

Reconstitution of the Histologic Characteristics of a Giant Congenital Nevomelanocytic Nevus Employing the Athymic Mouse and a Cultured Skin Substitute

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This study addresses the development of an animal model for human giant congenital nevomelanocytic nevi (GCNN). Skin grafts were made from 1) non-involved split-thickness skin from a 12-month-old GCNN patient, 2) nevus split-thickness skin from the same GCNN patient, 3) nevus full-thickness skin, and 4) cadaveric human split-thickness skin. For groups 1) and 2), human epidermal and dermal cells were enzymatically isolated and expanded in tissue culture. Composite grafts were made by placing the cultured dermal cells into a collagen-glycosaminoglycan (GAG) matrix, followed by placement of the epidermal cells onto the opposite, laminated side of the matrix. All grafts were placed onto full-thickness wounds of athymic mice and biopsies were obtained from 6 to 38 weeks later for light microscopy including S-100 immunoperoxidase staining, and electron microscopy.

The GCNN cultured skin mice (group 2) developed black, raised skin in the healed wounds. None of the group 1 mice

developed lesions, grossly or histologically. All of the nevus full-thickness mice retained the nevus grossly. Histopathologic examination at 38 weeks of the black, raised plaques of group 2 demonstrated a reconstituted dermis similar to group 3. Nevus cells were larger and more epithelioid in the upper dermis, as seen with true GCNN. These nevomelanocytes were not seen in the dermis at 24 weeks, suggesting that the nevus cells migrated from the epidermal component of the cultured graft to the dermis during this time frame (24–38 weeks). The melanocyte identity of these cells was confirmed with S-100 immunoperoxidase staining and electron microscopy.

These findings are unique to this composite cultured graft system. The ability to culture specific types of melanocytes and place them into skin substitutes on athymic mice provides a basis for the study of GCNN and melanocyte biology *in vivo*. *J Invest Dermatol* 97:649–658, 1991

Giant congenital nevomelanocytic nevi (GCNN) have significant consequences for both patient and family. The involvement of this lesion on the face or substantial areas of the trunk may lead to severe aesthetic deformities and subsequent psychosocial problems for the child. More importantly, GCNN are precursor lesions of malignant melanoma, and have a relatively high potential for transformation. Excision in infancy is often necessary, especially

for the larger GCNN. The full-thickness tissue loss of these major operative procedures can involve 30–40% or more of the total body surface area, and often must be staged due to the paucity of adequate donor sites from which to obtain normal skin. Also, some truncal sites of involvement (including perineum) and other anatomical areas, such as the face and orbit, are extremely difficult to excise and cover.

The frequency of transformation to malignant melanoma has not been clearly defined, as various studies have reported a frequency ranging from less than 1% to 42% [1–5]. After careful evaluation of the literature and reviewing their experience in England and Wales, Quaba and Wallace [6] estimated the risk to be 8.5% within the first 15 years of life, in lesions that cover greater than 2% of the total body surface area. Kaplan [7] averaged a number of studies and determined a 14% incidence over a patient's lifetime. He also stated that 60% of these malignancies developed in the first decade of life.

There are no reliable criteria for determining which congenital nevomelanocytic nevi will transform. "Large" or giant congenital nevi (> 20 cm) definitely have an increased incidence, but there is no reliable way to assess an individual nevus' potential for transformation. This is compounded by the fact that early diagnosis is difficult when malignancy develops in the dermal component, and spread to lymphatics and blood vessels can occur with no clinical change in the lesion.

The differentiation pathway and evolution of both GCNN and acquired melanocytic nevi (AMN) are controversial. Many authors

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Abbreviations:

AMN: acquired melanocytic nevi

DEJ: dermo-epidermal junction

GAG: glycosaminoglycan

GCNN: giant congenital nevomelanocytic nevi

HF: human fibroblasts

HK: human keratinocytes

feel that AMN represent genetically controlled proliferations of melanocytes at predetermined sites on the body. Unna's *Abtropfung* theory (dropping off and migration of melanocytes from the dermo-epidermal junction) has been replaced by the concept that new nests of nevomelanocytes are surrounded by a stroma. This results in an elevation of the plane of the dermo-epidermal junction and clinically as a papular compound or dermal nevus [8,9].

Congenital melanocytic nevi may represent a similar phenomenon that occurred during in utero development. An alternate theory could explain the development of GCNN and dermal melanocytomas such as blue nevi. Because all melanocytes are believed to derive from primitive neuroectodermal melanoblasts, GCNN may occur through differentiation and migration of pluripotential dermal nerve sheath precursor cells [10].

The purpose of this study was to develop an animal model for evaluating GCNN. Two techniques were employed to transfer the patient's nevus to the athymic mouse. First, we grafted the full-thickness nevus and followed its behavior over time. Second, by co-culturing the nevomelanocytes with fibroblasts and keratinocytes, and placing them into a skin substitute, we followed their development. We took advantage of the ability to culture both human dermal and epidermal cells, place them onto a collagen-GAG matrix, and have this skin substitute graft consistently "take" and persist on the athymic mouse [11-14]. This grafting technique has also been used successfully in limited clinical studies treating massive burn patients [15-16] and hopefully will be a major adjuvant in treating GCNN patients. The ability to place a specific individual's skin cells into this model allows for study of both normal and lesional skin from patients with GCNN. Because the athymic mouse can live and retain this reconstituted nevus for up to two years, the possibilities for characterization and manipulation of these nevomelanocytes are many.

MATERIALS AND METHODS

Conditions for Graft Placement Four conditions for skin grafts were used: 1) cultured composite grafts from uninvolved split-thickness skin of GCNN patient ($n = 6$), 2) cultured composite grafts from split-thickness samples of nevi from the same GCNN patient ($n = 6$), 3) full-thickness non-cultured samples of nevus from the same GCNN patient ($n = 8$), and 4) non-cultured cadaveric split-thickness skin (xenograft, $n = 5$). Conditions 3) and 4) are included as long-term controls to study the behavior of skin on athymic mice. From two to four animals were biopsied at 6, 20, and 38 weeks post-placement for histologic and electron microscopic analysis. Gross examination was carried out with all remaining animals at each time point.

Preparation of Grafts Cultured composite grafts were made from separate, parallel tissue cultures of human epidermal and dermal cells isolated from a 12-month old patient (Fig 1) with a giant congenital nevomelanocytic nevus (GCNN), and placed onto a collagen-glycosaminoglycan (C-GAG) membrane (Fig 2A). This C-GAG membrane is constructed to have an open pore structure ($50-150 \mu$) in order to promote ingrowth of fibrovascular tissue from the wound bed. The top portion of this membrane has been laminated with a layer of collagen-GAG, thus forming a planar surface for more consistent attachment of the cultured human keratinocytes [17-20].

Split-thickness skin was obtained from the GCNN patient during one of her excision and grafting operations. This skin was taken from the nevus itself (anterior thigh) and from unaffected areas [lateral calf (2 cm^2)] that would normally be used for reconstructive grafting purposes. Full-thickness nevus was also obtained from pathology specimens after adequate samples were examined by surgical pathology. All tissue was obtained with informed consent from the patient's parents for research purposes as specified by the UCSD Medical Center's Institutional Review Board.

