Intraosseous BMP implants in rabbits

Inhibitory effect on bone formation

Charlotte Jeppsson[1], Mathias Boström[2] and Per Aspenberg[1]

The bone harvest chamber is a model for rapid spontaneous bone healing in rabbits. We have previously shown inhibition of bone formation by using BMP-2 on a collagen carrier in this intraosseous model, despite bone formation when depositing BMP-2 on a similar carrier subfascially in the same animals. The doses were 12 and 0.6 μg/ 5 mm^3 chamber volume. As these findings conflicted with most other experiments dealing with the skeletal response to BMP-2, we repeated the previous experiments with variations. We studied: 1) a lower BMP-2 dose, 2) a different type of BMP (BMP-7/OP-1), 3) a different carrier (hydroxyapatite), 4) a different chamber construction allowing contact with extraskeletal tissue and 5) BMP-2 on the original collagen carrier in an acutely inserted chamber in rats. We also studied the border between the BMP-2 implant and the preexisting bone to see whether BMP-2 caused premature differentiation of the callus so that proliferation was stopped and a bone cyst formed. The low dose of BMP-2 reduced tissue ingrowth and tended to reduce bone formation. BMP-7 showed the same inhibitory effects as BMP-2. BMP-2 on a hydroxyapatite carrier also inhibited bone formation in the chamber. In the chamber that allowed contact with extraskeletal tissue, we observed no effects of BMP-2. The border between the BMP-2 implant and the preexisting bone did not look like a cyst wall. BMP-2, from the same batch, on a similar collagen carrier, regularly increased bone formation in the acutely inserted bone chamber in rats, thereby excluding major defects in the BMP-2 implants. The inhibition in this specific model is a consistent finding and not due to an overdose, a specific BMP-type, a specific carrier or premature callus differentiation.

[1]Department of Orthopedics, Lund University Hospital, SE-221 85 Lund, Sweden. Tel +46 46-17 15 26. Fax -13 07 32;
[2]Hospital for Special Surgery, New York, NY, USA. Correspondence: Dr. P. Aspenberg
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There are high expectations for the clinical use of BMPs in stimulation of bone formation. Several studies report their efficiency for healing critical size defects in rodents and nonhuman primates (Tourimi et al. 1991, Yasko et al. 1992, Cook et al. 1994). Experiments in spontaneously healing bone defects are rare, but increased strength of osteotomies treated with BMP-2 in rabbits has recently been reported (Turek et al. 1997). We found inhibition of bone and tissue formation when introducing BMP-2 into a spontaneously healing skeletal defect in a titanium chamber in rabbits (Jeppsson and Aspenberg 1996). As these findings conflicted with previous experiments concerning BMPs, we then varied the experimental conditions to find a reason for this inhibition. One reason might be that the dose of BMP-2 was too high in this enclosed environment. Therefore we repeated the experiment with a lower dose of BMP-2 to see if that would induce bone formation where higher doses were inhibitory. We also wondered whether other members of the BMP family had the same inhibitory properties as BMP-2, and therefore repeated the experiment using OP-1. Another reason for the inhibitory effects could be that the collagen carrier in this specific situation interfered in a negative way with the BMP. Therefore we repeated the experiment, with hydroxyapatite as a carrier instead. The chamber model used so far differs from other models in that the BMP-2 implant is strictly intraosseous. Hence, no cells of extraosseous origin have access to it. Further, this closed environment restricts diffusion of BMP-2 away from the implant. Therefore we also designed a new chamber variant, allowing contact with extraskeletal tissue.

The BMPs are usually considered to be differentiation factors. Since there are some reports that BMP in high doses may cause cyst formation (Sciadini et al. 1995), a possible explanation of the inhibitory effects is that the callus derived from the bone surrounding the BMP-2 implant differentiates into bone, before sufficient proliferation has occurred. This would explain why hardly any tissue was present in the cham-

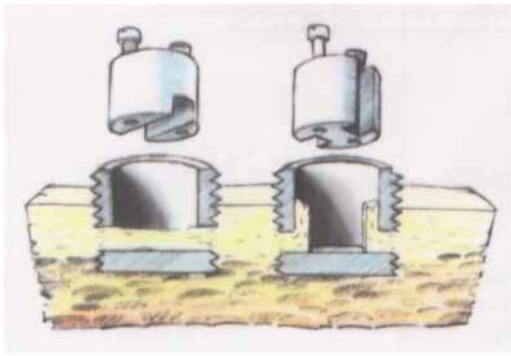

Figure 1. The Bone Harvest Chamber (to the left) which may be transformed to the Vertical Bone Harvest Chamber (to the right) by exchanging the inner core from one that connects the two ingrowth openings with one another, to one that separately connects each ingrowth opening with the surrounding tissue.

