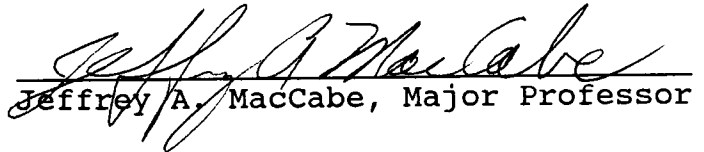
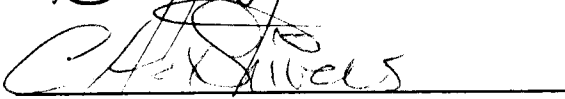


To the Graduate Council:

I am submitting herewith a thesis written by Eleanor Gallo entitled "Development of Interdigital Tissue as Revealed by Conditions That Alter Its Fate." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Zoology.


Jeffrey A. MacCabe, Major Professor

We have read this thesis
and recommend its acceptance:

Accepted for the Council:


Vice Provost
and Dean of The Graduate School

DEVELOPMENT OF INTERDIGITAL TISSUE AS REVEALED
BY CONDITIONS THAT ALTER ITS FATE

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Eleanor Gallo

May 1989

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ABSTRACT

The effect of Janus green B (JGB) on avian limb cell migration was studied by excising small portions of interdigital tissue from between all digits and culturing these explants with 10 $\mu\text{g/ml}$ JGB. All interdigital cell migration was inhibited by this dose. In a similar experiment wedges of interdigital tissue from between digits III and IV were incubated in 1.0-10.0 $\mu\text{g/ml}$ JGB. The results gave a typical dose response curve in which almost complete inhibition occurred with the high doses of JGB and lower doses gave less inhibition.

A second series of experiments was undertaken to study interdigital cell migration in vivo. However, it was discovered that transplantation of interdigital tissue resulted in ectopic chondrogenesis. This change of fate of these cells from death, and possibly migration, to chondrogenesis thus became part of the focus of this study.

In addition to transplantation, simple incisions were cut into III-IV interdigital tissue. Wounding interdigital tissue resulted in ectopic chondrogenesis. The more severe the wounding the more digit-like the extra cartilage. Transplantation of a complete interdigital zone resulted in the development of a complete extra digit and simple incisions resulted in the development of a partial digit.

To determine if exposure of the injured surfaces to amniotic fluid (AF) caused the ectopic chondrogenesis, amniotic fluid was removed and replaced with sterile phosphate buffered saline (PBS) prior to doubly incising interdigital tissue. This failed to inhibit the ectopic chondrogenesis. Exposing interdigital tissue to AF in vitro also failed to inhibit chondrogenesis. These results suggest changes within the interdigit, possibly to nutrient or oxygen availability, are responsible for the formation of ectopic digits.

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I. INTRODUCTION

The development of a multicellular organism involves growth, differentiation, and morphogenesis, beginning with a fertilized egg and ending with the adult form of the organism. Although cell death seems inconsistent with the goal of increased size and complexity, it is nevertheless important both in the embryo and the adult (Saunders and Fallon, 1967). In adult tissue there is a balance between cell proliferation and cell death such that tissue size is maintained (Hinsull and Bellamy, 1981). A developing organism increases in size so that, in general, cell growth greatly exceeds cell death. Nevertheless, during specific stages of development massive localized cell death occurs. Examples are the degeneration of the Wolffian or Mullerian ducts during sexual differentiation (Hinchliffe, 1981); cell death in the anterior, posterior, and interdigital necrotic zones (ANZ, PNZ, and INZ respectively), and opaque patch (OP) in limb development (Hinchliffe, 1981; Hinchliffe and Ede, 1973; Hurle and Climent, 1987; Dawd and Hinchliffe, 1971); and the reabsorption of the amphibian tail during metamorphosis (Lockshin, 1981).

