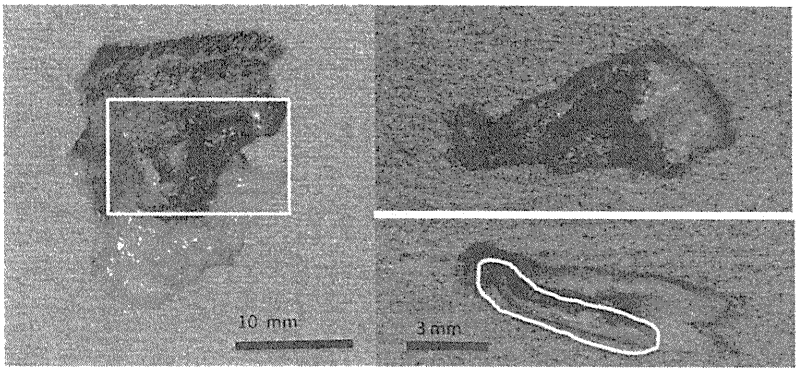
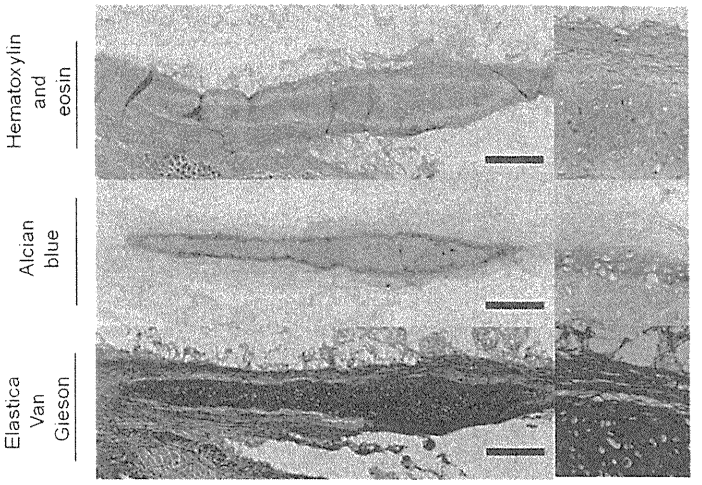


(図 4)イヌ背部より回収した組織の組織学的評価



Scale bar: 100 μm

D. 考察

再生医学の概念は 1990 年代より広まり，その概念は定着して久しいと言える．再生医学は，少ない侵襲で組織・細胞を採取し，増殖させ，相対的に高い治療効果を得る，というところに目標を置いて研究・検討が繰り返されている．その際に，増殖効率の高い細胞群として，各種幹細胞やそれに準じた細胞群が用いられることが多い．しかし，そういった細胞群を使用するにあたっては，高い倫理的配慮や計画性が必要となるため，厚生労働省から「ヒト幹細胞を用いる臨床研究に関する指針」(平成 18 年 7 月 3 日告示第 425 号，平成 22 年 11 月 1 日改正第 380 号)が告示されており，それらを臨床応用するにあたってはこの指針に準ずるものでなければならない．そのため，高い安全性を確保できる環境整備とプロトコールがなければ幹細胞やそれに準じた細胞群を臨床応用することはできない．

本学付属病院は病院内に CPC を建設・設置した．当院 CPC は同区画内に作業スペースを 2 ヵ所設計しており，それぞれ CP1 と CP2 としている．そして，CP1 にはクラス 100 の空気清浄度を保持できるアイソレーターを設置している．当アイソレーターは空気

清浄度を高く保つ機能の他に，アイソレーター内を過酸化水素で燻蒸，殺菌することが可能であり，高い衛生度と滅菌性に優れている．しかも，検体採取する手術室は同一施設内であり，とくに中央手術室は同一の階層にあるため採取された検体の滅菌性が高いまま処理することが可能であると期待している．

また臨床応用に際して，取扱う細胞自体やその生成物を最終産物として体内に注入・移植することになるが，その最終産物自体の安全性も高く保たれなければならない．そのためにはGMPに準拠した基準のもと全ての行程を管理しなければならない．つまり，製造管理として培養行程，品質管理として原料や資材から製品の保管，試験行程などの管理を行い，それを明記するプロトコールと手順書の作成しなければならない．

そのため，安全性の高いプロトコールを作成し，その妥当性を証明することが現時点で必要と考えている．そこで，現在われわれは培地に添加するサイトカインやサプリメントを医薬品に置換することで安全性を高めようとしている．また，ヒト以外の動物における検証法の確立し，プロトコールの妥当性の評価を行うことを計画している．

われわれの従来の方法では軟骨分化誘導にresearch useのサイトカインや添加物を使用している．具体的にはアスコルビン酸やbFGF(詳細は研究方法4．参照)を用いている．それらの製品はある程度の滅菌性は得られているものの，GMPに準拠した製造法で製造していないものや，準拠していてもその保証ができないものが多い．そこで，通常われわれが診療で使用している医薬品はGMPに準拠した方法で製造されており，滅菌性が高い．しかし，その一方で溶媒やpH調製剤が含まれているため，単純に置換することは難しいと考えている．現時点では医薬品を用いた培地においても，軟骨分化を誘導することは可能であり，重症免疫不全マウスへの移植において組織再構築能も確認した．しかし，その一方で分化誘導がかかる期間が従来法と比較し長期であることが問題と考えている．医薬品に置換していないサイトカイン，サプリメントとしては，insuline like growth factorなどである．今後はインスリンなどの添加を試みるとともに，それらの適正濃度の評価が必要であろう．

また，重症免疫不全マウスへの皮下移植実験で得られた組織も更なる評価が必要だと考えている．結果で示したとおり，それぞれ軟骨組織の再構築は認めるものの，顆粒状であったり，軟骨膜様組織が菲薄であったりする．おそらくこれは軟骨膜細胞の影響と予想している．軟骨膜細胞が軟骨膜を再構築する機能に個体によるバラつきや年齢による変化があるかもしれない．再構築される組織は治療効果に直結するため，今後も検討を要する．

今回，大動物実験においても組織の再構築を確認することができた．今ま

では，組織再構築能は重症免疫不全マウスにおいて評価していた．しかし，免疫機能が再構築される組織の性状や大きさを左右することは容易に想像される．実際にヒトへの臨床応用する際にも，免疫が存在する環境への移植，または投与となる．そのため，免疫が及ぼす影響は評価しなければならない．今回の結果により，イヌにおいて自家耳介弾性軟骨由来の軟骨膜細胞を移植することで均一な弾性軟骨の再構築が得られることが確認された．しかし,今回は従来重症免疫不全マウスへ移植している細胞量と同等かそれよりも多い量をイヌ背部皮下へ投与しているにもかかわらず，再構築された組織は小さいものであった．その原因としては，免疫の有無が関与している可能性は否定できない．しかし，その一方でイヌ耳介軟骨・軟骨膜細胞の特性解析も不十分であることが判明した．具体的には，軟骨分化を誘導しても培養上清の粘稠性が変化しないことなどが今回わかった．原因としては，動物種が異なるため，添加したサイトカインやサプリメントが適正に働いていない可能性を予想している．

今後は移植細胞量と再構築される軟骨組織量の相関関係や新規足場材料の検討も必要と考えている．しかし，現段階ではイヌにおける大動物実験では定量的な変化まで検証するには不確実であると考える．そのため，大動物実験に限らず，様々な手法を組み合わせ総合的に判断していくしかないと考えている．

尚，現在当院でプロトコールの作成に着手している計画は当科のものしかない．しかし，その一方で様々な計画を同時に運用することも可能であり，その場合は複数の計画とプロトコールが干渉してはならない．そのため，当院では再生細胞治療センター運営委員会(以下 CPC 運年委員会)が存在し，全職員を対象とし定期的に講習会を施行している．その場で相互の計画を周知し，プロトコールが干渉することのリスクを低減するように努めている．そのため，CPC 運営委員会主催の講習会において当科も発表を複数回行っている．具体的には，CPC 運営に関する事項を運営委員会側から院内に周知すると同時に当科のプロトコールを提示し周知している．(別添資料①参照)

E. 結論

われわれは，本学付属病院内に建設・設立された再生細胞治療センターにおいて，軟骨・軟骨膜由来細胞の培養技術を臨床応用することを目指している．それにあたり，GMP に準拠したプロトコールの作成と厚生労働省への申請，プロトコールの妥当性を示すための基礎実験を施行中である．現在，安全性を高めるために医薬品を用いた培地の検証を行っている．また，そういったプロトコールの評価方法を確立するため，イヌを用いた大動物実験を行っている．今後は培養期間の短縮を図るとともに，大動物において