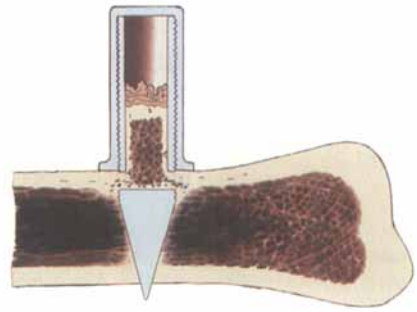

Figure 2. The Bone Conduction Chamber used for rat experiments.

bers. Therefore we modified the original experiment to see if we could show differentiated mature bone at the border between preexisting bone and the site for the BMP-2 implant. Finally, BMP-2 from the same batch on the same carrier was implanted in a different type of chamber in rats. This model has previously shown increased bone formation in response to BMP-2 on a hydroxyapatite carrier (Aspenberg et al. 1996a), and served as a positive control to exclude defects of the BMP-2-collagen implant.

Material and methods

Chambers

The Bone Harvest Chamber (Figure 1) is a 7 mm high and 6 mm wide titanium cylinder, which is threaded so that it can be screwed into the proximal medial tibial metaphysis of adult rabbits (Albrektsson et al. 1983). This provides a standardized spontaneously healing skeletal defect. The cylinder contains a piston-like core with a 1 × 1 × 5 mm groove facing the bottom of the cylinder. This groove is co-axial with holes in the outer cylinder, providing a continuous canal through the entire device for tissue ingrowth. The cylinder will osseointegrate with the surrounding bone in 6 weeks, but one end sticks out of the bone. From this end, the core can be pulled out, exposing the tissue in the ingrowth canal which can be harvested without disturbing the surrounding bone. The chamber can be used for repeated harvesting, and bone formation in it has been shown to be sensitive to various forms of disturbance (Goodman 1994), yet bone ingrowth remains stable over as many as 20 consecutive harvests (Thoren et al. 1995).

The Vertical Bone Harvest Chamber (Figure 1) is created by replacing the piston-like core in the bone harvest chamber by a core in which the horizontal groove has been replaced by two parallel vertical grooves, measuring 1 × 1 × 7 mm. These two vertical grooves thus connect the ingrowing openings in the bottom of the chamber with the subcutaneous tissue outside the top. The openings are not connected with each other.

The Bone Conduction Chamber (Figure 2) consists of a threaded titanium cylinder, formed from two half-cylinders, held together by a hexagonal closed screw cap. One end of the implant is screwed into the bone. The cylindrical bone ingrowth chamber in the implant has a diameter of 2 mm, and is 7 mm long. There are two ingrowth openings at the bone end of the chamber. Thus, the ingrowing tissues enter the cylindrical space at the cortical level. This space extends far out into the subcutaneous region, and the ingrown bone-derived tissue can fill the chamber, without competition from other tissues. Without an osteoconductive material in the space, new tissue, most of it bone, will fill a small part of the chamber within 6 weeks (Aspenberg and Wang 1993). The chamber can be filled with porous materials having possible osteoconductive properties, and the ingrowth of new bone or other tissues into the material can be measured on histological slides. The advantage of this model is that bone ingrowth can be measured in millimeters. Various osteoconductive materials have shown different bone ingrowth distances (Aspenberg and Wang 1993).