Proliferative human keratinocytes (HK) and fibroblasts (HF) were isolated from the split-thickness skin with 0.025% collagenase (Worthington Biochemical; Freehold, NJ.) + 10% v/v bovine

pituitary extract (BPE). After 30-45 min in the collagenase solution, the epidermis was mechanically separated from the dermis. The epidermal cells were then isolated into a single cell suspension by treatment with 0.025% trypsin and 0.01% EDTA solution for an additional 5 min [21]. Trypsin was neutralized with 10% fetal bovine serum. Various cells, including human keratinocytes and melanocytes, can be isolated with this technique and passed along in the culture period. The epidermal cells were resuspended and expanded in number in 75-cm² polystyrene tissue culture flasks (Corning Glass Works, Corning, NY) with modified MCDB 153 containing 0.2 mM/l calcium [22], increased amounts of selected amino acids [23], 10 ng/ml epidermal growth factor (EGF) (Gibco Laboratories; Grand Island, NY), 5 μ g/ml insulin, 0.5 μ g/ml hydrocortisone, 0.1 mM/l ethanolamine, 0.1 mM/l phosphoethanolamine, 0.5% vol/vol bovine pituitary extract, and penicillin (10,000 units/ml)-streptomycin (10,000 μ g/ml)-amphotericin B (25 μ g/ml) (PSF) (Gibco Laboratories) as an antibiotic-antimycotic agent.

The dermis was completely dissolved by further treatment for 2-3 additional hours with the collagenase solution. HF isolated by this method were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories) with identical amounts of EGF, insulin, hydrocortisone, and PSF; plus 10% vol/vol fetal bovine serum (FBS). Both HK and HF were incubated at 37°C, 5.0% CO₂, and saturated humidity.

Approximately 2 weeks after primary cultures were begun, and after two further passes, HF (5×10^5 cells/cm²) were placed onto the open-pored side of a 10 \times 10 cm collagen-GAG membrane. This was kept for 2 d in DMEM + 10% FBS, with daily medium changes. At this time, the collagen-GAG membrane was turned over and placed into MCDB 153 and HK (5×10^5 cells/cm²) were inoculated onto the laminated side. Medium was changed daily, and in 4 d grafts were ready to place onto the mice (Fig 2B). For the last



Figure 1. Giant congenital nevomelanocytic nevus. Child is 12-month old girl with nevus covering approximately 40% of her total body surface area.

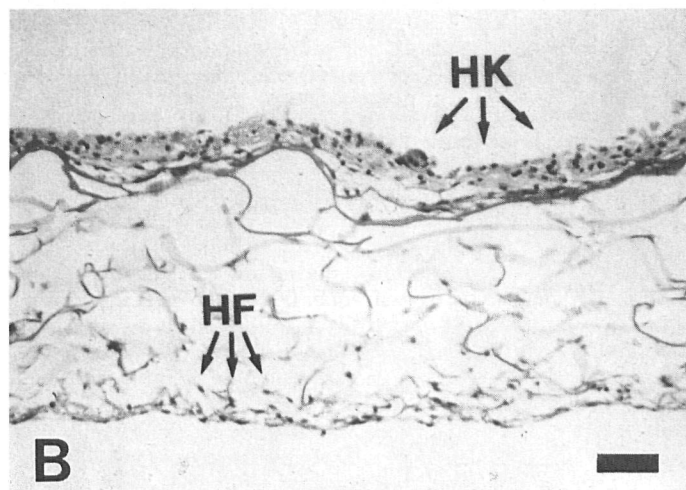
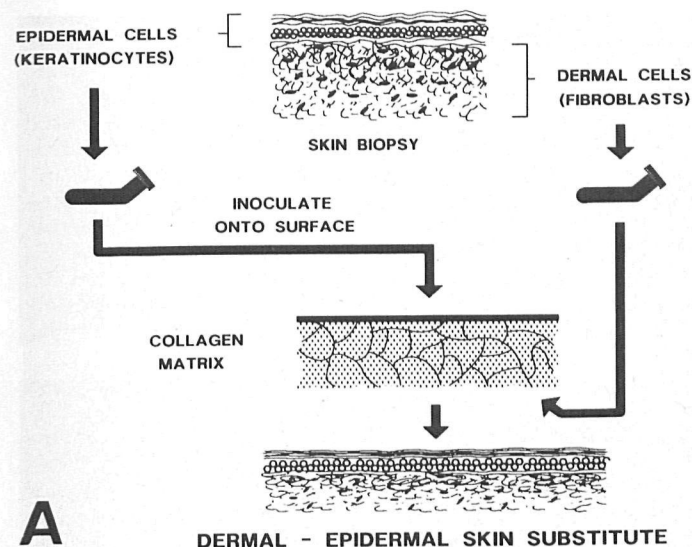


Figure 2. A. Preparation of cultured skin substitute. Keratinocytes and fibroblasts are enzymatically isolated from a skin biopsy and placed into parallel tissue culture systems. When enough cells have been grown, the fibroblasts are placed on the open, bottom side of the collagen-GAG membrane, followed 2 days later by the keratinocytes on the opposite, laminated surface. After four additional days in culture, the grafts are ready for placement. B. Pre-placement cultured composite skin substitute. Histology of collagen-GAG membrane with human fibroblasts (HF) on the wound bed side of the graft and human keratinocytes (HK) on the upper, exposed side of the graft. Scale bar indicates 0.1 mm (hematoxylin-eosin, magnification $\times 80$).

48 h of culture period the calcium concentration was increased to 0.3 mM. The last 24 h in culture, EGF and BPE were removed and human recombinant basic fibroblast growth factor (100 ng/ml) (Amgen Biologicals, Thousand Oaks, CA) was added to help stimulate vascular ingrowth once placed onto the wound.

Athymic Mouse Model All animal studies in this experiment were approved by the UC San Diego Animal Subjects Committee. Seven- to eight-week-old athymic mice (Balb/c-nu/nu, Simonsen Laboratories, Gilroy, CA) were kept in a Duoflo (Bioclean Lab Products, Inc, Maywood, NJ) laminar flow hood and all handling was accomplished with sterile technique. All cages, water, mouse chow, and bedding were autoclaved prior to mouse inhabitation. Room temperature was maintained at 25–27°C and there were 12 h of light daily. No perioperative deaths were encountered and no animals were lost to infection over the 38-week course of the experiment.

Mice were anesthetized with 10.0 mg Avertin (tri-bromo ethanol in tert-amyl alcohol, Aldrich Chemical Co. Inc., Milwaukee, WI) by intraperitoneal (IP) injection. A 2 × 2 cm full-thickness excision was created on the left lateral side of each mouse, sparing the panniculus carnosus. Grafts from the four conditions were placed into this defect and N-terface (Winfield Laboratories, Richardson, TX), a non-adhering dressing, was placed onto the graft to minimize shear damage. Xeroform petrolatum gauze (Sherwood Medical: St. Louis, MO) was then placed and a tie-over dressing was employed by using silk sutures. Three Band-Aid adhesive bandages (Johnson & Johnson, New Brunswick, NJ) covered the dressings and kept the animals from disturbing the wounds. Mice were injected IP with 1.0 ml sterile saline with 3.0 mg ceftazidime (Fortaz, Glaxo, Research Triangle Park, NC) for 10 d postoperatively. Sulfamethoxazole and trimethoprim (Biocraft Laboratories, Elmwood Park, NJ) was added to autoclaved drinking water for the first four postoperative weeks. Mice were anesthetized with Penthrane (Abbott Laboratories, Chicago, IL) at various time points for obtaining biopsies. At time of sacrifice, a lethal dose of Avertin was injected IP and entire graft including panniculus carnosus was removed for evaluation.