Cell death and its regulation are encoded in the genome as are growth, morphogenesis, and their regulation (Saunders and Fallon, 1967). The trigger to initiate the

cell death program may be internally or externally derived, although little is known about the actual signals which initiate cell death (Saunders and Fallon, 1967; Hinchliffe, 1981). One hypothesis is that cells divide a specified number of times after which they die (Hinsull and Bellamy, 1981). Another hypothesis is that cells which are programmed to die produce specific "cell death" proteins just prior to death (Pollack and Fallon, 1974). In some instances hormones have been shown to stimulate cell death (Hinsull and Bellamy, 1981). Prior to sexual differentiation both male and female rudimentary reproductive structures are present in vertebrate embryos (Hinchliffe, 1981). In males, testosterone causes the Wolffian ducts to differentiate into the epididymus and vas deferens, and Mullerian Inhibiting Hormone causes the Mullerian ducts to regress (Hinchliffe, 1981). In females estrogen causes the Mullerian ducts to differentiate into the oviducts and without testosterone to maintain them, the Wolffian ducts regress (Hinchliffe, 1981). In amphibians high levels of thyroxine, which stimulate muscle growth in the legs, stimulate cell death during reabsorption of the tail at metamorphosis (Lockshin, 1981).

Cell death in the embryonic limb, especially in the chick embryo, has been studied in detail (Fallon and Saunders, 1968; Kelley, 1970; Dawd and Hinchliffe, 1971; Hinchliffe and Ede, 1973; Hinchliffe and Thorogood, 1974;

Pautou, 1975; Hurle et al., 1981; Rowe and Fallon , 1982; Hurle and Climent, 1987), yet there are still many unanswered questions. The major areas of cell death in the developing limb include the ANZ, PNZ, OP, and INZ (Hinchliffe, 1981).

The INZs are areas of cell death which free and shape the final form of the digits (Saunders and Fallon, 1967; Kelley, 1970; Pautou, 1975; Hinchliffe, 1981; Hurle et al, 1981; Hurle and Climent, 1987). They appear later in development than the ANZ, PNZ or OP. A variety of species have INZs: avian species, humans, mice, rats, lizards, and turtles (Hinchliffe, 1981; Saunders and Fallon 1967; Hurle and Climent, 1987). In contrast, amphibia do not have INZs (Fallon and Cameron, 1977), suggesting the INZ's first phylogenetic appearance occurred during the evolution of reptiles from ancestral amphibians.

Although many species have INZs, the extent of cell death varies from species to species. Extensive interdigital cell death occurs in the chick embryo while in the duck it occurs to a lesser degree resulting in the characteristic webbed foot (Saunders and Fallon, 1967; Hinchliffe, 1981). The only exception in the duck is the cell death which occurs between the first and second digits where there is no webbing in the adult foot; in this case, interdigital necrosis is as extensive as that between digits I and II in the chick footplate (Hinchliffe, 1981).

In Rallidae species (Coot and Moorhen) which have lobes along the margins of the toes rather than complete webbing, the INZs are thinner than those in the chick (Hurle and Climent, 1987). One mutant of chick embryos, talpid³ (ta³), is distinguished by the absence of INZs in the forelimb and a reduction or absence of these in the hindlimb resulting in total soft tissue syndactyly in the wing and complete to partial syndactyly in the foot (Hinchliffe and Thorogood, 1974). These observations support the idea that INZs function to separate the digits (Hinchliffe and Thorogood, 1974). In one transgenic mouse strain a pRSV-CAT DNA sequence inserted into a gene causing abnormal development in which homozygous recessive mice were born with syndactylous toes on all feet (Overbeek et al., 1986). Overbeek et al. (1986) do not indicate whether or not a decrease in cell death was associated with the syndactyly.

After the interdigital tissue is removed by cell death, necrosis continues along the anterior and posterior margins of the digits. This persistence of cell death is believed to shape the final form of the digits (Hinchliffe, 1981; Hurle et al., 1981; Hurle and Climent, 1987). Kelley (1970) observed that while some interdigital mesenchymal cells are dying, others continue to divide. He suggested that the viable cells could possibly migrate to the digital

areas and thus contribute to the formation of the digits (Kelley, 1970).