も，より安定的に評価できる方法を検証していきたい．

F. 健康危険情報

総括研究報告書に記載の通り．

G. 研究発表

なし

H. 知的財産権の出願・登録状況

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

当院再生細胞治療センターを利用した本学GMP/TRの拠点創設

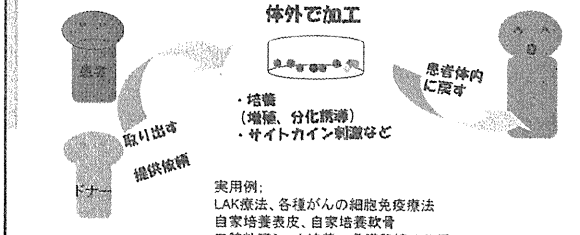
本プロジェクトの目的

(1)先端医科学研究センターから生まれた研究成果を元に、
横浜市立大学附属病院にて患者を対象とした臨床研究を行うこと、
すなわち、***本格的なTR(translational research)***の実践のために
必要な再生細胞治療センターの基盤をさらに強化し、TR拠点を確立すること

(2)再生医療や細胞治療に関わる臨床研究の実施にあたり必須となる、
当院再生細胞治療センター(以下、CPC)を
GMP(Good Manufacturing Product)準拠レベルで
運営するために必要な情報を集積し、
本学関係者に提供し、還元するシステムを構築すること

再生医療と細胞治療

1)今までにない、新しい概念の治療
2)TR(translational research) 実践の場



実用例:
LAK療法、各種がんの細胞免疫療法
自家培養表皮、自家培養軟骨
口腔粘膜シート培養ー角膜移植の代用
骨髄間葉系細胞の培養ー　骨欠損部位への埋め込み

『ヒト幹細胞を用いる臨床研究に関わる指針』に該当する対象疾患

対象疾患は下記要件に適合するものに限定されている

**(1)重篤で生命を脅かす疾患、身体の機能を著しく損なう疾患
又は一定程度身体 の機能若しくは形態を損なうことにより
QOL(生活の質)を著しく損なう疾患であること**

**(2) ヒト幹細胞臨床研究による治療の効果が、
現在可能な他の治療と比較して優れていると
予測されるものであること。**

(3) 被験者にとってヒト幹細胞臨床研究の治療により得られる利益が、不利益を上回ると十分予測されるものであること。

ー 大学病院でなければ、実践出来ない医療

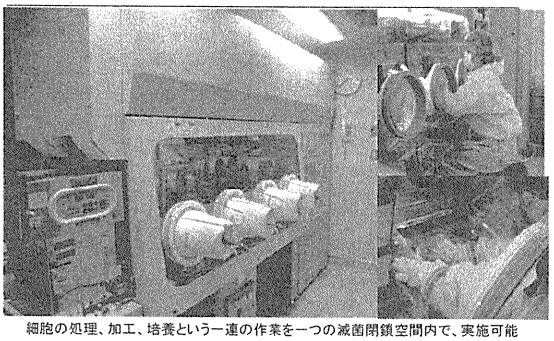
細胞治療・再生医療を『患者診療』として健全に行うためには

- 専用設備と手順の規定
- 教育訓練をうけた人員
- 細胞の取り違え、交叉汚染の徹底防止
- 感染症伝搬の防止
- 全行程のモニタリング、経過観察

→ GMP (Good Manufacturing Product)準拠

病院内に 製薬会社の薬品製造工場を造るような
高いレベルの技術&ノウハウが要求される。
＋各種指針、ガイドラインの厳しい規制

附属病院4階:
再生細胞治療センター内:細胞取り扱い作業エリア
アイソレーターシステム(第二世代)の導入



細胞の処理、加工、培養という一連の作業を一つの滅菌閉鎖空間内で、実施可能

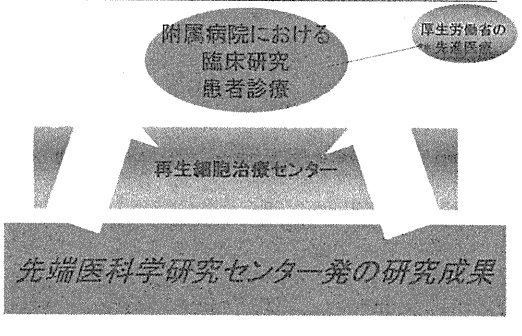
神奈川県内公的病院唯一のセルプロセッシングセンターとしての取り組み

(1) 本研究成果で得られた全てのノウハウを本学の「知的財産」として、運用するための基盤を整備する

(2) 本学関係者の再生医療、細胞治療等の実施希望者に対して、実地での教育訓練の場を提供する
(例:アイソレーターシステムの使用方法、臨床研究の実施に関わる院内システム)

(3) CPC運営、再生医療に関する情報や各種ガイドライン・通達を集約し、情報を提供する

トランスレーショナルリサーチ拠点の創設



Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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発表者氏名	論文タイトル名	書籍名	出版社名	ページ	出版年
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Ⅳ．研究成果の刊行物・別刷

A Novel Craniofacial Osteogenesis Distraction System Enabling Control of Distraction Distance and Vector for the Treatment of Syndromic Craniosynostosis

Shinji Kobayashi, MD, Takeshi Nishiouri, MD,* Jiro Maegawa, MD,† Takashi Hirakawa, DDS,‡ and Toshihiko Fukawa, DDS§*

Background: Distraction osteogenesis is now an important clinical tool in craniofacial surgery. However, controlling the distance and vector of distraction in infants with syndromic craniosynostosis with good repeatability is a task that still proves difficult today. We have developed a new facial osteogenesis distraction system that combines the advantages of external and internal distraction devices to enable control of both the distraction distance and vector. This article describes the method and short-term results of this system.

Methods: Our distraction system uses both a conventional external distraction device and a newly developed internal distraction device. Postoperative control of the distraction vector is performed using the external device, whereas control of distraction distance is done with the adjustable-angle internal device. This system was used for 2 patients with Crouzon syndrome.

Results: The system enabled control of lengthening distance and vector, and no complications occurred during the procedures.

Conclusions: We developed a facial distraction system leveraging the advantages of external and internal distraction devices, which we then used to successfully control both lengthening distance and vector. The system would be particularly indicated in patients with severe scarring due to multiple follow-up surgeries and in patients requiring distraction of 20 mm or more.

Aligning the periorbital profile at 5 to 6 years old caused the maxilla to rotate counterclockwise, and we consider that a procedure combining Le Fort III osteotomy with Le Fort I and II osteotomies is required to prevent these rotations.

Key Words: Distraction osteogenesis, Le Fort III osteotomy, syndromic craniosynostosis, Crouzon syndrome

(*J Craniofac Surg* 2012;23: 422–425)

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The authors report no conflicts of interest.

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Distraction osteogenesis is now an important clinical tool in craniofacial surgery since having first been used to lengthen the human mandible.[1] Distraction techniques have been applied extensively to all components of the craniofacial skeleton, including syndromic craniosynostosis. Different authors have successfully used a variety of external and internal distraction systems to advance the midface.[2–6]

However, controlling the distance and vector of distraction in infants with syndromic craniosynostosis with good repeatability still proves difficult.[7] This is mainly because in infants, significant overcorrection is usually required, and the inferior orbital rim (orbitale) and point A distraction vectors are different. External and internal distraction devices are currently used to address this problem. On one hand, external distraction devices generally allow control and adjustment of horizontal and vertical movements of the midface but cannot deliver adequate distraction forces for lengthening, so that the distraction distance is limited, and sufficient with good repeatability facial overcorrection proves difficult.[7,8] On the other hand, internal distraction devices deliver a strong extrusion force but do not allow control of the postfixation distraction vector.[9] To overcome the issues of both devices, we have developed a new facial osteogenesis distraction system capable of controlling both distraction distance and vector, and this article describes the method and short-term results of this system.

METHODS

Our system uses both external and internal distraction devices. Externally, a conventional halo-type external distraction device (RED System; Martin LP, Jacksonville, FL)[5] was used, whereas internally, a newly developed distraction device with a three-dimensional adjustable angle to interlock with the halo-type distraction device was used. The concept behind this system is to control the vector of postoperative lengthening using the external distraction device and to control the lengthening distance with the adjustable-angle internal distraction device.