BMP implants and controls

The recombinant BMP-2 was a gift from Genetics Institute (Cambridge, Massachusetts, USA). It was given as a 2 μg/μL solution and kept at –80 °C. As the control, a buffer from Genetics Institute was used. The BMP-2 and control solutions were diluted with

sterile water to the desired concentration. The collagen carrier was sterile type-I collagen (Helistat®, Colla-Tec Inc. Plainsboro, NJ, USA). The collagen was cut into pieces, filling out the chamber canals in the dry state. The BMP-2 solution was pipetted on the collagen and the pieces were freeze-dried. The OP-1 was a gift from Stryker Biotech (Natick, Massachusetts, USA). It was given as dried sterile collagen granules, with OP-1. Dried sterile collagen granules without OP-1 were given as the control. Prior to implantation, the dried collagen granules were moistened with saline solution, turning them into a formable mass, with a concentration of 0.5 µg OP-1/mm^3. The hydroxyapatite was produced by collecting the femoral and tibial diaphyses from female 200 g Sprague-Dawley rats. The bones were immediately cleaned of periosteum and marrow. They were washed in water, defatted in chloroform methanol 1:1 for 20 minutes, rinsed several times in methanol, air-dried, ground and sieved to a particle size of 0.35–0.65 mm. Thereafter, the washing was repeated twice. The powder was then heated with water to 270 °C at an autogenic pressure of 55 bar for 4 h, whereafter it was washed in water, acetone and ethanol. Prior to use, the powder was air-dried. This removes the organic components of the bone, without altering the mineral (Aspenberg et al. 1996b).

Surgical procedures

The rabbits were anesthetized with an intravenous injection of Hypnorm 0.9 mL/kg (Phentanylum 0.2 mg/mL + Fluanisonum 10 mg/mL) and a local injection of Xylocaine 20 mg/mL, 0.5 mL bilaterally. Bone harvest chambers were implanted bilaterally in all rabbits. It was carried out by exposing the proximal, medial tibial metaphysis of the rabbit, just anterior to the medial collateral ligament. A 6.5 mm cortical window was excised with a hollow drill. The cylinder of the bone harvest chamber was screwed into the bone with a special wrench, so that the ingrowth openings were located at the level of the tibial cortex. After 6 weeks of osseointegration, the contents of the bone harvest chamber were harvested and discarded. The experiments then started and the chambers were harvested every other week, including 2-week periods with active substances, followed by 2 weeks as a rest period, with no treatment.

The rats were anesthetized with peritoneal injections of 0.6–0.7 mL of a solution containing 15 mg pentobarbital and 2.5 mg diazepam per mL. Under aseptic conditions, longitudinal incisions were made bilaterally over the anteromedial aspect of the proximal tibial metaphyses. After incising and raising the periosteum, the medial and posterior- lateral cortices were pierced with a 1.0 mm spike, just anterior to the insertion of the medial collateral ligament. The hole created in the medial cortex was manually enlarged with a 3.2 mm drill. The collagen, with or without BMP-2 was placed in the chamber, which was then screwed into position so that the bone ingrowth holes were placed at the level of the cortical bone, and the pointed end of the implant was engaged through the opposite cortical bone. The wounds were closed in layers. At the end of the experiment, the rats were killed with an overdose of pentobarbital.

All participating rabbits. Letters A, B, C, D refer to the order in which each rabbit participated in the various experiments. Thus, e.g., rabbit no. 2 first participated in the adjacent bone experiment (A), then in the BMP-2 30 ng experiment (B), followed by the hydroxyapatite experiment (C), and finally in the Vertical bone harvest chamber experiment (D)

Rabbit no.	BMP	OP-1	Hydroxy-apatite BMP-2	Vertical BHC BMP-2	Adjacent bone BMP-2
Dose, µg	0.03	2.5	2.4	2.4	0.8 / 4
1					/A
2	B		C	D	/A
3	B		C	D	/A
4					/A
5	A		B	C	
6	B				/A
7	B				/A
8				A	
9			B	C	A
10			B	C	A
11			B	C	A
12					A
13	A	B			
14	A	B			
15	A	B			
16	A	B			
17	A	B			

Experimental design

17 skeletally mature rabbits were used. They were a crossbreed between lop-eared and white native breed. As the chambers were repeatedly harvested, some of the rabbits participated in more than one experiment according to the Table.

I. Low BMP-2 dose (10 rabbits). The rabbits received collagen with 30 ng BMP-2 in one chamber and collagen with the control solution in the contralateral chamber. After harvest, the chambers were left empty and harvested again after 2 weeks.

II. OP-1 in Bone Harvest Chamber (5 rabbits). In one chamber, the rabbits received 2.5 µg OP-1 on collagen granules and in the contralateral chamber they received control collagen granules. The chambers were harvested after 2 weeks.