Another suggested function of the INZ is to limit the number of digits which form (Hurle and Climent, 1987). Inhibition of cell death induced by JGB can result in ectopic cartilage formation in the interdigital tissue (Fernandez-Teran and Hurle, 1984). This ability of cell death to limit digit number has also been attributed to the ANZ and PNZ (Hinchliffe, 1981). Avian species which have three digits in the wing and four in the foot have ANZs and PNZs, while the mouse and rat which have five digits do not have ANZs or PNZs (Hinchliffe, 1981). The ta³ chick embryo, which develops polydactylous limbs, lacks ANZs, PNZs and INZs (Hinchliffe and Thorogood, 1974; Hinchliffe, 1981). From these results it seems the ANZ, PNZ, and INZs are all involved in limiting the number of digits formed.

Along with a description of INZs, details which are known about the actual mechanism by which death occurs at the cellular level should be examined. Historically it was believed that cells programmed to die synthesized lysosomal enzymes which the cells used to commit suicide (Saunders and Fallon, 1967). It is now accepted that cell death is a more complex process. Certain cell types undergo autophagy and autolysis, such as the epithelial cells in chick mesonephros regression and in Mullerian duct regression, and in non-muscle tissues in the amphibian tail during

metamorphosis (Hinchliffe, 1981; Saunders and Fallon, 1967; Lockshin, 1981). In other tissues, cells which are programmed to die deteriorate (without the aid of lysosomal enzymes) and fragment. This fragmentation stimulates neighboring cells to differentiate into macrophages and become phagocytic. The macrophages then ingest and digest many dead and dying cells (Saunders and Fallon, 1967; Hinchliffe and Ede, 1973; Dawd and Hinchliffe, 1981), although it is not known whether macrophages actively kill cells or simply clean up the debris. Tissues which utilize this type of cell death include mesenchymal cells of the necrotic regions of the limb (Hinchliffe, 1981; Hinchliffe and Ede, 1973; Dawd and Hinchliffe, 1971; Saunders and Fallon, 1967) and amphibian tail muscle during metamorphosis (Lockshin, 1981; Hinchliffe, 1981).

Specific teratogens have aided the study of embryonic development by disrupting one-to-several processes in the embryo. One teratogen, JGB, has been used to study cell death during the development of the chick embryo (Lazarow and Cooperstein, 1953; Braun, 1966; Stein et al., 1969; Fallon 1972a & b; Kieny et al., 1976; Pautou, 1978; Fernandez-Teran and Hurle, 1984; Hurle et al., 1986; MacCabe, 1986).

The deleterious effects of JGB on developmental processes in the chick embryo may be attributed to the action of JGB within cells (Lazarow and Cooperstein, 1953;

Braun, 1966). JGB is a vital stain for mitochondria which interferes with cellular respiration (Lazarow and Cooperstein, 1953; Braun, 1954, 1966). The dye binds to a specific lipoprotein in the mitochondria which results in the uncoupling of oxidative phosphorylation (Lazarow and Cooperstein, 1953; Braun, 1966) and thus decreases energy production and anabolism in the treated cells (Braun, 1966; Stein et al., 1969; Kieny et al., 1976).

One of the more apparent effects of treatment is the subsequent soft-tissue syndactyly and hypophalangy in the hindlimb (Braun, 1966; Stein et al., 1969; Kieny et al., 1976; Pautou, 1978; Hurle et al., 1986). JGB-induced syndactyly is associated with an inhibition of cell death in the interdigital membranes as seen by a decrease in macrophage differentiation or function. An in vitro study of the effect of JGB on interdigital tissue showed that JGB may inhibit both macrophage function early and cell death during exposure (MacCabe, 1986).

Further investigation indicates the effect of JGB may be far more complex than simply the inhibition of cell death. In 1965, Deleanu hypothesized that Janus green could effect the epithelial-mesodermal interactions resulting in a reduction of the macrophage number and in an inhibition of cell death in the interdigital mesoderm. Later studies revealed JGB does alter the epithelial-mesodermal interface (Hurle et al., 1986; Fernandez-Teran

and Hurle, 1984; Pautou, 1978; Kieny et al., 1976). This information and the fact that JGB causes syndactyly and hypophalangy in the embryonic chick foot indicates that the interaction of the ectoderm and mesoderm is necessary for normal regression of interdigital tissue and for outgrowth of the digits (Hurle et al., 1986; Fernandez-Teran and Hurle, 1984; Pautou, 1978; Kieny et al., 1976).