SURGICAL TECHNIQUE

The surgical procedure used to treat syndromic craniosynostosis involved performing a Le Fort III osteotomy via a coronal incision of the scalp and driving a 2-mm Kirschner wire (K-wire) through the zygomatic bones. After fixing the anterior portion of the adjustable-angle internal distraction device by inserting it into the K-wire, the posterior portion of the device was then attached to the temporal bone with screws. Meanwhile, a total of 4 left and right surgical wires were used for traction of the external distraction device. The surgical wires were attached to the external distraction device after being passed through the mini plate attached to the inferior border of the anterior nasal aperture and the transmaxillary K-wire. After the procedure, the distraction distance was controlled by advancing the

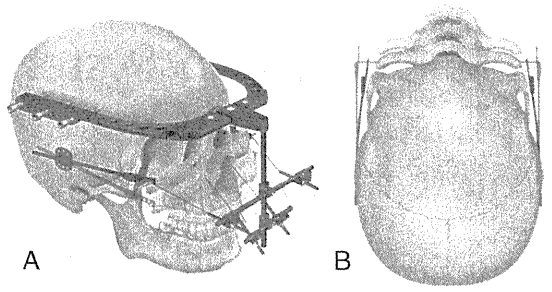

FIGURE 1. A, Maxillary distraction technique with internal and external devices is illustrated. The internal distraction device has an adjustable angle, whereas the external distraction device enables control of the distraction distance via the surgical wires. B, The angle of the internal distraction device's fixation position on the temporal bone can be altered by 5 to 15 degrees.

midface using the adjustable-angle internal distraction device with the K-wire as the axis, whereas the distraction vector was controlled using the 4 left and right surgical wires attached to the external distraction device (Figs. 1A, B). Immediately after distraction, the internal device was left in place, and the external device was removed along with all of the surgical wires. The internal device was subsequently removed after a consolidation period of approximately 2 months. The internal distraction device was simply removed after extracting the K-wire from the stab incision on the each cheek and making a 5-cm temporal incision. The removal took around 20 to 30 minutes because no coronal incision of the scalp was required.

CLINICAL REPORTS

Patient 1

This patient was a 6-year-old boy with Crouzon syndrome (Fig. 2). He had a bony and cartilaginous deviated nose. A deficiency of 15 mm at the incisors was measured by cephalometric analysis. The patient was taken to the operating room for Le Fort III osteotomy and placement of our systems. The fixation points of the internal devices for this system were the body of the zygoma anteriorly and the temporal bone posteriorly (Fig. 3). Distraction was initiated on postoperative day 5, and midface advancement was performed at a rate of 1 mm/d to achieve advancement of 15 mm at

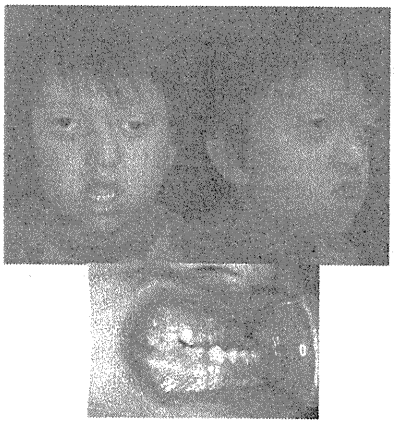

FIGURE 2. A 6-year-old boy with Crouzon syndrome seen preoperatively. He shows severe maxillary retrusion and a class III soft tissue profile.

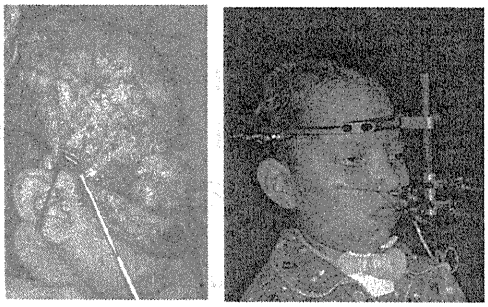

FIGURE 3. Note the position of the rigid external and internal distraction devices. Lengthening was performed by adjusting the internal distraction device's retroauricular shaft portion with a screwdriver and the external distraction device.

the occlusal plane. Then, additional advancement of 13 mm to overcorrect the maxillary position was performed.

Two months after the operation, the internal devices were removed. The patient's profile is shown approximately 6 months after the operation (Fig. 4). The patient's periorbital profile including the inferior orbital rim (orbitale) was good, but there was anticlockwise rotation of the maxilla (Figs. 4 and 5).

Patient 2

This patient was an 8-year-old girl with Crouzon syndrome. She had mental retardation. She underwent Le Fort III osteotomy and application of our systems for the correction of midfacial retrusion (Fig. 6). The fixation points of the internal devices for this system were the body of the zygoma anteriorly and the temporal bone posteriorly (Fig. 7). After a 5-day latency period, anterior distraction was performed at a rate of 1 mm/d for 25 days to overcorrect the maxillary position. Two months after the operation, the internal devices were removed. The patient's profile is shown approximately 6 months after the operation (Fig. 8). The patient's periorbital profile including the orbitale was good, but there was anticlockwise rotation of the maxilla (Fig. 9).

DISCUSSION

Rachmiel et al[10] and Staffenberg et al[11] demonstrated the feasibility of maxillary midface distraction in animals. The first human craniofacial distraction was first reported in 1992 by McCarthy et al.[1]

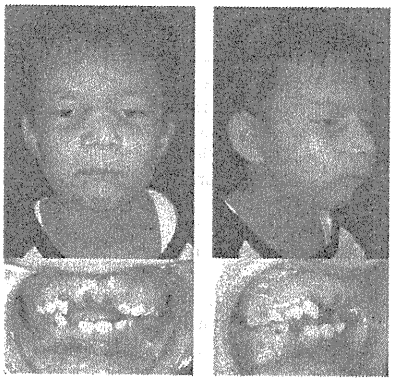

FIGURE 4. Six months after the procedure. Note the correction of the midface hypoplasia, reduced exorbitism, and overall improvement in facial appearance with planned overcorrection. Occlusion was rotated counterclockwise.

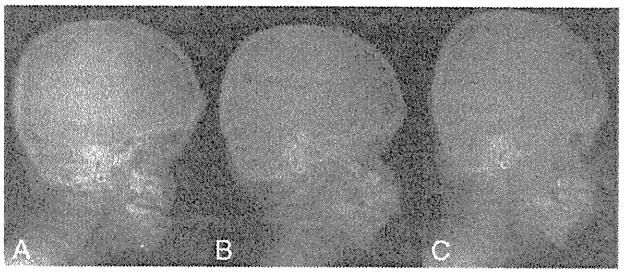

FIGURE 5. Serial lateral cephalograms taken preoperatively (A), at the end of the consolidation phase (B), and 6 months after surgery (C).

FIGURE 6. An 8-year-old girl with Crouzon syndrome seen preoperatively. She had mental retardation.

These authors successfully achieved mandibular hypoplasia. Subsequently, midface distraction has been used for Le Fort I[4,5] and Le Fort III[3,12] level of advancements. However, controlling the distance and vector of distraction in infants with syndromic craniosynostosis with good repeatability is difficult.[7] We have developed a facial distraction system leveraging the advantages of external and internal distraction devices, which we then used to successfully control both distraction distance and vector orientation.

Both external and internal distraction devices have their advantages and disadvantages.[7] The advantage of external distraction devices is that they enable the distraction vector to be controlled and easy to attach and remove. On the other hand, a drawback of the external device is that its distraction distance is generally limited,[13] although there is a good report that mean distraction was 26 mm.[14] From a psychologic perspective, it is recommended not to distract to the final adult maxillary position, as suggested by others.[15] Instead, because midface growth is not desirable in pediatric syndromic craniosynostosis, overcorrection is essential, and the midface should be advanced as much as possible.[16,17]

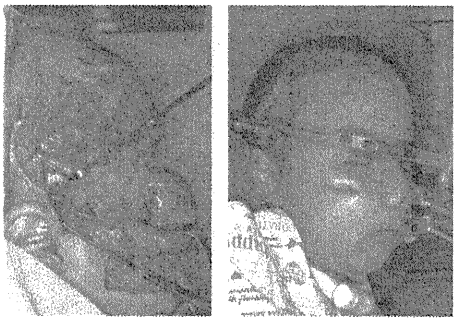

FIGURE 7. Note the position of the rigid external and internal distraction devices. Lengthening was performed by adjusting the internal distraction device's retroauricular shaft portion with a screwdriver and the external distraction device.