III. BMP-2 on hydroxyapatite (6 rabbits). Both chambers in each animal were filled with hydroxyap-

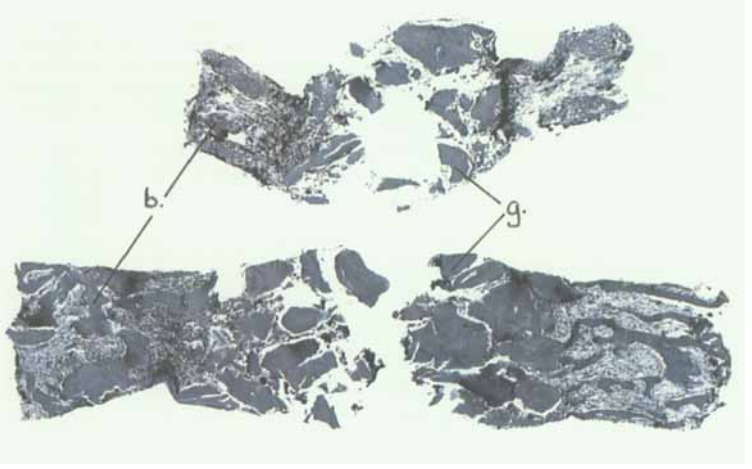

Figure 3. Paired specimens from the Bone Harvest Chambers in a rabbit showing the OP-1 exposed specimen at the top, and contralateral control at the bottom. Centrally in both specimens there are unresorbed collagen carrier granules (g) and at both ends there is ingrowing new bone (b), although much less in the OP-1 specimen.

atite granules. 4 µL of 0.6 µg/µl BMP-2 solution (2.4 µg) was pipetted onto the hydroxyapatite in one chamber and 4 µl of the control solution onto the hydroxyapatite in the contralateral chamber.

IV. Vertical bone harvest chamber with BMP-2 (7 rabbits). The inner core of the bone harvest chamber was changed to a vertical canal core. Each rabbit received 2.4 µg BMP-2 on collagen in both canals in one chamber and control solution on collagen in both canals in the other. After 2 weeks, the vertical canals were harvested and left empty for 2 more weeks.

V. Adjacent bone (10 rabbits). After 2 weeks without implants, the chambers were opened and only the middle third of the bone rod that had formed in the canal was cut out with a specially designed instrument. The defect created was filled with BMP-2 on collagen, either 4 µg (6 rabbits) or 0.8 µg (4 rabbits) in one chamber and control solution on collagen in the contralateral chamber. After 2 more weeks, the full length of the bone rod was harvested.

VI. Rat Bone Conduction Chamber. 10 male Sprague-Dawley rats (320–350 g) were obtained from Möllegaard (Köge, Denmark) and kept in the animal facilities (22 °C). Each animal received bone conduction chambers, implanted bilaterally in the proximal tibiae. Each rat received 2 µg BMP-2 per 25 mm^3 on collagen in one chamber and control solution on collagen in the other. After 6 weeks, the animals were killed and the specimens collected.

Evaluation

The specimens were fixed in 4% buffered formalin, decalcified in Parengy's solution, embedded in paraffin and cut into 5-µm sections, enabling the entire length of the specimen to be visualized on one slide (Figure 3). The sections were stained with hematoxylin and eosin. The decalcifying solution from the experiment with the low BMP-2 dose was analyzed for calcium content, using atomic absorbption. In this experiment, the rabbits were also given 99-technetium methylene diphosphonate (^{99}Tc MDP, approximately 45 MBq) as a bolus intravenous injection 3 hours before harvesting the BHC. After harvesting, the gamma emission of the specimen was measured and corrected for time-dependent decay.

Histology

In the experiment with low BMP-2 doses and in that with BMP-2 on hydroxyapatite, histomorphometry was performed with a microscope connected to a computerized video digital table system at a screen magnification of ×125. The entire specimen and the bone areas were circumscribed with a pen on the digitizing table and the bone area was expressed as the percentage of the total area of the section. All measurements were performed by one person only in each experiment, with the slides numbered at random and the identity tags covered.