The ectoderm, including the apical ectodermal ridge (AER), of the developing limb is adversely effected by JGB. The number and size of the gap junctions in the AER are reduced, but do not completely disappear as in normal interdigital AER regression (Hurle et al., 1986; Fernandez-Teran and Hurle, 1984). The AER flattens prematurely and does not regain its normal structure even after the JGB is reduced (Fernandez-Teran and Hurle, 1984; Fallon, 1972b). The JGB-induced inability of the AER to direct proper outgrowth and normal formation of the digits may be due to the disruption of the structure and therefore the function of the AER (Hurle et al., 1986; Fernandez-Teran and Hurle, 1984; Pautou, 1978; Kieny et al., 1976). This is supported by the observation that removal of the AER results in a truncated limb. (Rowe and Fallon, 1982). The effect of JGB on the ridge may therefore result in the observed hypophalangy.

Specific developmental events are slowed or inhibited by JGB-treatment resulting in partially formed, malformed,

or absent structures (Mayerson and Fallon, 1984; Fernandez-Teran and Hurle, 1984). Feather germs which developed prior to JGB-treatment were normal while those developing at the time of treatment developed more slowly. Feather germs which had not yet begun to develop at the time of treatment did not develop at all (Mayerson and Fallon, 1984). Interdigital cells which are programmed to die do not if JGB is administered prior to the onset of cell death (Fernandez-Teran and Hurle, 1984; Fallon, 1972a). Interdigital cells which are allowed to recover from the teratogenic effects of JGB do not die presumably because the time frame for them to die has passed; these recovered cells appear normal like the digital cells (Fallon, 1972a). Presumably, these cells can then differentiate similar to their neighboring cells, such as in the case of ectopic cartilage nodule formation in JGB-treated interdigital tissue (Fernandez-Teran and Hurle, 1984).

The above-mentioned effects of JGB on developing embryonic tissue indicate that developmental events occur during specific time frames. If these events are hindered the consequences may be: i) delayed development; ii) inhibition of a particular developmental event; or iii) if the event is in a cascade of developmental events, that series of events may be halted resulting in a

malformed structure (Mayerson and Fallon, 1984; Fernandez-Teran and Hurle, 1984).

The experiments described in this paper suggest that in addition to inhibiting cell death, JGB may inhibit the migration of mesodermal cells out of the interdigital regions as suggested by Kelley (1970). In addition, experiments show that slight wounding of interdigital tissue can have a dramatic effect on its differentiation. This has important implications for the study of physical causes of birth defects.

II. MATERIALS AND METHODS

Fertile White Leghorn chicken eggs, obtained from the University of Tennessee Poultry Farm, were incubated in a 37.5-38.0 °C humidified incubator for three days. At this time 2 mls of albumin were removed from each egg, and windows were cut into the shells and sealed with parafilm (American Can Co.). The eggs were returned to the incubator and incubated until 6 1/2 days (stage 29) of development, after which one of the following experiments was performed.

Janus Green B In Vitro

Wedges of interdigital tissue were dissected from between all digits of stage 29 (6-6 1/2 day) chick embryos. The proximal portion of the interdigital wedges were excised (Fig. 1) and cultured in 10 µg JGB/ml Eagle's Minimal Essential Medium (MEM, Gibco Laboratories) + 10% calf serum (CS) in 10 µl well microtest plates. This is approximately equivalent to the in vivo dose that produces syndactyly (10 µg injected into the amnion, which has a 1.0 ml volume at 6.5 days of incubation).