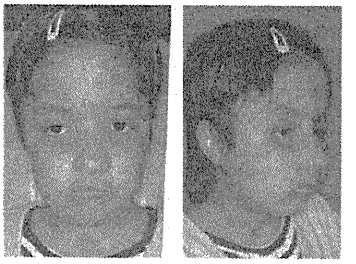

FIGURE 8. At 6 months after surgery. Note the correction of the midface hypoplasia, reduced exorbitism, and overall improvement in facial appearance with planned overcorrection. Occlusion was rotated counterclockwise.

Meanwhile, internal distraction devices do not permit control of distraction vectors but do allow control of distraction distance and are capable of delivering greater elongation than external devices by way of posterior extrusion, thus enabling overcorrection in infants.[18,19] Another benefit is that they are relatively inconspicuous because they are implanted in the patient. On the other hand, attachment of internal devices is more complex and time consuming than that of their external counterparts, and deviations in the left and right plates can cause facial asymmetry.[19] Another disadvantage is the need to typically perform a coronal incision of the scalp to remove the device. Using biodegradable materials in the attachment plates is one means of resolving this problem.[20] The delicate suture of zygomaticomaxillary sutures also means that fractures can occur in infants during distraction. Although cases of rescue with external distraction devices have been reported,[7] rescue is not possible using conventional internal devices.

The present system was developed to overcome the above-mentioned problems. The advantages of this system are that (1) it can achieve a distraction distance of at least 25 to 30 mm, thereby enabling overcorrection and, consequently, reducing the number of procedures; (2) the internal distraction device with three-dimensional adjustable angle enables postoperative control of distraction vectors even if the plates are not attached symmetrically, and it can also be applied in patients with congenital asymmetry; (3) anterior fixation of the internal device is done with a K-wire so that no area of the body of zygoma is required for fixation, and the device can be attached even if the zygomatic bone is hypoplastic; (4) the external distraction device can be removed immediately after completion of distraction without the need for a consolidation period; (5) the transmaxillary K-wire can prevent fractures due to weak zygomaticomaxillary sutures; and (6) removal is straightforward without the need for a coronal incision of the scalp. The system therefore has a number of

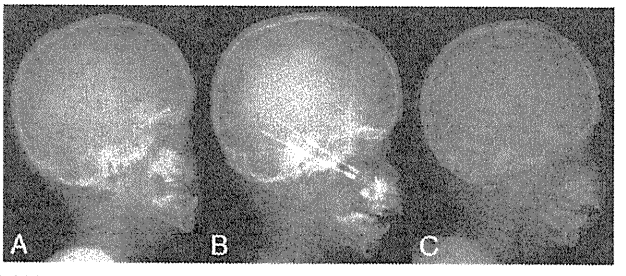

FIGURE 9. Serial lateral cephalograms taken preoperatively (A), at the end of the consolidation phase (B), and 6 months after surgery (C).

advantages and can be indicated in virtually all forms of syndromic craniosynostosis. We believe that it would be particularly well suited for patients with severe scarring because of multiple follow-up surgeries and patients requiring distraction of 25 mm or more.

One issue that needs to be addressed in the future is that, when overcorrection is performed in infants, the greater the distraction distance, the bigger the difference in the vectors of the orbitale and point A becomes,[13] making it difficult to set cephalometric targets. The patients in the present study also exhibited counterclockwise rotation of the maxilla and upward migration of point A further than was desired because we gave priority to alignment of the periorbital profile including the orbitale. We anticipate that the distraction distance and vector of the orbital level and occlusal levels would also differ in patients with short facial height typical of Apert syndrome. Resolving these issues requires a procedure integrating Le Fort III osteotomy with Le Fort I and II osteotomies and/or a study of downward facial rotation with the transmaxillary K-wire as the axis with a splint and elastic traction. In the future, careful medium and long-term follow-up of these patients is required.

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Reconstruction of human elastic cartilage by a CD44$^+$ CD90$^+$ stem cell in the ear perichondrium

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Despite the great demands for treating craniofacial injuries or abnormalities, effective treatments are currently lacking. One promising approach involves human elastic cartilage reconstruction using autologous stem/progenitor populations. Nevertheless, definitive evidence of the presence of stem cells in human auricular cartilage remains to be established. Here, we demonstrate that human auricular perichondrium, which can be obtained via a minimally invasive approach, harbors a unique cell population, termed as cartilage stem/progenitor cells (CSPCs). The clonogenic progeny of a single CD44$^+$ CD90$^+$ CSPC displays a number of features characteristic of stem cells. Highly chondrogenic CSPCs were shown to reconstruct large (>2 cm) elastic cartilage after extended expansion and differentiation. CSPC-derived cartilage was encapsulated by a perichondrium layer, which contains a CD44$^+$ CD90$^+$ self-renewing stem/progenitor population and was maintained without calcification or tumor formation even after 10 mo. This is a unique report demonstrating the presence of stem cells in auricular cartilage. Utilization of CSPCs will provide a promising reconstructive material for treating craniofacial defects with successful long-term tissue restoration.

stem cell identification | flow cytometry | cell transplantation | regenerative medicine | plastic surgery

Craniofacial injuries and abnormalities affect millions of patients worldwide, highlighting the need for novel therapeutic strategies (1). Conventional approaches to treat these defects rely on reconstructive materials, such as autocartilage and bone grafts or synthetic compounds (2–6). Collecting autologous tissue, however, places a significant burden on donor sites, and invariable absorption of the transplanted autografts often leads to treatment failure (7, 8). Moreover, owing to the limited amount of available autografts, severe craniofacial anomalies such as Treacher Collins syndrome and Nager syndrome still remain incurable (9, 10). The implantation of synthetic materials is associated with a number of potential complications, such as inflammation, extrusion, calcification, and abnormal skin (5, 6). To overcome these problems, many researchers have attempted to reconstruct elastic cartilage using cell-based engineering approaches that provide a sufficiently large volume of reconstructive material.

Several cell populations are potential sources for human elastic cartilage reconstruction (11–14). Human auricular chondrocytes, which is the only example already clinically applied, are highly chondrogenic and can produce significant levels of cartilage extracellular matrix (ECM). However, donor site morbidity is commonly observed and, more importantly, poor tissue maintenance remains a significant issue. It is expected that the use of stem cells will address these issues, because self-renewing stem cells can lead to permanent restoration of tissues characterized by high and continuous self-renewal (15). Although bone marrow mesenchymal stem cells (MSCs) were thought to be a promising resource, their use was associated with many serious problems, including low chondrogenic potential, vascularization, and mineralization (16, 17). Various tissue-derived MSCs, such as adipose-derived stem cells, also do not produce enough cartilage ECM to support 3D structures (18–20). The identification of an alternative source of stem cells with high chondrogenic potential is crucial to realize elastic cartilage regenerative therapy.

Previous papers have suggested that some of the cells derived from rabbit auricular (ear) perichondrium appear to be a stem or progenitor population, on the basis of in vitro multidifferentiation assays or label-retaining cell assays (21–23). However, considerable heterogeneities between these studies have precluded definitive evidence of stem cells in elastic cartilage, and the therapeutic potency of these stem/progenitor cells toward cartilage regenerative therapies remains to be determined. The goal of the present study was to determine whether human auricular perichondrium harbors cells with a stem cell character and to develop cell-manipulation techniques for reconstructing larger elastic cartilage with successful long-term tissue restoration aimed at future clinical use. Here, we show that human auricular perichondrium contains a promising stem/progenitor cell population. By combinations of newly identified stem cell markers, we successfully isolated a clonal population of CD44$^+$ CD90$^+$ stem cells from human auricular perichondrocytes, hereafter referred to as cartilage stem/progenitor cells (CSPCs). CSPCs proliferate robustly, show multiple differentiation capabilities, self-renew, and participate in tissue reconstruction. Our layered culture system enables CSPCs to efficiently differentiate into mature chondrocytes. Subcutaneously (s.c.) injected human CSPC-containing perichondrocytes reconstructed over 2 cm of elastic cartilage, consisting both of perichondrium and chondrium layers, and restored these structures without any ectopic tissue formation even after 10 mo.