In the experiment with the vertical bone harvest chamber in rabbits and the bone conduction chamber in rats, the histomorphometry was performed with a microscope connected to a computerized video digital table system at a screen magnification of × 31. The entire specimen and bone area were circumscribed, and the width measured with a pen on the digitizing table. In order to obtain a mean tissue and bone pene-

tration distance, the area of the total tissue and the area beneath the ingrown bone were divided by the width of the specimen. The adjacent bone specimens were studied with morphology only.

Statistics

Data were analyzed with Student's paired t-test.

Results

I. Low BMP-2 doses

Macroscopically, the specimens usually filled the canals with bone formation at both ends in the experimental and control chambers. A tendency towards inhibition of bone formation and a significant inhibition of total tissue ingrowth were found. The mean total tissue area in the control side was 4.2 mm^2, and in the BMP-2 side it was 3.0 mm^2. The pairwise difference (experiment-control) in total tissue area had a 95% confidence interval from –2.3 mm^2 to –0.07 mm^2 ($p = 0.04$). The mean bone area percentage in the control side was 21 %, and in the BMP-2 side it was 17%. The pairwise difference (experiment-control) in bone area percentage had a 95% confidence interval from –9 to +1 ($p = 0.1$). The mean technetium value in the control side was 18,300 counts per minute (cpm), and in the experimental side 10,200 cpm. The pairwise difference (experiment-control) in the specimens had a 95% confidence interval from –13,300 cpm to –2,900 cpm ($p = 0.007$). The mean calcium content in the control side was 146 µg and in the BMP-2 side it was 84.5 µg. The pairwise difference (experiment-control) in calcium content had a 95% confidence interval from –110 µg to –1.3 µg ($p = 0.05$). The calcium content of the specimens and the measured bone area had a linear correlation, $r^2 = 0.79$ ($p = 0.0001$).

II. OP-1 in bone harvest chamber

Macroscopically, the collagen granules were generally not absorbed, and the specimens were hard to remove from the bone harvest chamber in one block, especially on the OP-1 side (Figure 3). The mean total tissue area of the controls was 4.5 mm^2, and in the OP-1 side it was 2.7 mm^2. The pairwise difference (experiment-control) in total tissue area had a 95% confidence interval from –3.1 mm^2 to –0.4 mm^2 ($p = 0.02$).

The mean bone area percentage in the control sides was 18%, and in the OP-1 sides it was 1.7%. The pairwise difference (experiment-control) in bone area percentage had a 95% confidence interval from –29 to –2.2 ($p = 0.03$).

III. BMP-2 on hydroxyapatite

Macroscopically, the specimens usually filled the canal. An inhibition of both bone and tissue formation was found in the BMP-2 side, compared to the control side.

The mean total tissue area in the control sides was 3.4 mm^2 and in the BMP-2 sides it was 1.9 mm^2. The pairwise difference (experiment-control) in total tissue area had a 95% confidence interval from –2.3 mm^2 to –0.8 mm^2 ($p = 0.003$). The mean bone area percentage in the control sides was 11% and in the BMP-2 sides it was 1.7%. The pairwise difference (experiment-control) in bone area percentage had a 95% confidence interval from –12 to –5.4 ($p = 0.001$).

IV. Vertical bone harvest chamber

No effect of BMP-2 was found concerning the bone ingrowth distances. Macroscopically, the specimens filled the canals with bone formation in the end facing the ingrowth opening at the bottom of the canal. The mean bone distance in the control sides was 0.40 mm and in the BMP-2 sides it was 0.17 mm. The mean pairwise difference (experiment-control) in bone area had a 95% confidence interval from –0.6 mm to +0.1 mm ($p = 0.15$).

V. Adjacent bone

We did not find cyst formation with mature bone at the borders of that part of the specimen that had received BMP-2. A tendency towards cyst formation was found in the specimens from the rabbits that had received 4 µg in the middle third. In 2 of these 6, the chambers were almost empty at harvest, although only one third of the specimen had been resected at the time of implantation. The specimens receiving 0.8 µg usually showed bridging bone in both BMP-2 specimens and controls. Thus no conclusive effect of BMP-2 was found.

VI. Rat bone conduction chamber

In this model, BMP-2 increased bone ingrowth and total tissue ingrowth into the chamber (Figure 4). The mean bone ingrowth distance in the control sides was 1.3 mm and in the BMP-2 sides it was 3.5 mm. The mean pairwise difference (experiment-control) in bone ingrowth distance had a 95% confidence interval from 1.5 mm to 2.8 mm ($p = 0.0001$). The mean total tissue ingrowth in the controls was 2.4 mm and in the BMP-2 sides 3.9 mm. The mean pairwise difference (experiment-control) in total tissue ingrowth had a 95% confidence interval from 0.9 mm to 2.2 mm ($p = 0.0005$).