For examining a dose-response relationship only interdigital tissues from between digits III and IV were used while varying the JGB concentration (10.0, 5.0, 2.5,

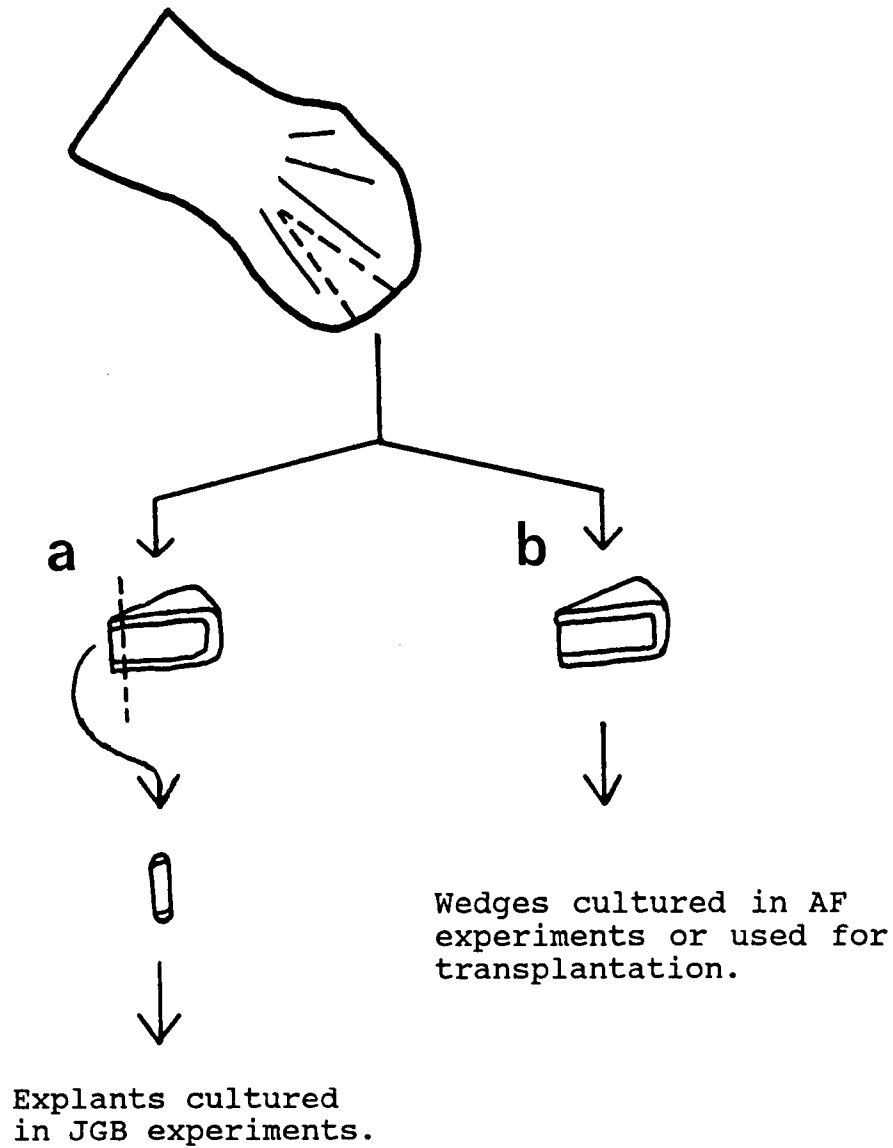


Fig. 1. Diagrammatic illustration of the portions of interdigital tissue used in the experiments. (a) The proximal portion of the interdigital wedges were cultured in the JGB experiments. (b) Whole interdigital explants were cultured in the in vitro AF experiments or used for transplantation.

and 1.0 $\mu\text{g/ml}$). Controls were cultured in MEM + 10% CS. All tissues were incubated at 37-38 °C in a humidified incubator with 5% CO₂.

The areas of the tissues in contact with the bottoms of the culture wells were determined by outlining the perimeter of the tissues using a Vanox Olympus microscope with a camera lucida before and after 24 hours of incubation. Measurements of these areas were calculated using the Bioquant System IV computer program (R & M Biometrics, ver. 3.16.88) with a Hipad Digitizer (IBM) and a Wild dissecting microscope with a camera lucida. The Student's t-test was performed on the data from the dose-response experiment using a $p = 0.05$ significance level.

