the nasal bones to the posterior nuchal line. The periostium was sedated with Lidocaine (2%) and removed. An 8-mm trephine dental bur (ACEuropa, Lda) was used to mark the defect site and a 0.7-mm drill (Synthes) was used to remove the bone to realize the craniotomy. An implant was press-fitted into the defect site. Six rats received a sham implant, and seven rats received a CTEC. The overlaying tissue was sutured back in layers. After 4 weeks, implants were removed and processed histologically as described below. Animals were housed at the Central Laboratory Animal Institute (Utrecht University, Utrecht, The Netherlands), and experiments were approved by the local animal care and use committee.

Histological Staining and Light Microscopic Analysis. Samples were fixed in 0.25% glutaraldehyde (Merck) in 0.14 M cacodylate buffer and dehydrated using sequential ethanol series. Scaffolds were embedded in methyl methacrylate (LTI), and sections were processed on a histological diamond saw (Leica SP1600). Sections were etched with an HCl/ethanol mixture and sequentially stained to visualize cartilage and bone. Cartilage formation was visualized by 0.04% thionin (Sigma) in 0.1 M sodium acetate (Merck), which stained cells blue and glycosaminoglycans pink. Bone formation was visualized by 1% methylene blue (Sigma) and 0.03% basic fuchsin (Sigma), which stained cells blue and bone pink. Histological sections were analyzed by using a light microscope (E600 Nikon). For mineralization studies, ESC-derived EBs or MSCs were grown on tissue culture plates in osteogenic medium for 21 days, fixed and incubated with 5% silver nitrate (Sigma) under a UV lamp, until black staining was observed.

Histomorphometry. Histomorphometry was performed on particles that were explanted at different time points and on the inner circle of cranial implants. Low-magnification images were made from two to three sections per sample. Scaffold, bone, and cartilage were pseudocolored, and image analysis was performed with KS400 software (Zeiss Vision). A custom-made program (University of Utrecht) was used to measure percentage of cartilage or bone compared to scaffold area.

Statistical Analysis. Statistical calculations were performed with SPSS 14.0 software. Histomorphometric data for particles were not normally distributed. Therefore, we used nonparametric tests to compare the amount of cartilage and bone in time (Kruskal–Wallis) and the amount of cartilage and bone at different time points (Wilcoxon signed-rank test). For the cranial implants, the samples of the sham group showed little to no bone in-growth in the inner circle, whereas bone was formed in all tissue-engineered samples. Because of the lack of variation in the sham group, we calculated the mean of the three images for each sample and a Mann–Whitney *U* test was used to detect a difference between the two groups.

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