Results

Human Perichondrocytes Are Highly Proliferative. We hypothesized that auricular perichondrium contains stem/progenitor cells that are highly proliferative, are able to differentiate into multiple lineages, can self-renew, and can contribute to tissue reconstruction. To test this hypothesis, we performed experiments using auricular cartilage remnants from microtia patients (Fig. 1 *A* and *B*). The patients included 19 males and 11 females, with a mean age of 10.6 ± 1.4 y (mean ± SD). There were no significant differences in the following experiments on the basis of their disease classification or complication. Auricular cartilage remnants were manually separated into the perichondrium layer, the interlayer, and the chondrium layer (Fig. 1*C* and Fig. S1, *Left*). After digesting each layer, the cells were cultured to yield perichondrocytes, interlayer cells, and chondrocytes, respectively. To examine their

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The authors declare no conflict of interest.

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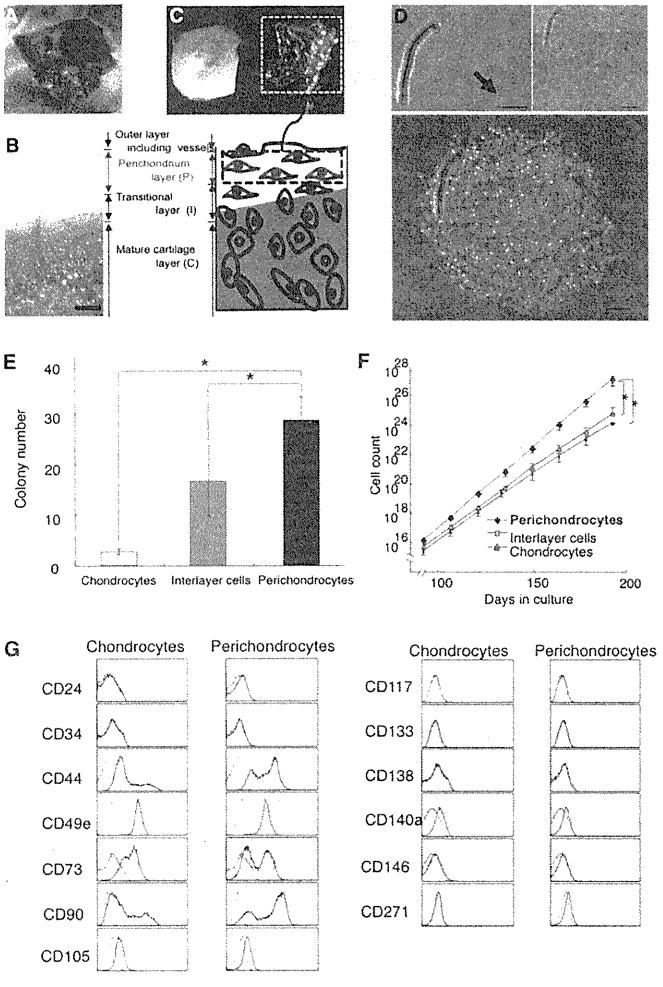

Fig. 1. Isolation and cultivation of human perichondrocytes. (*A* and *B*) Gross morphology and Alcian blue staining of auricular cartilage remnants from microtia patients. (Scale bars, 4 mm and 200 μm.) (*C*) Separation of the perichondrium layer from auricular cartilage. Separation was confirmed by Alcian blue staining of the separated chondrium layer (C), interlayer (I), and perichondrium layer (P) (also Fig. S1). (Scale bars, 200 μm.) (*D*) Clonal colony formation using cells derived from each layer after 4 wk (*Bottom*) of culture. Arrows indicate a single cell on day 1. (Scale bars, 500 μm.) (The figure is a composite of multiple panels.) (*E*) The colony-forming efficiency of cells derived from each layer. Each colony had to include more than 50 cells. Data are shown as the mean ± SD ($n = 9$). *$P < 0.001$. (*F*) Growth curves for cells derived from each layer after 196 d of culture. Data are shown as the mean ± SD ($n = 5$). *$P < 0.01$. (*G*) Flow cytometry analysis of the expression of cell-surface markers related to various stem cells on chondrocytes (*Left*) and perichondrocytes (*Right*). Isotype control antibodies were used for control samples (black dotted line). Perichondrocytes showed higher expression levels of CD44 and CD90 than was observed for chondrocytes.

colony-forming capacities, we cultured cells at low density (52 cells/cm^2). After 4 wk, single perichondrocytes formed colonies that contained over 50 cells (Fig. 1*D*). We then counted the semiclonal colonies. Perichondrocytes, interlayer cells, and chondrocytes formed 23.9 ± 4.5, 9.9 ± 6.8, and 2.3 ± 0.4 colonies, respectively (Fig. 1*E*). These results show that perichondrocytes possessed the highest clonogenicity.

Next, we analyzed the long-term proliferating capability to determine the proliferative rates of perichondrocytes. Perichondrocytes morphologically resembled fibroblasts and maintained this appearance after long-term culture (Fig. S1, *Right*). The proliferation rate of perichondrocytes and chondrocytes was determined from growth curves after 196 d of culture (Fig. 1*F*). On the basis of the growth curves, the doubling time of perichondrocytes was calculated to be 2.6 d compared with 3.0 d for chondrocytes. Thus, perichondrocytes contain highly expandable clones and proliferate 13% faster than chondrocytes.

Cell-Surface Marker Characterization of Human Perichondrocytes. To characterize the cell-surface marker profile of human CSPC populations, we performed flow cytometry. The hematopoietic stem cell markers CD34 and c-kit (CD117), and the MSC markers CD44, CD73, CD90, CD105, CD133, CD140a, CD146, and CD271 (24–28) were analyzed (Fig. 1*G*). Most marker expressions of perichondrocytes were similar to that of chondrocytes. However, CD44 and CD90 expressions showed significant differences between them. Higher expressions of CD44 and CD90 were observed in perichondrocytes compared with those of chondrocytes (CD44, 58.8 ± 8.6% and 35.4 ± 7.3%; CD90, 63.0 ± 1.7% and 46.5 ± 3.9%), indicating a potential candidate for clonal isolation of stem cells.

In Vitro Elastic Cartilage Differentiation of Human Perichondrocytes. It is crucial to obtain a highly chondrogenic population with plenty of cartilage ECMs following in vitro cultivation before cell transplantation into a lesion. To develop suitable culture conditions for cell transplantation, we focused on the use of a layered culture system combined with several cytokines, which have been shown to enhance hyaline cartilage regeneration in vitro (29). Cultured human perichondrocytes were layered to differentiate them into mature chondrocytes in the presence of bFGF (basic fibroblast growth factor) and IGF1 (insulin-like growth factor 1) (Fig. 2*A*). The perichondrocytes gradually differentiated into chondrocytes on the basis of the layering and began to produce proteoglycan and type II collagen (Fig. 2*B*). Several mucopolysaccharides were secreted from the perichondrocytes, making the culture media viscous and matrix-like (Fig. 2 *C* and *D* and Movie S1). This is important because high viscosity encourages cells to stay near the site where transplanted.

Then, to quantify and compare the chondrogenic potential of the perichondrocytes with that of chondrocytes, we performed real-time PCR analysis of genes related to elastic cartilage differentiation. Under differentiation conditions, we observed increased expression of a number of chondrium markers, including versican (*CSPG2*, 4.2-fold), elastin (*ELN*, 9.6-fold), alpha 1 type II collagen (*COL2A1*, 2.1-fold), and fibrillin 1 (*FBN1*, 17.2-fold), whereas expression of the perichondrium marker alpha 1 type I collagen (*COL1A1*) decreased to 0.18-fold of the original level (Fig. 2*E*).

We also performed ELISAs to evaluate the secretion of elastic cartilage ECM proteins. The concentrations of secreted ECM proteins in perichondrocyte cultures under differentiation conditions were 17.5 ± 4.3 μg/mL proteoglycan, 235.6 ± 19.9 μg/mL elastin, and 61.8 ± 7.5 μg/mL collagen. Surprisingly, similar concentrations of secreted proteins were observed for chondrocytes (19.0 ± 1.3, 234.0 ± 16.3, and 55.8 ± 4.9 μg/mL, respectively), highlighting the high chondrogenic potential of the perichondrocytes (Fig. 2*F*).