Figure 4. Paired specimens from the experiment with Bone Conduction Chambers in the rat. Control specimen to the left and BMP-2 exposed to the right. The ingrowth openings are at the base of the specimen. Arrow indicates bone ingrowth border.

Discussion

In previous experiments, we found inhibition of bone formation when BMP-2 was placed in the bone harvest chamber (Jeppsson and Aspenberg 1996). These findings were probably not caused by too high a BMP concentration, because the inhibition was still found when the BMP-2 dose was lowered to less than one hundredth of the dose suggested for clinical use. The consistent finding of inhibition when using different carriers implies that an unfavorable reaction between the BMP-2 and the collagen carrier is not responsible for inhibition. Furthermore, the inhibition is not a specific BMP-2 effect or dependent on the formulation we used, because it was also found when OP-1 was placed in the bone harvest chamber. Moreover, as BMP-2 on hydroxyapatite has been shown to enhance bone formation in the rat chamber model (Aspenberg et al. 1996a), it is unlikely that the carrier is responsible for the negative effect on bone formation in the bone harvest chamber in rabbits.

BMPs have pleiotropic effects based on concentration-dependent thresholds (Reddi 1994). We tried to mimic a situation with a concentration gradient of BMP-2, by modifying the bone harvest chamber so that it allowed diffusion of the BMP-2 out of the chamber, but then no BMP-2 effect was found. Nor did we find evidence of premature differentiation and cyst formation when studying the preexisting bone adjacent to the BMP-2 implant (Sciadini et al. 1995). When implanting BMP-2 on collagen in the rat chamber, the stimulatory effect on bone formation was obvious. The only difference compared to the bone harvest chamber model, except species, was that the implantation of the BMP-2 was done at the same time as the rather traumatic insertion of the chamber into the bone.

The present study was planned to elucidate the effects of BMP dose, type, carrier and some implant site characteristics. At the same time, we started a study of BMP-2 effects in a chamber subjected to micromotion. The latter study showed that repeated tissue deformation together with BMP-2 in the chamber leads to bone formation, whereas deformation alone, or BMP-2 alone, were inhibitory (Boström et al. 1998). The seemingly contradictory findings of the present study can be explained against this background. The rat bone conduction chamber is screwed into the bone when the experiments starts. This insertion trauma could correspond to the effects of mechanical perturbation in the micromotion study. Apart from the bone harvest chamber model, all implantations of a BMP with carrier entail considerable trauma.

We suggest that some effect of trauma or mechanical stimulus is necessary for BMPs to induce bone in such experiments. Furthermore, BMPs can induce apoptosis during embryonic development (Zou and Niswander 1996). One might speculate that, in the absence of major tissue deformation or trauma, BMPs may cause programmed cell death in adult animals as well. This would explain why tissue and bone formation were inhibited in the bone harvest chamber, where BMP implantation induces only minimal tissue trauma. It may also explain the findings in the experiment concerning the adjacent bone. In these cases, inhibition by BMP-2 was not reproducible, although it was sometimes present. These implantations were difficult to perform and therefore probably more traumatic.

The need for trauma in bone induction may also be related to the difficulties in inducing bone by intramuscular BMP injections. It would be interesting to try to replace physical trauma by biochemical factors known to be produced in tissue exposed to trauma. The combination of a BMP and such a factor could be used for an injection in fractures that fail to unite, without the need to produce mechanical tissue trauma.

Apart from the absence of major trauma, there may be yet another prerequisite for BMPs to inhibit healing. In the limb bud, BMP-2 and OP-1 cause apoptosis of undifferentiated mesenchymal cells. However,

once cells have condensed and started to attain a cartilaginous phenotype, the BMPs instead cause proliferation (Marcias et al. 1997). In the bone harvest chamber, healing also starts with only undifferentiated mesenchymal cells, which may similarly undergo apoptosis in response to BMPs. In contrast, other types of skeletal defects contain a wider variety of cells at different differentiation stages and there a proliferative response ensues.

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