Light Microscopy Tissue samples were fixed in 10.0% formalin, embedded in paraffin, sectioned at 3 μ m thickness, mounted onto slides, and stained with hematoxylin-eosin.

Immunoperoxidase Tissue was mounted on heat-fixed paraffin, sectioned, fixed with xylene and alcohol, and blocked with 0.6% hydrogen peroxide-methanol. S-100 immunoperoxidase staining was carried out by an indirect technique and counterstained with Mayer's hematoxylin.

Electron Microscopy Samples for transmission electron microscopy were fixed in Trump's fixative and 1% osmium tetroxide, dehydrated, infiltrated and embedded in epoxy resin, thin sectioned, mounted onto grids, and stained with lead citrate and uranylacetate. Microscopy was performed on a transmission electron microscope (Hitachi, Nissei Sangyo America, Mountain View, CA) operated at 75 kV accelerating voltage.

RESULTS

Biopsies were taken over the 38-week duration of this experiment for all of the four conditions. The six-week results were obtained by taking a 5 × 5 mm biopsy after the animals were anesthetized. The remainder of the results are derived from animals that were killed, with the entire graft taken. Results are presented in a chronologic fashion, with three major time points at 6 weeks, 20–24 weeks, and 34–38 weeks post-placement. Random grafts were tested for "take" by staining them with a fluorescein-labeled antibody specific for human HLA-ABC antigens [12]. Not all grafts were tested, but the random grafts evaluated were all positive for persistence of human cells.

Gross Appearance (Fig 3) Full-thickness nevus mice (group 3) retained the gross morphologic characteristics of the surgical specimen over the entire length of the experiment. These grafts contracted to approximately 60% of their original size, and retained pigmentation and hair follicles similar to that found on the patient. Group 4 (human allograft), retained its appearance on the mouse with some increased pigmentation, as seen with all allografts placed on athymic mice previously in this laboratory [11–14]. Contraction rates were somewhat greater than group 3. All of the group 1 (cultured, uninvolved skin from GCNN patient) mice showed typical morphologic characteristics of cultured skin grafts. The contraction was approximately 35% of original size. The resultant skin was of a color similar to that of the adjacent mouse skin, and had good shear strength and elasticity. No mouse at any time showed pigmented areas that resembled the dark, raised GCNN. In contrast, four of

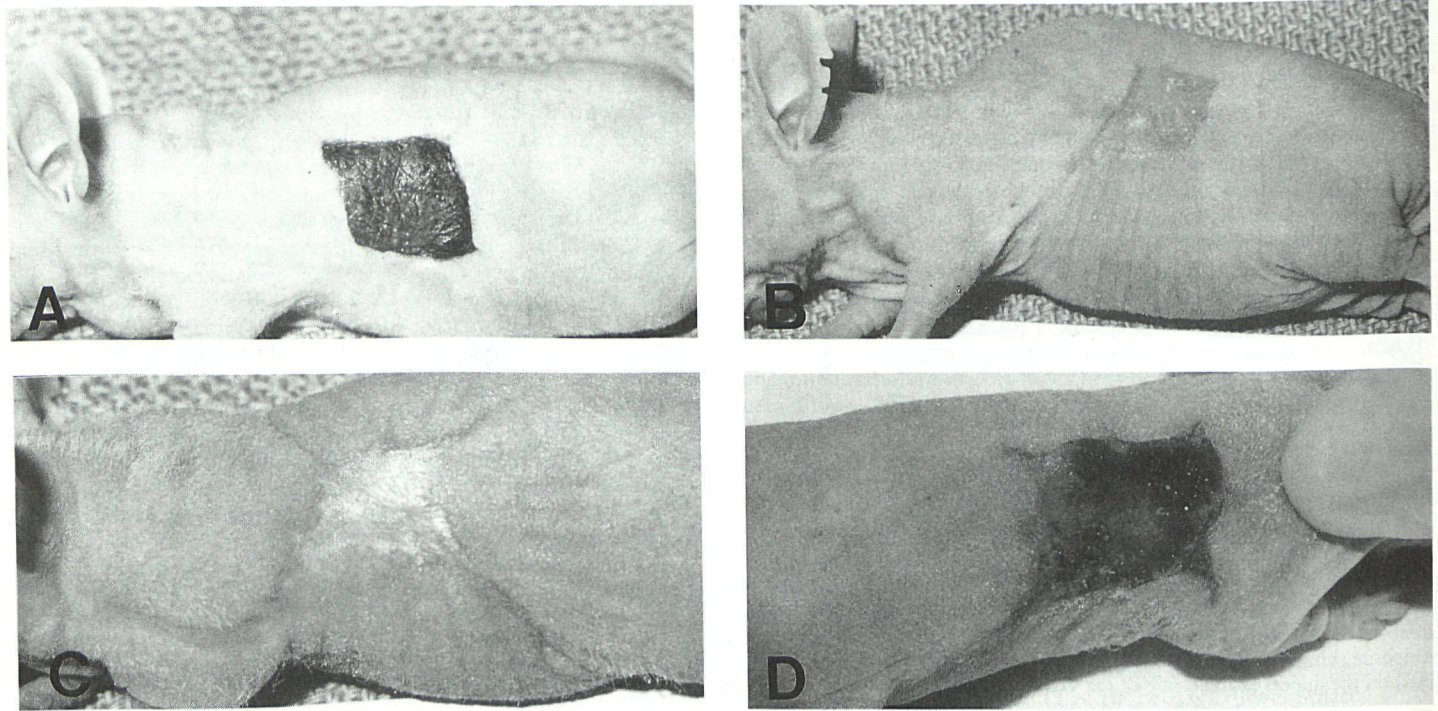


Figure 3. Gross appearance of grafts on athymic mice, 34–38 weeks post-placement. A) Full-thickness nevus skin graft; group 3. B) Human split-thickness xenograft; group 4. C) Cultured composite graft from uninvolved skin of nevus patient; group 1. D) Cultured composite graft from nevus skin; group 2.

the six mice in group 2 (cultured GCNN skin) showed black pigmentation of the entire grafted area. This was first apparent at one week post-grafting, and by three weeks post-placement the majority of the grafted area had a very dark, raised appearance. At no time in the study did any mouse develop a visible tumor or nodule.