Human Perichondrocytes Reconstructed Elastic Cartilage Containing Both Perichondrium and Chondrium Layers. Human perichondrocytes were expanded, subjected to cartilage differentiation conditions, and s.c. injected into nonobese diabetic [(NOD)/SCID] mice. We performed the same experiments with human chondrocytes (Fig. 3 *A–H*). Histochemical analyses demonstrated that perichondrocytes differentiated into mature chondrocytes and formed an elastic cartilage rich with proteoglycans and elastic fibers (Fig. 3 *I–O*). Immunohistochemistry revealed that regenerated cartilage contained a Col I$^+$ capsule enveloping a Col II$^+$ chondrium layer (Fig. 3*P*). These results indicated that perichondrocytes contain a putative stem/progenitor population with regenerative capacity.

Elastic cartilage composed of two layers was maintained even 6 and 10 mo after perichondrocyte transplantation (Fig. S2 *A–L*). During the growth and maturation period, it is generally ac-

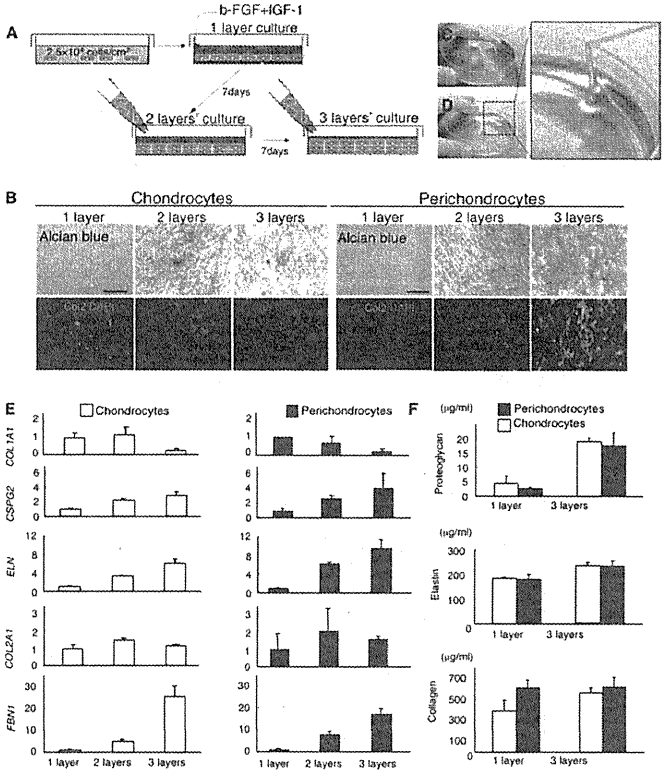

Fig. 2. Human perichondrium-derived cells show a highly chondrogenic profile. (*A*) Schematic diagram of the layered culture system used for chondrogenic induction. (*B*) Cytochemistry staining of chondrocytes (*Left*) and perichondrocytes (*Right*) after the cells were cultured in the layered system. Perichondrocytes differentiated into chondrocytes that produced proteoglycans (Alcian blue) and type II collagen (Col2, Green). (Scale bars, 200 μm.) (*C* and *D*) Aspiration of untreated (*C*) and treated (*D*) culture media. The secretion of several mucopolysaccharides from the perichondrocytes after layered induction made the media viscous and matrix-like (Movie S1). (*E*) Real-time PCR analyses of gene expression profiles related to elastic cartilage in chondrocytes (*Left*) and perichondrocytes (*Right*) after the cells were cultured in the layered system. Data are shown as the mean ± SD ($n = 3$). (*F*) ELISAs specific for proteoglycans, elastic fibers (elastin), and collagen secretion from chondrocytes (*Left*) and perichondrocytes (*Right*) after the cells were cultured in monolayers or a trilayered culture. Data are shown as the mean ± SD ($n = 3$).

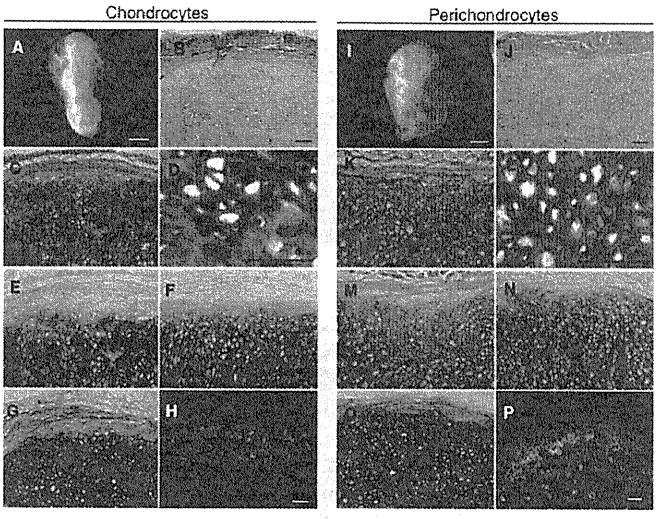

Fig. 3. Tissue reconstitution capability of human perichondrocytes. (*A* and *I*) Chondrocytes (*Left*) and perichondrocytes (*Right*) formed cartilage-like tissues 3 mo after s.c. transplantation. (Scale bars, 1 mm.) (*B* and *J*) Hematoxylin/eosin, (*C*, *D*, *K*, and *L*) Alcian blue, (*E* and *M*) Toluidine blue, and (*F* and *N*) Safranin O staining showed that the reconstructed tissue contained mature chondrocytes in lacunae that produced high levels of proteoglycan. (*G* and *O*) Elastica Van Gieson staining showed that the reconstructed cartilage contained elastic fibers. (*H* and *P*) Immunohistochemistry revealed that perichondrocyte-derived cartilage (*Right*) contained type I collagen (Col1)$^+$ capsules that enveloped a type II collagen (Col2)$^+$ chondrium layer. (Scale bars, 200 μm.)

cepted that the cell density of the chondrium layer decreases (30). Consistent with this, we found that the cell density decreased from 1.6 to 0.4 × 10^6 cells/mm^2 presumably due to cell maturation (Fig. S2*M*). The dry weight of perichondrocyte-derived cartilage is heavier than that of chondrocytes with statistical significance (Fig. S2*N*). Fibrous tissue, blood vessels, bone, or tumors did not develop during the 10-mo observation period. In practical applications for congenital anomalies, larger elastic cartilage should be reconstructed. To achieve this goal, we transplanted larger volumes of perichondrocytes (injection volume = 3 mL; ~2 × 10^7 cells) after chondrogenic induction. After 2 mo, over 2 cm of elastic cartilage was generated (Fig. S3*A* and Movie S2). Reconstructed tissue consisted of homogenously distributed mature chondrocytes with plenty of cartilage ECMs (Fig. S3*B*). These results are particularly striking, as we were able to control the size of the reconstructed cartilage by adjusting the injection volumes.

Transient Amplification and Transition to a Dormant State of CD44$^+$ CD90$^+$ Cells. On the basis of FACS analyses, we investigated the distribution of CD44$^+$ CD90$^+$ cells in primary or regenerated tissues to characterize the putative stem cells. Immunohistochemistry revealed that in primary auricular cartilage, a rare population of CD44$^+$ CD90$^+$ cells specifically resided in the perichondrium layer, but not in the chondrium layer (Fig. 4*A*). The ratio of CD44/CD90 double positive cells was 0.84 ± 0.25% in the perichondrium layer. For regenerated cartilage, CD44$^+$ CD90$^+$ cells were retained in the perichondrium layer throughout the 10 mo (Fig. 4*B*). Interestingly, the ratio of CD44$^+$ CD90$^+$ cells in the perichondrium was initially amplified up to 18.02 ± 7.06% at 1 mo and then diminished to 0.62 ± 0.05% at 10 mo, the same as observed in primary auricular cartilage remnants (Fig. 4*C*). CD44 or CD90 single positive cells also experienced the same distributional change (Fig. 4*D*). These distributional changes seemed to depict the transient amplification of stem/progenitor cells at an initial state of tissue regeneration and transition to a dormant state at a later phase.

Prospective Isolation of Stem Cells from Human Auricular Perichondrocytes. To provide definitive evidence of stem cells in the auricular perichondrium, flow cytometry was used to fractionate the perichondrocytes into four subpopulations on the basis of the expression of CD44 and CD90 (Fig. 5*A*). We then cultured the cells from each fraction at low density (52 cells/cm^2) to identify the fraction that contained the cells that efficiently formed clonal colonies. After 21 d of culture, the CD44$^+$ CD90$^+$ cells clearly formed the most colonies (Fig. 5 *B* and *C*). These data suggested that both CD44 and CD90 were good cell-surface markers to enrich the stem/progenitor cells.

