A culture system for the live analysis of successive developmental processes and the morphological control of mammalian vertebral cartilage

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Abstract The mesoderm-derived segmental somite differentiates into dermomyotome and sclerotome, the latter of which undergoes vertebrogenesis to spinal cartilage and ultimately to vertebral bones. However, analysis and manipulation of the developing mammalian vertebrae in the same embryo has been infeasible because of their placental-dependent embryogenesis. Here, we report a novel culture system of the mouse embryonic tailbud, by which the developmental processes of mammalian vertebral cartilage are traceable and manipulatable in the same sample. The anaplastic segmental somites/sclerotomes in the tailbud of 13 gestational day (g.d.) embryo that are structurally continuous to the vertebral column underwent progressive vertebrogenesis when (1) the ectoderm-derived nascent epidermis was microsurgically removed prior to cultivation, and (2) the sample was incubated at the air-medium interface. After cultivation for 5 days, the size and shape of the instructed vertebral cartilage showed features comparable to well-differentiated body vertebra along with the expression of the cartilage marker collagen type II, suggesting that aggressive differentiation of the sclerotomal cell lineage was

achieved. In the presence of recombinant bone morphogenic protein (BMP) and Noggin, or adenoviral particles for extracellular epimorphin, dramatic alteration of the vertebral morphology ensued in the explants. Thus, this model system provides an approach to study the detailed molecular mechanisms of mammalian vertebrogenesis and enables pretreatment strategies of precartilaginous fragments for improving the efficacy of subsequent transplantation.

Keywords Vertebrogenesis · Live analysis · Cartilage · Epimorphin · Organ culture · Morphogenesis

Introduction

In vertebrate animals, spinal bones play vital roles as the physical anchorage and as the platform that guards bone marrow and the spinal cord (Lippert 1966; Maimoun et al. 2006), for this reason elucidation of the molecular mechanisms involved in mammalian vertebrogenesis has not only fascinated the scientific community but has implications for regenerative medicine. The mammalian vertebrogenesis is roughly divided into two processes: initiation of cartilage formation via differentiation/morphogenesis of somite/sclerotome and the subsequent endochondral ossification (Balling et al. 1992). These two steps have been thought to be governed by a number of signaling molecules that are spatiotemporally

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transmitted via complex cell–cell communications. The signaling molecules for vertebrogenesis include diffusible factors such as BMP, Noggin, sonic hedgehog (Shh) and Wnt, and cell surface signaling inducers including epimorphin, Ephrin, Eph and Delta, which activate distinct downstream transcription factors (Christ et al. 2000; Compagni et al. 2003; Marcelle et al. 1997). Since the extent of vertebral morphology is determined before the endochondral ossifications, and that non-ossified cartilage alone plays a mechanically-important role in the mature vertebral bone, many investigations have focused on the process of the differentiation and morphogenesis of somite/sclerotome. However, since mammalian embryogenesis occurs in the maternal uterus, gene manipulation to these embryonic tissues and time-course analyses are practically impossible. The avian *in ovo* implantation model (microsurgical transplantation of genetically manipulated-cells to an embryo developing inside the eggshell) overcomes part of this difficulty (Oka et al. 2006), however, biological relevance of this model to mammals is obscure and the pattern of time-dependent changes in the same sample remains a formidable challenge.

Implantation of chondrocytes has long been anticipated as a therapy for cartilage defects including chondrosis and arthropathy (Luhmann et al. 2007; Farr 2008). However, the use of cells pre-suspended in a physiological solution has been met with numerable difficulties with regard to their engraftment efficiency, and alternative implantation methods are critically needed (Fuchs et al. 2002). Therefore, we propose here that for effective filling of the defected part of the bone system, the size and shape of the cell masses comprised of chondrocytes or precartilaginous cells should be twisted to fit and engraft with the residual tissues. Artificial stimuli with spatial and temporal signaling molecules for cartilage morphogenesis, e.g., BMP/Noggin, Shh and/or epimorphin, may be employed for this purpose.

The tail bones of caudate mammals including mice are structurally continuous and equivalent to the vertebral column. During embryogenesis, bone units in the tailbuds are differentiated from segmental sclerotome, which are anaplastic but analogous to the vertebral column at the earlier differentiation state (Shinohara 1999a). The embryonic tailbud is relatively easy to handle and analyze since it is physically separated and developmentally independent from

other organs or bone structures. In the present study, we demonstrate a novel culture method that utilizes the mouse embryonic tailbud, by which time-course analyses and control of mammalian cartilage development are both possible.