Surgical Pathology Specimen from GCNN Patient The lesion showed typical features of a congenital melanocytic nevus (Fig 4). There was a band-like infiltrate of nevomelanocytes filling the entire reticular dermis, extending to the base of the specimen. At the dermoepidermal junction, there was basilar keratinocytic hyperpigmentation with no significant increase in the number of melanocytes. A small area of the superficial papillary dermis was free of nevus cells. In the papillary and superficial reticular dermis, there were nests and aggregates of large nevus cells (type A or epithelioid) with abundant melanin granules in their cytoplasm. These cells gradually became smaller in the mid-reticular dermis (type B or lymphoid nevomelanocytes). In the deep reticular dermis, the cells were small and some were spindle shaped (type C or nevoid). Melanin granules were not visible at this level. Nevus cells were seen oriented around adnexal structures, blood vessels, nerves, and at the base were seen singly between collagen bundles. Occasional type C nevus cells continued to have pigment granules.

S-100 Immunoperoxidase Study of Cell Suspensions Prior to Placement on Composite Grafts Once the dermal and epidermal cells were passed twice in culture, enough cells were present for placement onto the grafts. This expansion took approximately 2½ weeks. These cell suspensions were stained with S-100 immunoperoxidase antibody. The dermal cells of group 2 (GCNN cultured nevus) showed mostly fibroblasts, although a few cells appeared to be melanocytes. These melanocytes had very small nuclei and appeared to be non-proliferative (Fig 5A). The S-100 stains of the epidermal cells of group 2 clearly showed numerous keratinocytes, but also numerous, viable melanocytes. These melanocytes demonstrated S-100 staining in both their nuclei and cytoplasm (Fig 5B).

Six Weeks Post-Placement Cultured non-involved or “normal skin” from GCNN grafts (group 1) showed a healed wound, with re-establishment of the epidermis similar to our other cultured grafts at this time point. There was a decreased, but present, rete ridge pattern with some dermal fibrosis, and a lack of adnexal structures, as expected. There were no remnants of the collagen-GAG matrix, and the dermis was composed of rather uniform, plump fibroblasts, and small, well-formed blood vessels. There were no nevomelanocytes. Only a rare melanocyte was seen at the dermoepidermal junction (DEJ). Melanophages were not seen in the papillary dermis, either by light microscopy or transmission electron microscopy.

Cultured partial-thickness GCNN (group 2) mice showed histologic patterns that were consistent within the group, with only minor variations. All animals showed reformation of a normal-appearing epidermis with a characteristic rete ridge pattern. The four animals that grossly had the GCNN pigmentation return showed marked increases in basilar keratinocyte hyperpigmentation at the DEJ without an obvious increase in melanocytes at this level. In some sections there were small cells present at the DEJ producing abundant amounts of pigment, which had been transferred through visible dendrites to the surrounding keratinocytes. In the underlying dermis, there were cells with pigment in their cytoplasm. Some of these had uniform, small melanin granules, and the others showed clumping. Some of them may have been nevomelanocytes, the ones with uniform small particles of melanin, and the others may have been melanophages. Also, a few of the cells appeared to be bipolar or dendritic melanocytes with pigment extending as small granules into the arms of the bipolar structures. Transmission electron microscopic examination revealed melanophages in the papillary dermis, but no cells were found that were unequivocally nevomelanocytes.

Groups 3 and 4 retained their histologic features. That is, no change in the full-thickness nevus or the split-thickness xenograft were present after six weeks on the mouse. No infiltrate or other change was present in the underlying wound bed.

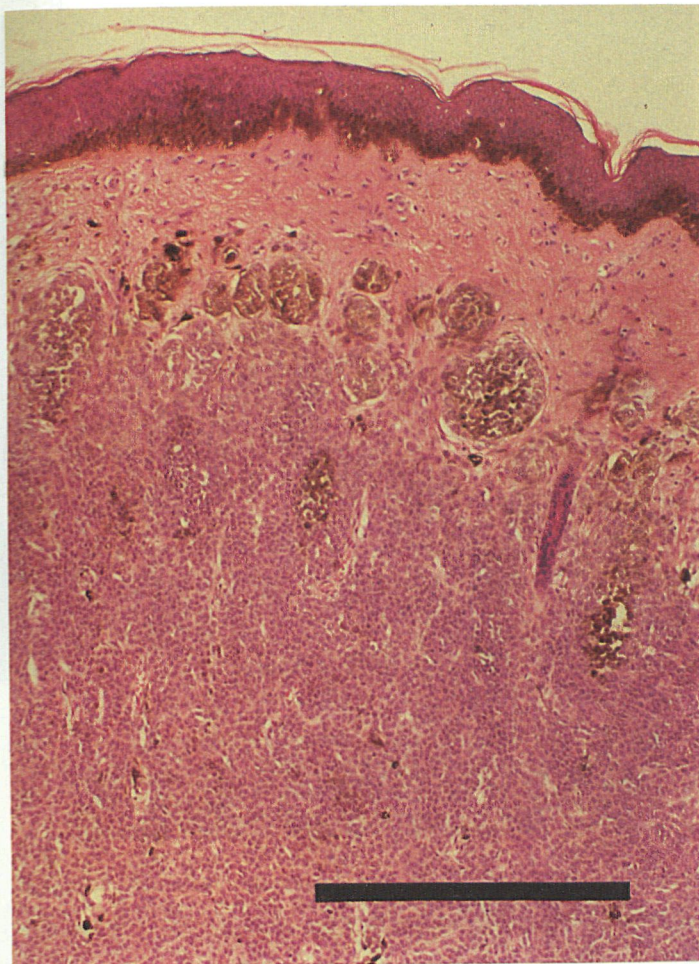


Figure 4. Histology of congenital nevomelanocytic nevus. Full-thickness specimen obtained at time of initial surgery shows typical features of congenital nevi. A band-like infiltrate of nevomelanocytes fills the reticular dermis, with an area of papillary dermis spared. Also, nests and aggregates of large nevus cells are seen in the papillary and superficial reticular dermis. Scale bar, 1 mm (hematoxylin-eosin, magnification $\times 40$).

Twenty to Twenty-Four Weeks Post-Placement The full-thickness nevus grafts (group 3) continued to show the characteristic congenital nevi features, but there appeared to be more fibrosis in the nevus itself (Fig 6A). Epithelioid melanocytes were seen more superficially, round lymphoid lymphocytes medially and smaller spindle cells or type C nevus cells in the deep dermis, but there appeared to be more collagen between the nevus cells than seen in the original specimen. This may represent a response to the grafting or a mechanism by which an ordinary nevus degenerates in an older person. No changes were seen in group 4.

Cultured normal grafts (group 1) showed a normal appearing epidermis without any suggestion of melanocytes. In the superficial dermis, there was some fibrosis and, again, no melanocytes appeared to be present. S-100 immunoperoxidase confirmed that there were no melanocytes present in the dermis.

Cultured nevus grafts (group 2) also had a normal-appearing epidermis but had considerably more hyperpigmentation at the DEJ, and there were numerous melanophages or possibly melanocytes in the superficial dermis. There were no obvious areas of reconstituted congenital nevus. S-100 immunoperoxidase showed a rare melanocyte at the DEJ with numerous melanophages in the superficial dermis, and a very rare positively staining cell in the dermis, which could be a nevomelanocyte. Again, no cells that were unequivocally nevomelanocytes could be found in the dermis with transmission

electron microscopy. Noted at this time on transmission electron microscopy were a few melanocytes at the DEJ contiguous with partially disrupted areas of the basement membrane. These cells appeared to be moving from the basal region of the epidermis through the basement membrane zone (Fig 9C).