Transplantation of Interdigital Tissue

Wedges of interdigital tissue were excised from between digits III and IV of 6-6 1/2 day (stage 29) chick embryos (Fig. 1, p. 12) and transplanted, after removal of a comparable amount of host tissue, to one of the following: (a) the III-IV interdigital area of another embryo in the same orientation (b) the original embryo in the same orientation, or (c) the thigh of the embryo. The embryos were left at room temperature to allow the transplants to adhere to the host and then incubated. Embryos were sacrificed after one, two or four days. The

legs were excised, fixed in 10% formalin, and either (a) stained with Victoria Blue or (b) paraffin embedded, serially sectioned at 7 μ m, and stained/counterstained with Alcian blue/nuclear fast red to examine cartilage patterns.

Simple Incisions in Interdigital Tissue

One or two incisions were made completely through the III-IV interdigital tissue of 6-6 1/2 day (stage 29) chick embryos which were then incubated at 37.5-38.0 °C in a humidified incubator. Embryos were sacrificed after one to four days incubation. The legs were excised and processed as described above.

Amniotic Fluid In Vivo and In Vitro

AF was removed from 6 1/2 day (stage 29) chick embryos (1.0 - 1.8 ml) and replaced with the same amount of sterile PBS. The III-IV interdigital tissue was doubly incised in three embryos. All embryos were incubated at 37.5-38.0 °C in a humidified incubator for one or four days after which the legs were excised, and processed as described above.

In a related experiment, III-IV interdigital tissue wedges were cultured in MEM + 10% CS + 10% AF, MEM + 10% AF, or MEM + 20% AF. Controls were cultured in MEM + 10%

CS. Cultures were incubated in a humidified incubator with air and 5% CO₂. Cultures were observed and media were changed daily for 6 days.

III. RESULTS

The Effect of JGB on Interdigital Mesodermal Cell Migration

Interdigital tissues were excised, placed into culture wells, and incubated for 24 hours in the presence and absence of 10 μ g/ml JGB. The areas of the tissues in contact with the bottom of the culture wells were outlined using a camera-lucida apparatus before and after the incubation period. The areas were then quantified using the method described in the materials and methods.

JGB adversely effected interdigital mesodermal cell migration in vitro. Control mesodermal cells migrated away from a block of explanted tissue while 10 μ g/ml JGB in the culture medium almost completely inhibited this migration (Table 1, Fig. 2). JGB-treated and control cells clearly behaved differently in culture. Untreated mesodermal cells attached to the bottom of the microtestplate wells, became fibroblastic, and migrated away from the interdigital tissue mass in a confluent monolayer. Mesodermal cells in JGB appeared not to attach as well, generally remained rounded in shape, and did not migrate appreciably from the interdigital tissue explant. These cells stained a steel-blue color in JGB-treated cultures.

The data also show differences in the migratory/spreading ability of the different interdigital regions.

Table 1: Migration of cultured cells (measured as an increase in area of the tissue in contact with the culture plate) from the interdigital tissue mass after a 24 hour incubation in the presence or absence of 10.0 μ g JGB/ml medium.

Interdigital Area	Mean Percent Increase in Area $\pm$ S.D.	
	JGB-Treated	Control
I-II	4.77 $\pm$ 17.74 (25)*	582.11 $\pm$ 479.54 (25)
II-III	16.00 $\pm$ 24.66 (35)	898.48 $\pm$ 501.98 (23)
III-IV	22.74 $\pm$ 57.58 (46)	1146.92 $\pm$ 562.41 (32)

*numbers in parentheses indicate sample size.