Materials and methods

Antibodies and reagents

The primary antibodies used for immunohistochemistry or western blotting include those against collagen type II (Collagen Research Center), smooth muscle actin (Sigma–Aldrich) and epimorphin ((Hirai 1994) and R & D laboratories). FITC-, Cy3- and HRP- labeled secondary antibodies were from Sigma–Aldrich. Recombinant form of human BMP-4 and mouse Noggin were from R & D laboratories and were used in cell culture at a final concentration of 2 and 10 µg/ml, respectively. Alcian blue, alizalin red S, propidium iodide and 4', 6-diamidino-2-phenylindole (DAPI) were from Sigma–Aldrich.

Organ culture of embryonic tailbud

All experiments using mice were conducted in conformity with the policies and procedures of the Institutional Animal Care and Use Committee of Kwansei Gakuin University. The tailbuds of 13 g.d. embryos from ICR mice (the day when the vaginal plug is found is counted as day 0) were isolated and the ectoderm-derived epidermal cell layer was microsurgically removed. The tailbuds were then placed on the transparent porous membrane (Nuclepore, diameter 13 mm, pore size: 8 µm) floating on DMEM/HamF12 medium supplemented with 10% FCS as described previously (Hirai et al. 1989a, 1992). The explants were photographed daily followed by wholemount skeletal staining or immunohistochemistry, or homogenized in lysis buffer for western blotting.

Wholemount skeletal staining

The mouse embryos and the tailbud explants from various culture periods were treated with 100% ethanol for 12 h and acetone for 1 day. Subsequently,

the samples were incubated with staining solution containing 0.015% alcian blue and 0.5% acetic acid in ethanol overnight. For some samples 0.015% alizalin red S was added to the staining solution. After dehydration with 100% ethanol the tissues were incubated in solution containing 2% potassium hydroxide and 20% glycerol for 3 days. Thus prepared specimens were kept in 20% glycerol.

Immunohistochemistry and western blotting

Immunohistochemistry and western blotting were carried out as described previously (Hirai et al. 1998). In brief, cryosections (10 μ m thick) or PVDF membranes onto which protein bands have been electrically blotted were incubated with the primary antibodies followed by the secondary antibodies. The nuclei were counter-stained with 10 ng/ml of propidium iodide or DAPI in sections, and β -actin was detected on the blots as the loading control.

Quantitative analyses

The development of cartilage was quantified as follows. The body vertebra or the tailbuds cultured for 0, 2 and 5 days were stained with alcian blue and photographed. Four sclerotome-derived cylindrical vertebral units or alcian blue-positive units at the exact central portion of the explants were carefully determined, and the width and height of each unit along with the distance between the adjacent units were measured. Quantitative analyses were performed at least three times independently and the values are expressed as mean $\pm$ S.D. Data were analyzed using Mann–Whitney *U*-test, and a *P*-value of < 0.05 was considered statistically significant.

Adenovirus infection

Some explants were infected with adenoviral particles carrying extracellular epimorphin. The ectoderm free-tailbuds from 13 g.d. embryo were pre-incubated at 37 °C in a medium droplet containing 10^8 pfu/ml of adenovirus that has been introduced with full-length epimorphin containing a N-terminal fusion of an exogenous signal peptide (Hirai et al. 2007). After 12 h infection, the explants were applied to the organ culture.

Results

Morphological characterization of anaplastic vertebra in the tailbuds

The tailbud of the 13 g.d. mouse embryo has a string of somites/sclerotomes of several developmental stages, which are structurally analogous and continuous to the body vertebral bones (Fig. 1A left). The segmental somites align from differentiating sclerotomes to immature presomitic mesodermal tissues along the rostral-caudal axis. The alcian blue staining demonstrated that the most caudal part of body sclerotomes already shapes as cartilaginous cylinders at this time-point, whereas sclerotomes/somites from the most rostral to central part of tailbud were also alcian blue-stained but they did not exhibit the cylindrical shape (Fig. 1A right and B left upper). These alcian blue-positive cells roughly overlapped with collagen II-positive areas in the tailbud (Fig. 1A right). The smooth muscle actin-positive dermomyotomes surround the sclerotome, and epimorphin, an important activator for initial developmental stage of cartilage formation (Oka et al. 2006), was detectable between these somite-derived compartments (Fig. 1A right). Quantitative analysis of the body and tailbud specimens revealed that the alcian blue-positive units in the immature tailbud are widely separated displaying a more flattened morphology in comparison to those in matured body and well-developed tail of 18 g.d. embryo (Fig. 1B).