Thirty-Four to Thirty-Eight Weeks Full-thickness nevus (group 3) continued to retain the characteristic pattern of the GCNN (Fig 6B). S-100 staining clearly showed positive nevomelanocytes in the pattern of a congenital nevus. Transmission electron microscopy of the superficial dermis revealed typical nevomelanocytes with abundant melanosomes (Fig 9A).

Cultured normal skin (group 1) had no changes from the previous time point (Fig 7A). S-100 stains showed no melanocytes or nevomelanocytes at the DEJ or in the dermis (Fig 7B).

Cultured nevus grafts (group 2) showed significant differences since the previous animals had been studied, 14 weeks before. The dermis had many round multinucleated cells, representing a reconstitution of the congenital nevomelanocytic nevus from which these cells were obtained. In the superficial dermis there were larger, more-epithelioid cells, a few of them multinucleated. The mid-reticular dermis had round cells producing pigment, and smaller spindle cells were present in the deep dermis (Fig. 8A). S-100 staining showed positivity of all of these cells in the dermis, extending to the fat (Fig 8B). This confirmed that these cells were indeed nevomelanocytes.

Transmission electron microscopic examination of samples taken from these two mice confirmed presence of nevomelanocytes in the mid-dermis, with prominent melanosomes (Fig 9B). At this point in time, these nevomelanocytes appeared very similar to those found in the full-thickness nevus (group 3) (Fig 9A).

DISCUSSION

The athymic or nude mouse is not a new model for the study of skin. This immunocompromised animal was discovered in 1962 by Grist in Glasgow and has been employed in studies of wound healing, skin metabolism, and reactions to disease [24]. Manning et al first described placement and long-term acceptance of human skin on these mice [25]. Other authors have transplanted xenografts, either as skin grafts or as subcutaneous implants to evaluate the biology of certain disease states. Among the uses of the athymic mouse is in the study of malignant melanoma. Efforts in this area have led to successful transplantation and persistence of human melanomas [26,27]. The athymic mouse has distinct advantages in the study of melanoma: transplanted tumors can be serially passed, histologically the tissue remains unchanged over time, surface antigens and tumor markers appear to be conserved, and treatment modalities show consistent effects not related to individual mice, but rather to the characteristics of the transplanted tumor [28]. Also, the cell cycles of these tumors appear to remain consistent over time [29,30], with such growth kinetics being an important aspect in the study of solid tumors.

The athymic mice that have retained transplanted subcutaneous implants of human melanoma can subsequently be studied for their melanocyte biology and response to chemotherapeutic agents, and in establishing detection markers. The disadvantages of these models include a 30–50% failure of “take,” and the inability to study the long latency period often found in melanomas. Also, though the histotypical features of melanoma are retained, this may not reflect the original biology of these cells.

Although melanomas have been transplanted onto athymic nude mice, there is currently no animal model that addresses giant congenital nevomelanocytic nevi. This paper describes two potential methods to study this lesion. First, the actual GCNN can be transplanted as a full-thickness skin graft onto athymic, immunocompromised mice. We had no graft failures in the eight animals that received the 2×2 cm full-thickness sections of nevus. These grafts retained all the histologic characteristics of the original nevus, including the growth of the hair present at time of transplant, over the

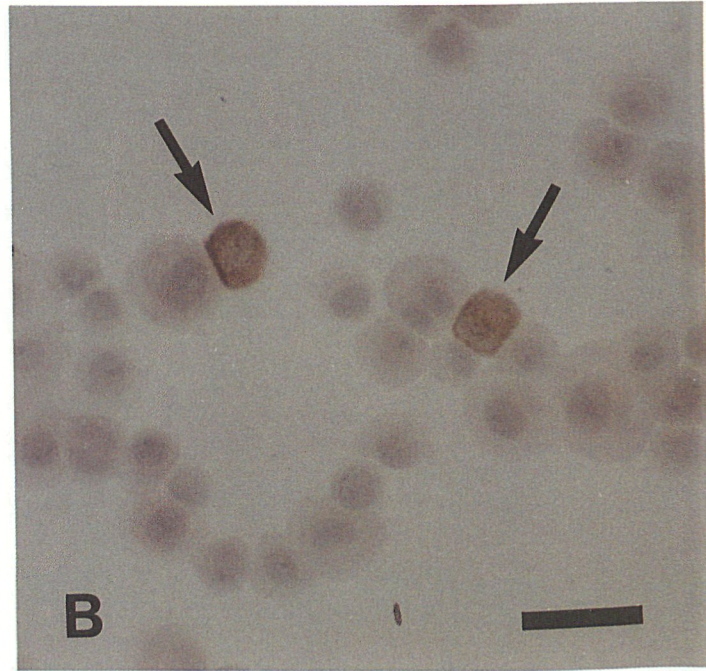
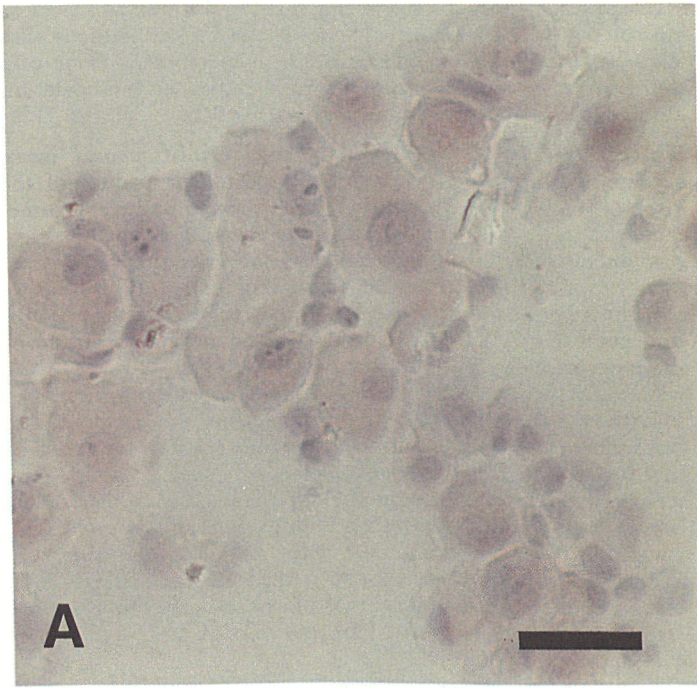


Figure 5. S-100 immunoperoxidase stain of pre-placement cells. *A*) Dermal cells after 2 weeks in culture and two passages. Majority of cells are viable fibroblasts with an occasional melanocyte, which appears non-proliferative. *Scale bar*, 0.1 mm (magnification $\times 150$). *B*) Epidermal cells after 16 d in culture and two passages. Numerous viable keratinocytes are seen, and also many viable melanocytes (*arrows*), which stain positive for S-100. *Scale bar*, 0.1 mm (magnification $\times 150$).