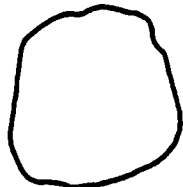
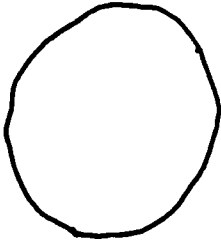
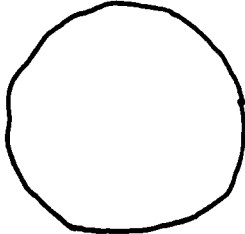
<u>Interdigital region</u>	<u>Area of tissue attachment before incubation</u>	<u>Area of tissue attachment after 24 hours of incubation</u>
a) I-II with JGB		
b) I-II Control		
c) II-III with JGB		
d) II-III Control		
e) III-IV with JGB		
f) III-IV Control		

Fig. 2. An example of the inhibition of interdigital cell migration in vitro by 10 μ g/ml JGB after 24 hours of incubation (a, c, e). Explants were surgically removed from between digits I and II (a, b), II and III (c, d), and III and IV (e, f). 50X.

The most anterior region (I-II) migrates the least and the most posterior (III-IV) migrates the farthest (Table 1, p. 17). All doses and all interdigital regions gave considerable variation in response from one culture to the next. The origin of this variability is not known.

JGB was shown to inhibit cell migration from a block of explanted mesoderm. The inhibitory effect was dose dependent (Table 2; Fig. 3). All doses tested (10.0, 5.0, 2.5, 1.0 μ g/ml) showed statistical differences ($p > 0.05$) in cell migration when compared with the control. While there is a statistical difference between cell migration in the control cultures and the 1.0 μ g/ml JGB cultures, there is also a notable overlap between their ranges due to the variability from culture to culture. This overlap may result from the variation in the ability of cells from different embryos to overcome the toxic effects of JGB. The control explants also demonstrated a range of migratory values suggesting genetic differences may account for some of the variance. Inbred chick strains are not generally available and were not used for these experiments. Some very low values for cell migration could be due to the explants having been positioned with ectoderm in contact with the microtestplate so that the mesoderm did not attach or attached late to the bottoms of the culture wells.

The treatment of interdigit III-IV with doses of JGB from 1.0-10.0 μ g/ml gave a typical dose-response

Table 2: Migration of III-IV interdigital mesodermal cells in various concentrations of JGB after a 24 hour incubation period.

JGB Dose ($\mu\text{g/ml}$)	Mean Percent Increase $\pm$ S.D.
10.0	22.74 $\pm$ 57.58 (46) ^{1,2}
5.0	11.24 $\pm$ 19.00 (12) ²
2.5	168.94 $\pm$ 167.39 (12) ²
1.0	748.28 $\pm$ 576.92 (14) ²
Control (0 $\mu\text{g/ml}$)	1146.92 $\pm$ 562.41 (32)

¹numbers in parentheses indicate sample sizes

²p > 0.05, using the t-test to compare values for the JGB-treated and control cultures