Organ culture of the mouse tailbud

The trunk of the intact tailbud is approximately 500 μ m in diameter and is incapable of survival when incubated in the bottom of a tissue culture plate bathed in medium probably because of oxygen or nutrient deficiency (Fig. 2A upper). When the tailbud was placed on a porous membrane made of polycarbonate and lifted to air-medium interface, the tailbud somites/sclerotomes that were wrapped by ectoderm-derived epidermis did not grow or differentiate, and eventually underwent apoptotic cell death. However, those localized near the cutting surface activated morphogenesis and a differentiation program exhibiting appreciable vertebral bone-like morphology (Fig. 2A middle). We found that microsurgical removal of the epidermal sheet from the entire surface of the tailbud

Fig. 1 The sclerotomes in the tailbud as undifferentiated vertebral cartilage of the body. **A** alcian-blue/alizarin red stained specimens of a 13 g.d. (left) and 18 g.d. (middle) mouse embryo. *Right panels*, alcian-blue stained 13 g.d. tailbud (**a**) and the immunohistological distribution of collagen type II (**b**), epimorphin (**c**) and smooth muscle actin (**d**) in the cryosection of the tailbud (green). The nuclei were counterstained with propidium iodide (PI) and the transverse sections were used for epimorphin (**c**) and smooth muscle actin (**d**). **B** Quantitative analysis of the size and shape of the cartilage. Alcian blue-stained vertebral units in the body (left lower) or tailbud (left upper) were photographed and measured for the width (**a**) and height (**b**) of each unit of four cartilages in the central part, as well as the distance between each unit (**c**). The tailbud cartilage exhibits a relatively spatulately shape- (small **b** value) and are independently positioned (large **c** value). The value of **b/c** in the 13 g.d. tailbud is statistically different from that in the body or matured tail of 18 g.d. embryo. Six specimens from three independent experiments were analyzed for each category. Data means $\pm$ SD. * $P < 0.05$

allows all of the somites/sclerotomes to form well-developed palpable cartilage strings within 4–5 days, and they keep growing for at least 10 days (Fig. 2A lower, B). Quantitative analysis of the explants and the alcian blue-stained cartilage frameworks demonstrated that the developed cartilage units resemble those of the body and the 18 g.d. embryonic tail in morphology, suggesting that tailbud sclerotomes achieve a normal developmental process in this culture system (Figs. 2C, 3A, B). Chondrocyte differentiation in the explants was further confirmed by the increased expression of collagen type II during

culture (Fig. 3A, C). Since explants are cultured on top of transparent membranes this enabled us to analyze time-dependent changes in cartilage morphology in each sample (Fig. 2B), while interrogating the molecular mechanisms underlying formation of the vertebrate cartilages with either genetic manipulation or chemical inhibitors. Interestingly, the cultured tailbuds were found to be occupied mostly by well-differentiated cartilage strings, whereas the smooth muscle actin-positive myotome and the surrounding mesenchymal cells (which were present at the beginning of culture) became undetectable by 5 day of explant culture (Figs. 1A right, 3A right upper). During tail organogenesis *in vivo*, these cells differentiate and form well-developed muscle and dermal tissues to surround the cartilage strings (Fig. 3B).

Exogenous addition of noggin, BMP-4 and epimorphin alters cartilage morphogenesis and functional differentiation

Shh, Wnt and BMP proteins are well known as central paracrine players in somite differentiation as well as subsequent sclerotome-derived cartilage formation (Sanchez-Camacho and Bovolenta 2009; Otto and Rao 2004). Among their signals, those activated by BMP and the biologically-inherent antagonist Noggin have been supposed to be crucial for the later stage of the cartilage formation and the bone regeneration process (Rosen 2006; Monsoro-Burq et al. 1996). The addition of BMP-4 triggered hypertrophy of the explanted rostral cartilage (Fig. 4A, B) along with increased expression of collagen type II (Fig. 4C). In some explants an unusual hypertrophy also ensued in the surrounding non-cartilage cells that prevented elongation of the cartilage string (Fig. 4A). In contrast, in the presence of Noggin chondrocyte differentiation was severely depressed and the shape and size of the cartilage units were significantly altered, albeit the string maintained its beaded structure (Fig. 4A, B). In addition, we found that the forced expression of the extracellular form of epimorphin in the explants elicited a distinct morphogenic response in the cartilage: each unit exhibited a large cartilage cluster and the junctions between each unit were constricted, which may account for the previously detected phenotypes with the avian *in ovo* model, that is, the ectopic expression of extracellular epimorphin promoted sorting/aggregation of precartilaginous cells