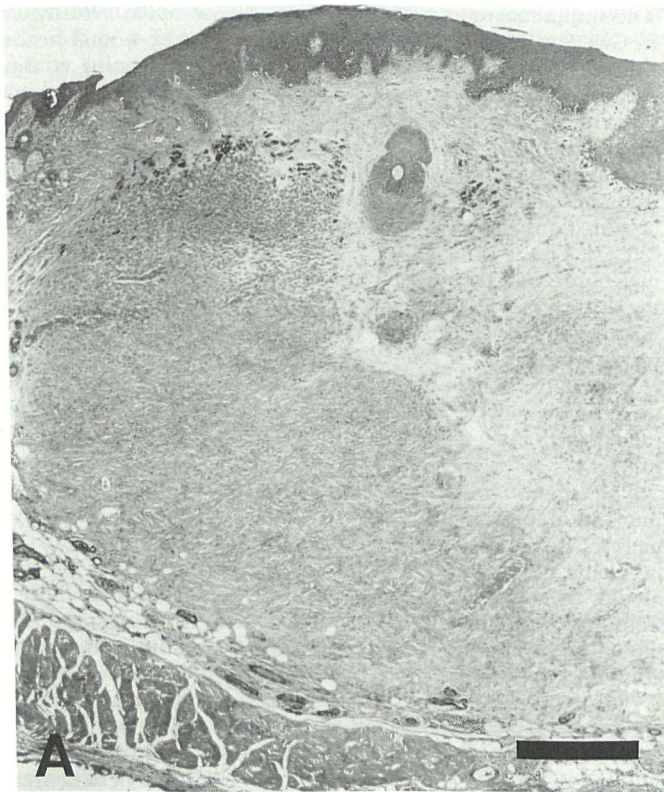


Figure 6. *A*) Histology of full-thickness nevus graft on the athymic mouse at 20 weeks post-placement. Characteristic GCNN features are present, but some fibrosis of the dermis is seen. *Scale bar*, 1 mm (magnification $\times 16$). *B*) Histology of full-thickness nevus graft on the athymic mouse at 34 weeks post-placement. The features of GCNN are still present, with somewhat more fibrosis of the dermis noted. Nests are also less distinct in the superficial dermis. *Scale bar*, 1 mm (magnification $\times 16$).

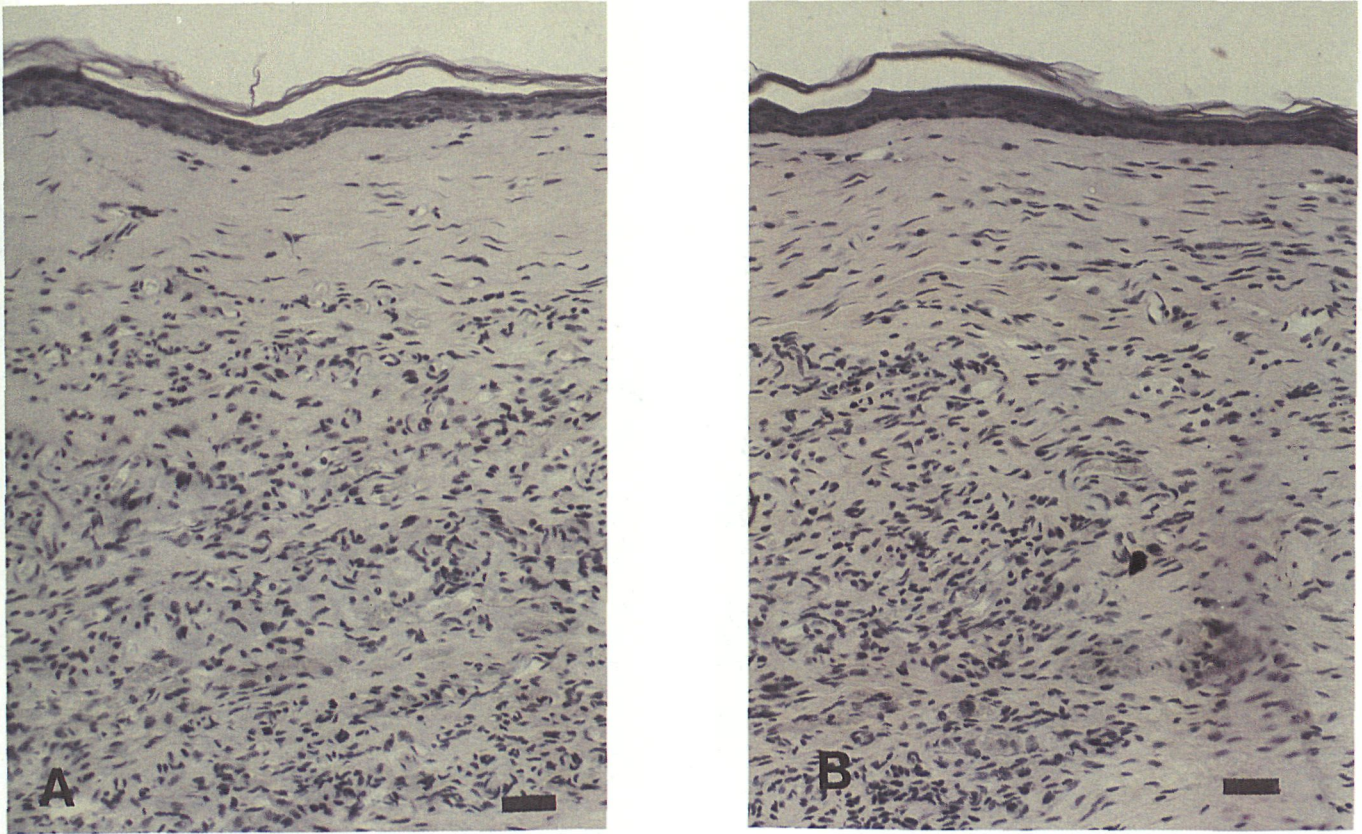


Figure 7. Histologies of cultured composite skin substitutes made from uninvolved skin of congenital nevomelanocytic nevus patient at 38 weeks post-placement on the athymic mouse (group 1). No nevomelanocytes are seen at the DEJ or in the dermis. Scale bar, 0.1 mm (magnification $\times 80$). A) Negative S-100 control. B) S-100 immunoperoxidase stain.

38-week course of this experiment. Therefore, the transplanted nevus can be studied in multiple ways. Transforming agents, including specific chemicals and ultraviolet light, could be employed to study the transformation potential of these lesions *in vivo*. Also, the nevomelanocytes can be maintained and passed onto other animals, as the life span of these mice is approximately 2 years. This model, however, shares the shortcomings of previous models for melanoma, as the latency period for transformation of congenital nevi to melanoma can be substantial and more than likely it is not a frequent event.

The second model generated in this study has other, potentially more significant features. That is, by culturing the cellular components of this congenital nevus, a model has been generated that can not only look at the biology of the nevomelanocytes, but also at the natural history of the lesion itself.