Then, we attempted to culture single cells in each well of 96-well plates, following clone sorting of cells on the basis of CD44/CD90 expression. After 21 d, we counted the large colonies (LCs > 100 cells), which potentially reflected a rapid rate of cell proliferation. The results showed that CD44$^+$ CD90$^+$ cells displayed a significantly greater capacity to extensively expand than was observed for CD44$^+$ CD90$^-$, CD44$^-$ CD90$^+$, or CD44$^-$ CD90$^-$ cells (Fig. 5*D*). Clones isolated from the CD44$^+$ CD90$^+$ fraction most efficiently formed LCs (8.3 ± 2.1%) comprising several hundred cells. The CD44$^-$ CD90$^+$ fraction-derived clones were also capable of forming LCs (3.8 ± 1.6%), but the efficiency of LC formation was statistically lower ($P < 0.05$, $n = 3$). Far fewer LCs were grown from clones derived from the

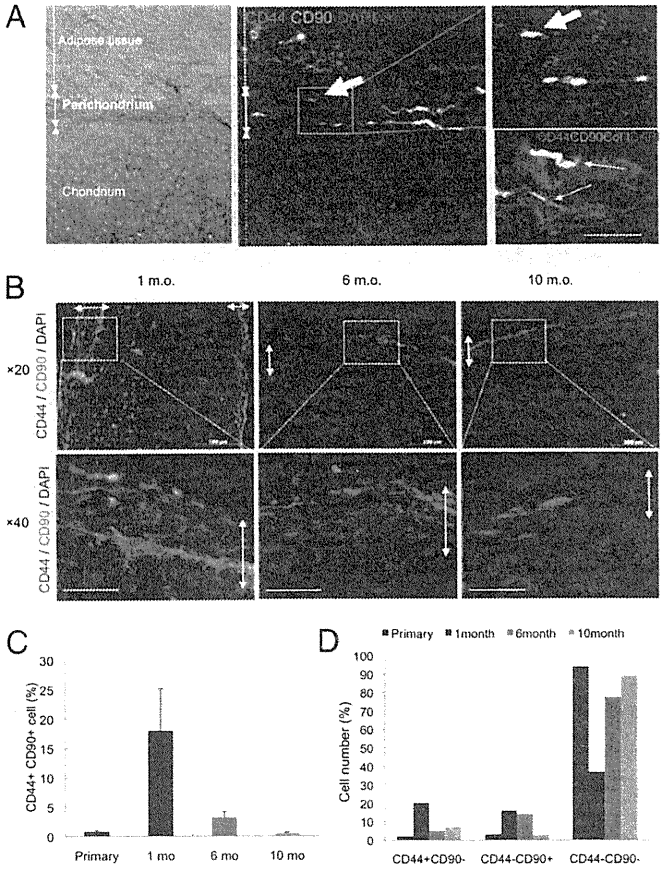

Fig. 4. Presence and retention of CD44$^+$ CD90$^+$ cells in primary or regenerated perichondrium. (*A*) Rare population of cells in human primary auricular perichondrium (Col I$^+$) specifically express CD44/CD90. Arrows indicate rare double positive cells existing in the outer perichondrium. (Scale bar, 50 μm.) (*B*) Immunohistochemical analyses of CD44 and CD90 showed apparently distinct distributions at multiple time points (1, 6, and 10 mo). Double-headed arrows show the perichondrium layers. (Scale bars, 100 μm.) (*C* and *D*) Quantification of CD44$^+$ CD90$^+$ cells (*C*) or CD44$^+$ CD90$^-$, CD44$^-$ CD90$^+$, and CD44$^-$ CD90$^-$ cells (*D*) in primary or regenerated perichondrium.

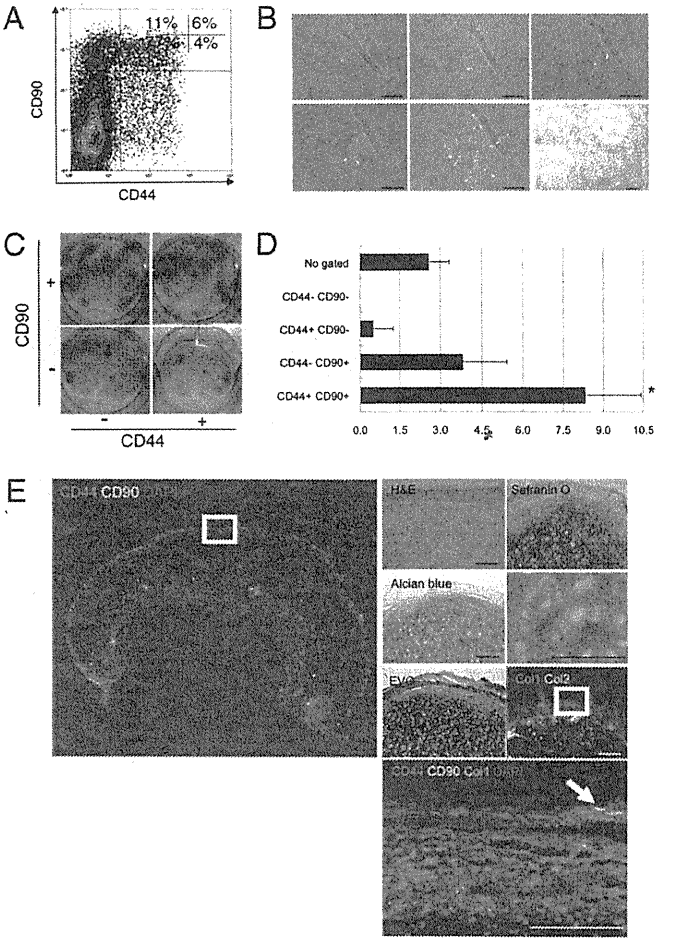

Fig. 5. Reconstruction of human elastic cartilage by a single CD44$^+$ CD90$^+$ stem cell in the human auricular perichondrium. (*A*) Perichondrocytes were fractionated via flow cytometry on the basis of the expression of CD44 and CD90. The percentages of each subpopulation are shown. (*B*) Clonal colony formation of CD44$^+$ CD90$^+$ cells. (The figure is a composite of multiple panels.) (*C*) Macroscopic observation of clonal colonies derived from multicolor sorted cells visualized using Giemsa staining. (*D*) Ability of sorted perichondrocytes to form large colonies (LCs). The *x* axis shows the percentages of cells that formed colonies. Data are shown as the mean ± SD ($n = 3$). *$P < 0.05$. (*E*) Reconstruction of human elastic cartilage by clonally cultured CD44$^+$ CD90$^+$ cells. Reconstructed cartilage consisted of mature chondrocytes with plenty of cartilage ECMs, as evaluated by histochemical staining. The arrowhead shows the retention of a CD44$^+$ CD90$^+$ cell in the reconstructed perichondrium layer. (Scale bars, 100 μm.)

CD44$^+$ CD90$^-$ subpopulation, whereas cells from the CD44$^-$ CD90$^-$ fraction formed no LCs.

The isolation and cultivation of definitive single-cell–derived CD44$^+$ CD90$^+$ cells enabled us to evaluate several criteria of stem cells. We first examined multipotency using several sorted clones. Compared with unipotential chondrocytes (Fig. 2*B* and Fig. S4*A*), all of the six isolated clones possessed chondrogenic, adipogenic, and oseteogenic potential (Fig. S4*B*).

The identification of multipotent CD44$^+$ CD90$^+$ stem cells with high proliferative potential suggested that these cells could provide a basis for the reconstruction of continuously self-renewing elastic cartilage. To test this approach, we transplanted clonally propagated CD44$^+$ CD90$^+$ cells. Three months after transplantation, elastic cartilage containing both ColI$^+$ perichondrium and Col II$^+$ chondrium were generated. Cells in the chondrium layer consisted of mature chondrocytes packed in lacnae. Surprisingly, CD44$^+$ CD90$^+$ cells were retained in the outer perichondrium layer, suggesting the presence of self-renewing stem cells in the regenerated elastic cartilage.

Discussion

Despite the implications of stem cells in auricular perichondrium from rabbit studies, no studies to date have identified a definitive single-cell–derived stem cell population in elastic cartilage (21). This is attributable to the low frequencies of highly clonogenic stem cells, a common challenge in the field of mesenchymal stem cell-related research (31, 32). Here, we have successfully identified and isolated a human stem/progenitor cell population (CSPCs) existing in the auricular perichondrium by combinations of CD44 and CD90 markers. Clonally propagated CD44$^+$ CD90$^+$ cells from auricular cartilage, on the basis of a number of criteria, appear to be a population of stem cells. The utilization of CSPCs from human auricular cartilage will not only improve our understanding of basic cartilage biology, but will lead to novel therapeutic strategies, including long-term tissue restoration, for patients with craniofacial defects.