Fig. 2 Establishment of the culture system for the mouse tailbud. **A** appearance of 13 g.d-embryonic tailbuds cultured for 2 (left) and 5 (right) days. The tailbud did not develop in the bottom of culture medium (upper). When the culture assembly was lifted to the air-medium interface, sclerotomes in the central tailbud activated programmed cell death ultimately forming a hollow tube structure, while those near the cutting surface grew outwards and underwent vertebrogenesis (insets of middle column). The complete removal of ectoderm-derived nascent epidermis resulted in successful vertebrogenesis of all the segmental somites/sclerotomes in the explant (lower). Bar, 300 μm. **B** The time course of development (from day 0 to day

9) of an epidermis-free tailbud cultured at the air-medium interface. The time-dependent morphological changes in the same sample were followable merely by microscopic observation. Bar, 300 μm. **C** Quantification of the tailbud sclerotome/cartilage in culture. The width (a) and the height (b) of the central cartilage units, and the distance between them (c) were measured. By day 5, they begin to resemble the mature vertebral cartilage of the body and of the 18 g.d. embryonic tail. Four (day 2) and six (day 5) specimens from three independent experiments were analyzed for each category. Data means ± SD. * $P < 0.05$ (vs. day 0)

and caused the structurally abnormal vertebral column (Oka et al. 2006) (Fig. 4D). These results suggest that artificial control of these signaling pathways for altering the morphology and differentiation of cartilage can be performed using this culture system.

Discussion

In the present study we showed a novel culture system for the mammalian tailbud, where developmental steps in the formation of vertebral cartilage

can be followed. The embryonic tailbud is comprised of several cell lineages including ectoderm derived-anaplastic epidermis and mesoderm derived-somite (Hirai et al. 1989b). At 13 g.d. embryos, somites from the rostral to the central part of the tailbud have already differentiated into sclerotome and dermomyotome, and sclerotome-derived precartilage elongates to enclose the neural tube-derived spinal cord. In contrast, those on the caudal side just begin to differentiate into sclerotome and dermomyotome. In our culture system, all of the cells derived from somite sclerotome grow and occupy most of the explant and form mature vertebrate cartilage columns within 4–5 days.

Fig. 3 Analysis of the tailbud cultured for 5 days. **A** *left upper*, The typical specimen stained with alcian blue. *Lower left*, the immunohistological distribution of collagen type II in the longitudinal section of the explant. The expression of smooth muscle actin (*right upper*) and collagen type II (*right lower*) in the transverse sections demonstrated that the explant was mostly occupied by mature cartilage and the dermomyotome-derived cells disappeared during culture. Bars, 300 μ m. **B** *upper left*, wholemount-staining of the tail of 18 g.d. embryo. The transverse sections were stained for smooth muscle actin (*upper right*) and collagen type II (*lower*). Bars, 500 μ m. Note that the morphology and the size of the collagen II-positive cartilage developed in culture (**A**) were similar to those developed in vivo (**B**), however, the difference was apparent in the number of cells or tissues surrounding the cartilages. **C** The increased expression of the cartilage marker collagen type II during culture. The explants were collected daily and analyzed by western blotting. Three independent experiments were performed and all of them showed similar expression patterns (collagen II was always separated into two protein bands of monomeric and dimeric/modified forms of α 1 chain). Representative images are shown. β -actin was tested for a loading control