The characterization of congenital nevomelanocytic nevi has been difficult. Its features are inconsistent, as the nevus cells are predominately found both in the epidermis and/or dermis. The risk of transformation has not been well defined and removal of the lesion is disfiguring and often not completely possible [31,32]. Nickoloff carefully examined 29 patients for the presence of nevus cells by employing S-100 protein and myelin basic protein immunoperoxidase stains [33]. He showed that only two cases were intra-epidermal and 21% had deep reticular dermal infiltration, whereas all 29 had focal involvement of the adventitia of the eccrine ducts or follicular epithelium in the midreticular dermis or lower. He felt

that the latter point was most consistent with the features attributed to congenital nevomelanocytic nevi. Mark et al [34] and Rhodes et al [35] classified features of the CNN differently and felt that involvement of the collagen of the middle or deep third of the reticular dermis was quite specific and sensitive.

Though the most characteristic features of CNN are not agreed upon, the evolution of these lesions over time is even less understood. The majority of pediatric lesions show a junctional pattern of nevus cells, whereas adult nevomelanocytic nevi are more often completely isolated in the dermis. Unna's "dropping down" concept arose from this observation, although no one has shown this to be the case with sequential biopsies in individual patients.

The second model presented here, that of a cultured composite dermal-epidermal graft composed of cells derived from a GCNN, might possibly address this issue. Both the dermis and epidermis were treated enzymatically to release the normal cells found in each component, and put into appropriate tissue culture conditions. The epidermal cells were placed into serum-free MCDB 153 medium, which is optimized for human keratinocytes (HK), but also supports human melanocytes. The melanocytes that remain in culture do not proliferate well, as the keratinocytes overgrow them [36]. The addition of 12-O-tetradecanoyl phorbol 13-acetate (TPA) to MCDB 153 preferentially stimulates the melanocytes and retards the HK [37]. Also, nevus cells have been shown to proliferate in tissue culture [38] and are for the most part indistinguishable from epidermal melanocytes. They also show similar characteristics *in vivo* [39].

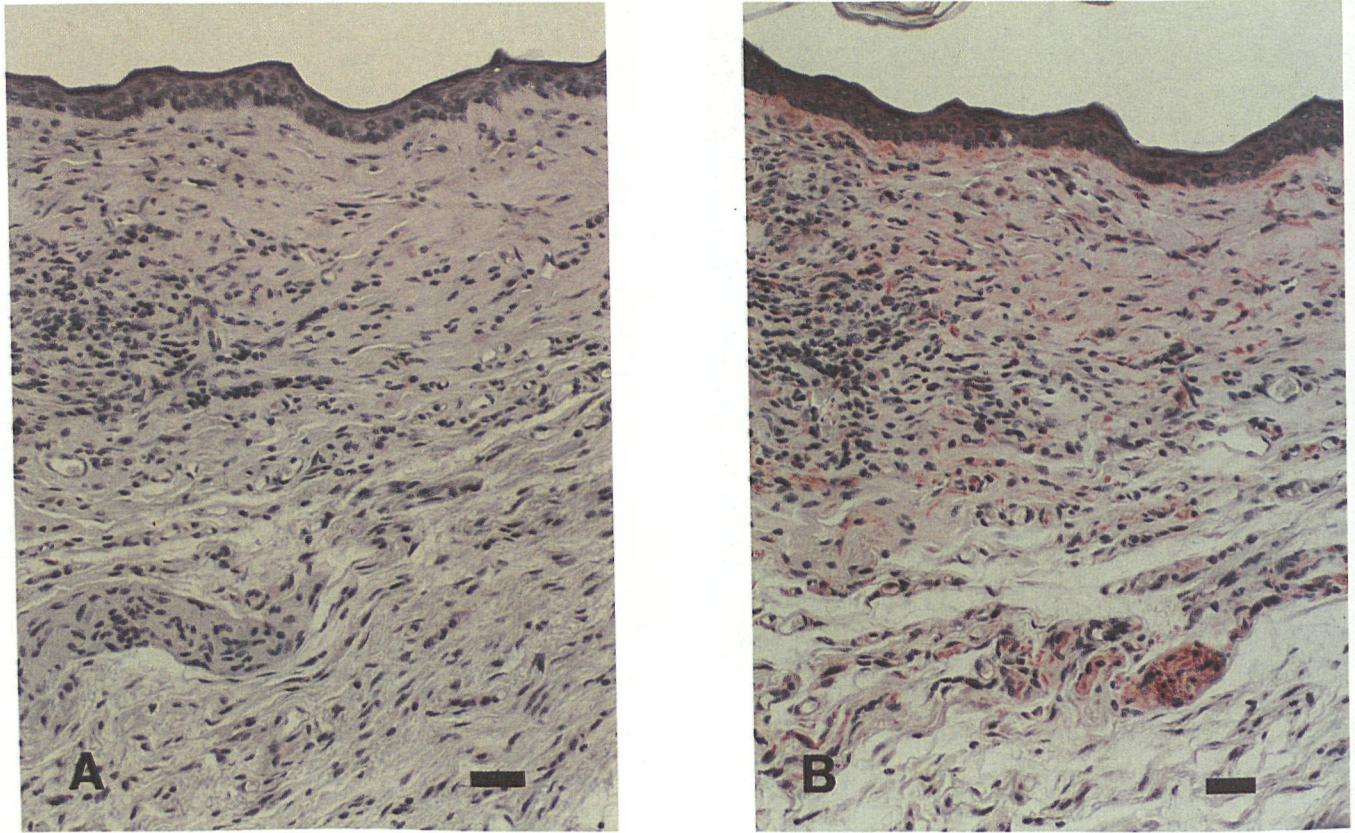


Figure 8. Histologies of cultured composite skin substitutes made from nevus skin of a congenital nevomelanocytic nevus patient at 38 weeks post-placement on the athymic mouse (group 2). Nevomelanocytes can be seen throughout the dermis. These cells are more epithelioid in the superficial dermis, and become smaller in the deeper dermis. These findings are consistent with a reconstitution of the histologic pattern of the congenital nevomelanocytic nevus from which these cells were obtained. Scale bars, 0.1 mm (magnification $\times 80$). A) Negative S-100 control. B) S-100 immunoperoxidase stain.

In our study, the melanocytes remained viable in MCDB 153 after two passes and were transplanted onto the cultured grafts along with the HK. This population of S-100 positive cells was taken from the actual nevus and, therefore, whether these cells were neval or epidermally derived melanocytes cannot be ascertained. The dermal component, though, had no apparent proliferative melanocytes. This is seen both by negative S-100 staining of dermal cells just prior to placement on the cultured graft and by a paucity of dermal melanocytes on the S-100 staining of grafts prior to 24 weeks post-placement. Electron microscopy duplicated these findings, and additionally showed that at 24 weeks melanocytes present were actively migrating through the DEJ.