Various tissue-derived MSCs have been investigated as possible cellular resources for elastic cartilage reconstruction. These cells, however, do not efficiently differentiate into chondrocytes and fail to produce elastic cartilage-specific ECM components, such as proteoglycans and elastic fibers (16, 18). Furthermore, bone marrow MSC-derived cartilage is associated with hypertrophy, vascular invasion, and ectopic mineralization (17). In

contrast to MSCs, CSPCs isolated from the auricular perichondrium displayed a highly chondrogenic profile that was similar to that observed for chondrocytes. However, similar to MSCs, these cells were able to differentiate into adipocytes and osteocytes, suggesting that they are a higher cell lineage than cartilage-committed progenitor cells, which are predetermined to form chondrocytes of elastic cartilage. In line with these expectations, CSPCs expressed CD44 and CD90, similar to MSCs, whereas they did not express the MSC markers CD133, CD140a, CD146, or CD271 (24, 33, 34). Thus, our isolated stem/progenitor cells are closely related to MSCs, yet are a distinct cartilage stem/progenitor cell population. The hierarchy of the mesenchymal cell lineage has not yet been clearly elucidated. A better understanding of this hierarchy may allow CSPCs to be used as a therapeutic resource for applications other than elastic cartilage reconstruction. Moreover, it may lessen the risk of certain adverse effects, such as ectopic ossification, which is a serious problem associated with MSCs.

Continuously self-renewing tissues are successively restored by stem cells. Hematopoiesis provides a well-known paradigm of a stem cell-dependent, steadily self-renewing system. Using postnatal stem cells may significantly alter approaches to cell-based engineering. We found that extensively expanded perichondrocytes that had been subjected to a layered culture system were able to form elastic cartilage after s.c. transplantation. Similar to normal auricular cartilage, the reconstructed cartilage was composed of two layers: perichondrium and chondrium. Transplants successfully restored their original structure even after 10 mo without ectopic tissue formation, indicating that perichondrocytes may be a better resource than MSCs. Our study suggests that the grafting of perichondrocytes should allow long-term restoration of reconstructed tissue owning to self-renewing CSPCs in the perichondrium layer.

Further evaluations of the extracellular matrix components or mechanical evaluations of the regenerated cartilage will be necessary to show the clinical relevance of perichondrocytes (35, 36). However, the use of autologous auricular perichondrocytes represents several important advantages compared with the harvesting of chondrocytes so far. First, harvesting perichondrocytes requires a minimally invasive procedure, whereas collecting chondrocytes from the auricle places a significant burden on donor sites. Perichondrocytes can be obtained from a thin fibrous layer of the auricle even from microtia patients. Second, their high proliferative potential enables us to shorten the culture period. Finally, it is expected that perichondrocytes will enhance morphological preservation through continuous self-renewal because they contain a definitive stem/progenitor population (CSPCs). This is important especially in children, as permanent tissue restoration through many decades is an essential requirement for treating craniofacial anomalies.

There are currently no published reports describing the successful clinical use of a stem cell population for human elastic cartilage reconstruction. Taken together, our innovative discoveries provide three different modes of treatment strategies using an autologous stem/progenitor cell population, as shown in Fig. S5: (*i*) Direct cell injection and (*ii*) Two-stage transplantation without a scaffold. First, direct cell injection, which consists of simple procedures, is ready for clinical application. The application is limited to small or simple-shaped lesions, but this method makes it possible to reconstruct self-renewing elastic cartilage after extended expansion of patients' own cells. For larger and more complex deformities like microtia, we propose two-stage transplantation without a scaffold that is also clinically applicable. In this procedure, a sufficient volume of elastic cartilage is obtained from primary cell transplantation into a non–weight-bearing area like the lower abdomen. Then, a generated cartilage block is used for the framework designed for a given patient's deformities and is then transplanted into a lesion. The optimization of technical aspects (e.g., culture duration, injection volumes, and techniques for sculpting the framework) and further studies concerning safety should lead to the establishment of promising treatment options for currently incurable craniofacial anomalies.

Methods

Isolation and Cultivation of Human Perichondrocytes. We obtained elastic cartilage samples from microtia patients following the approved guidelines set by the ethical committee at Yokohama City University (approval no. 03–074). We stripped off the adipose tissue and microscopically separated the cartilage into three layers: the chondrium layer, interlayer, and perichondrium layer. Dissected tissues were cut into small pieces and digested for 2 h at 37 °C in PBS containing 0.2% collagenase type II (Worthington) with shaking. After passing through a 100-μm nylon mesh (BD Falcon), the cells were washed three times with PBS. Cell suspensions were cultured in Dulbecco's modified Eagle medium and Ham's F-12 medium (DMEM/F-12; Nissui Pharmaceutical) supplemented with 10% FBS (Moregate) and 1% antibiotic antimycotic solution (AMS; Sigma) in 5% CO_2 at 37 °C.

FACS Analysis and Cell Sorting. We immunolabeled cells with 1 mg of fluorescence-conjugated mouse antihuman monoclonal antibodies or isotype-matched IgGs (Table S1) for 30 min at 4 °C. The fluorescence-labeled cells were analyzed and separated with a MoFlo cell sorter (DakoCytomation).

Cell Multipotency in Vitro. For chondrogenic induction, each sample was seeded at a density of 2.5×10^4 cells/cm^2. For the 48 h after seeding, cells were cultured under our standard culture medium. Thereafter, cells were cultured for 5 d using DMEM/F-12 containing 10% FBS, 1% AMS, L-ascorbic acid 2-phosphate, dexamethasone, insulin-like growth factor-1, and fibroblast growth factor-2. After 7 d of monolayer induction, detached cells from the same donor-derived subculture were adjusted to a density of 2.5×10^4 cells/cm^2 and seeded on the monolayer culture. These steps were repeated twice with an interval of 1 wk. We also tested the adipogenic and osteogenic potential of perichondrocytes as previously described (37).

Gene Expression Analysis. For reverse transcription-polymerase chain reaction (RT-PCR) experiments, we designed primers (*SI Methods*) using Primer3 software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The following quantitative PCR (qPCR) primers were used in TaqMan gene expression assays: *COL1A1*, Hs00266273_ml; *COL2A1*, Hs00164099_ml; *CSPG2*, Hs01007933_m1; *ELN*, Hs00355783_ml; and *FBN1*, Hs00171191_m1 (Applied Biosystems).

ELISA. We quantitatively determined the chondrogenic potential of perichondrocytes by measuring proteoglycan, elastin, and collagen production using ELISAs. We subjected supernatants from the cell culture dish to Blyscan, Fastin, and Sircol assays (Biocolor) (38).

In Vivo Transplantation. Cells that had been subjected to chondrogenic differentiation were scraped with a cell lifter. The scraped cells were collected into a 2.5-mL syringe (Terumo) equipped with a 23-gauge injection needle (Terumo). An appropriate volume of the culture was s.c. injected into NOD/SCID mice (Sankyo Laboratory). The mice were bred and maintained in accordance with our institutional guidelines for the use of laboratory animals.

Histochemical and Immunohistochemical Analysis. We stained the sections and/or cultured cells with HE, AB, Toluidine blue, Safranin O, EVG, Alizarin Red S (Muto Pure Chemicals), or Oil Red O (Sigma). For immunohistochemical analysis, the tissue sections and cultured cells were immunolabeled with primary antibodies, rabbit antihuman type I collagen monoclonal antibodies (Monosan), and mouse antihuman type II collagen polyclonal antibodies (Chemicon), at 4 °C overnight. After washing, the sections and/or cells were incubated with Alexa Fluor 488- and/or Cy3-conjugated secondary antibodies (1:800; Molecular Probes) specific for the appropriate species for 1 h at room temperature. The samples were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and analyzed with a LSM510 laser-scanning microscope (Zeiss).

Statistical Analysis. Data are expressed as the mean ± SD from at least three independent experiments. Differences between three or four groups were analyzed using the Kruskal–Wallis test by ranks, and post hoc comparisons were made with Mann–Whitney *U* test with Bonferroni correction. Two-tailed *P* values <0.05 were considered significant.

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