The most important criteria for successful development of the cartilage in culture was removal of the nascent epidermis prior to incubation. Within the intact tailbud, the surrounding epidermis elicits an apoptotic response in sclerotomal cells and the tissue eventually develops hollow epidermal tubes containing cell debris, implying that ectoderm may secrete factors that severely hamper vertebrogenesis in culture. Such candidates may include Noggin and chordin, intrinsic antagonists of BMP, or Wnt, which have been shown to be secreted from ectoderm and regulate the direction of somite differentiation and its derived tissues (Fisher and Halpern 1999; Ikeya and Takada 2001). Indeed, this study revealed that addition of a recombinant form of Noggin dramatically inhibited cartilage development in epidermis-free explants, however, Noggin did not induce apoptotic cell death as seen in culture of the intact tailbud. In addition, the sclerotomes near the cutting surface of the explants underwent cartilage differentiation even without the removal of the epidermis. We also noticed that the epidermal layer partly loses the inhibitory effect when it was cut in a lengthwise direction and not completely separated from the explant (data not shown). An alternative possibility for the harmful effect of epidermis on vertebrogenesis in our culture model is that the nascent epidermal tissue develops during culture and seals an outer surface on the sclerotome, which obstructs expansion of the space for cartilage

Fig. 4 The effect of exogenous stimuli with signaling factors for vertebrogenesis. **A** Alcian blue-stained tailbuds cultured in the absence (*left*) or presence of 2 $\mu\text{g}/\text{ml}$ BMP-4 (*middle*) or 10 $\mu\text{g}/\text{ml}$ Noggin (*right*) for 5 days. In the BMP-treated explants, outgrowth and jerky movements of non-cartilage cells were often observed (arrow). Bar, 300 μm . **B** Quantitative analyses of the tailbud sclerotome/cartilage cultured with BMP-4 or Noggin. BMP-4 and Noggin led to cartilage hypertrophy and developmental defects, respectively. Three specimens from three independent experiments were analyzed for each category. The width (*a*) and height (*b*) of central

cartilage units, and the distance between them (*c*) were measured at day 5. Data means $\pm$ SD. **C** Expression of collagen type II in the explants cultured with or without these stimuli for 5 days. The major two protein bands correspond to monomeric and dimeric/modified forms of collagen $\alpha 1$ chain. **D** The forced expression of the extracellular form of epimorphin with adenoviral particles for signal peptide-connected epimorphin (sig-EPM adeno) led to the overexpression of intact (34 kD) and sugar-modified form (37 kD) of epimorphin (*left*), concomitantly with the enlarged cartilage units and the constricted seams (*right*). Bar, 300 μm

morphogenesis. This would be likely due to blockage of nutrient inflow from the medium. Given that embryonic epidermis from this stage of development normally differentiates upwards in a rapid fashion rather than laterally, it therefore gives rise to a compact stratified epidermis containing a tight junction exhibiting granular layer and a highly keratinized horny layer (Hirai et al. 1989b; Troy et al. 2007).

While removal of the epidermis no doubt provides the requisite cues for cartilage development, this, in turn hampers the growth and differentiation of dermomyotome into muscle and dermal tissues. Smooth muscle actin-positive cells that are abundantly present at the beginning of the culture become scarcely apparent in only a thin layer of perichondrium which has been shown to possess potential to differentiate into chondrocytes (Kronenberg 2007). Presumably, cells originated from dermomyotome cannot survive without factors from epidermis. On the other hand, in the presence of BMP, some types of cartilage that form in culture become surrounded by thick layers of cells often containing subpopulations that spontaneously expand and contract as smooth muscle cells. Although all of the somite-derived components are known to interact with each other and these interactions are thought to be important for their successful differentiation and structural connection in vivo (Brand-Saberi and Christ 2000), our culture system revealed that a certain number of these tissue interactions may be dispensable, at least for the onset of aggressive cartilage formation. However, more analyses would be necessary to further elucidate the physiological relevance of interactions between sclerotome and ectoderm/myodermatome for the tailbud organogenesis.

Vertebrogenesis of the tailbud in the mouse shows some differences to that in the cervical, thoracic and lumbar vertebra (Shinohara 1999a, b). Moreover, human beings have evolutionarily lost most of what is considered tail, and the remaining portions of this vertebral region are fused to each other to form the coccygeal vertebrae that are thought to be functionally redundant. Nevertheless, this culture system opens a way to study molecular mechanisms underlying sclerotomal differentiation and the formation of vertebral cartilage, provided that the tailbone demonstrates mechanical, morphological and developmental similarities with spinal vertebrae in human.

As a consequence of these detailed analyses of vertebrogenesis, this culture system may also depict

experimental strategies to regulate the condition of cartilage fragments or chondrocyte cell masses for their implantation. For example, local and temporal gene expression followed by application of the explant culture system may generate a cartilage fragment with the appropriate shape and morphology. These approaches are now under investigation.

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