The outcome of this study is that, over time, grafts from a cultured congenital nevomelanocytic nevi can recreate their original histologic pattern on the athymic mouse. The melanocytes that were present at the time of placement onto the graft were originally carried through culture with the epidermal cells. They went on to form junctional theques, with spreading of dendritic segments and melanin to adjacent keratinocytes. Finally, some of these cells appear to have migrated through the basement membrane of the DEJ and reconstituted the histologic appearance of nevomelanocytes. This process took place after multiple cell replications in vitro (approximately $20\times$) and greater than five months on the athymic mouse. The evolution of this nevus on the athymic mouse raises a

question asked by multiple authors, "Are these nevus cells inherently different than 'normal' epidermal melanocytes or does the environment influence their eventual expression?" Perhaps further understanding of the melanocytic differentiation pathway will help in understanding the role of nevus cells in determining their eventual outcome. Specifically, how do these cells that migrate to the dermis avoid being irreversibly committed to the epidermis [8-10,40]?

Lastly, this model was designed to be able to amplify the number of mice that could be used to study various aspects of GCNN. By making grafts from cells that have been expanded 100 to 1000 times, as many grafts as necessary can be made and placed onto the mice. This allows for large numbers of studies, requiring minimal amounts of tissue from an individual patient. An interesting factor in this model is the multiple replications of the melanocytes in vitro. How this rapid turnover changes the ultimate biologic behavior of these cells is unknown, but surely quite different than the rather slow rate found with melanocytes in situ.

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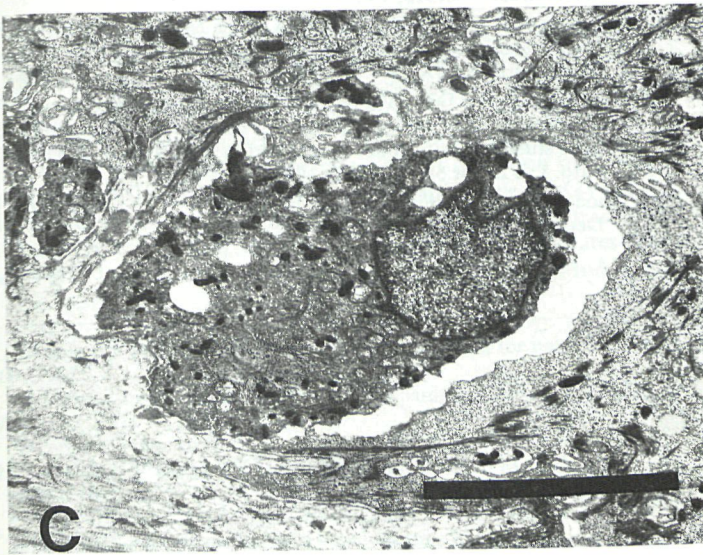
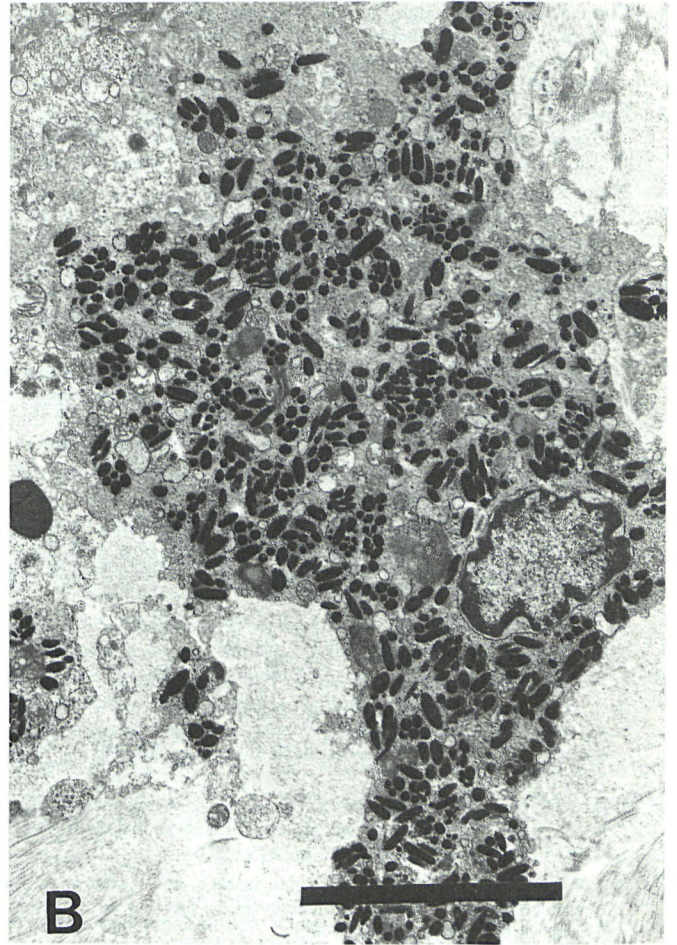
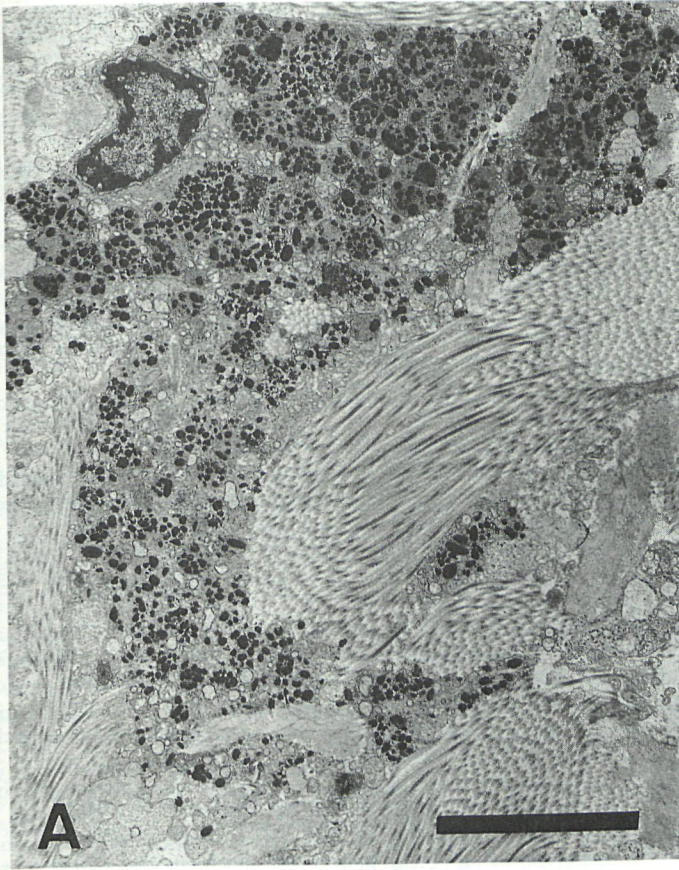


Figure 9. Transmission electron microscopy. *A*) Nevomelanocyte found in the mid-dermis of full-thickness nevus specimen from child with GCNN. Scale bar, 5 μ m (magnification $\times 5100$). *B*) Melanocyte found in the mid-dermis of athymic mouse at 38 weeks post-placement of composite cultured skin substitute made from nevomelanocytic nevus cells. The pigmented cells found at this level have the histopathologic characteristics of nevomelanocytes from the patient, including prominent melanosomes. Scale bar, 5 μ m (magnification $\times 6400$). *C*) Melanocyte at the dermoepidermal junction at week 20. Contiguous area of the basement membrane shows disruption. Scale bar, 5 μ m (magnification $\times 6300$).

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