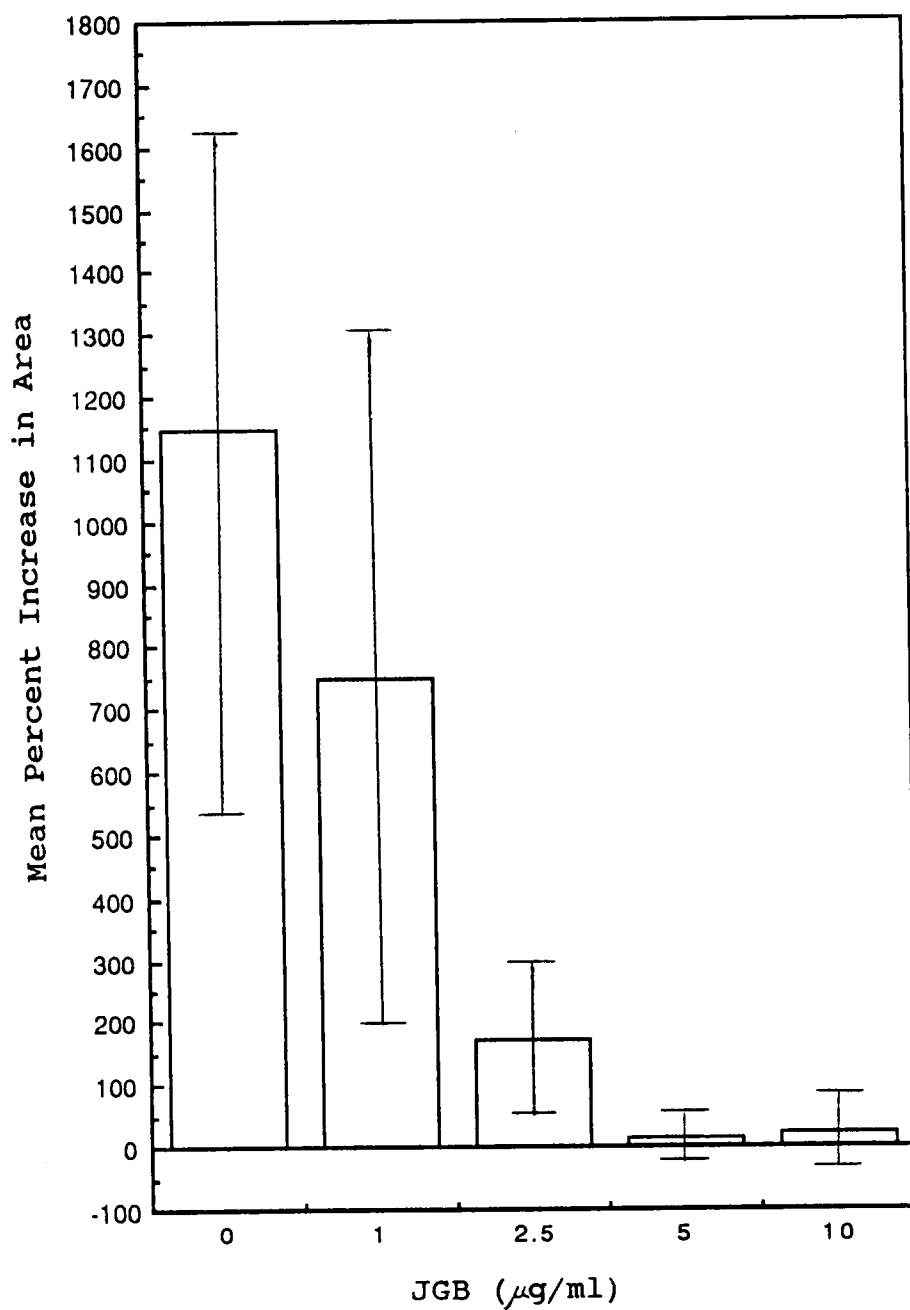


Fig. 3. Migration of III-IV interdigital mesodermal cells in various concentrations of JGB after 24 hours of incubation. Bars indicate range of migratory values. Note overlap of ranges between the 1.0 $\mu\text{g/ml}$ JGB dose and the control (0.0 $\mu\text{g/ml}$).

relationship. Almost complete inhibition of cell migration occurred at 5.0 and 10.0 $\mu\text{g/ml}$, whereas 1.0 and 2.5 $\mu\text{g/ml}$ gave less inhibition (Table 2, p. 20).

At 5.0 and 10.0 $\mu\text{g/ml}$ JGB mesodermal cells generally did not attach well, remained rounded, and did not migrate. At 2.5 and 1.0 $\mu\text{g/ml}$ JGB mesodermal cells appeared less elongated than the controls, somewhat intermediate in shape between the fibroblastic control cells and the rounded high-dose JGB-treated cells. These cells attached and migrated, but not as effectively as controls without JGB in the medium.

The Effect of Transplantation on Interdigital Differentiation

In a separate series of experiments complete interdigital wedges of tissue were excised and either transplanted to a separate host (from which the corresponding piece had been removed) or replaced in the donor embryo. The embryos were allowed to develop for an additional one to four days.

Surgically implanting a wedge of III-IV interdigital tissue in the same or a separate host embryo resulted in ectopic cartilage formation (Table 3, Fig. 4). After 24 hours of incubation the distal portion of the experimental interdigit resembled the distal portion of a prospective

Table 3: Frequency of ectopic chondrogenesis resulting from transplantation of III-IV interdigital tissue.

Host/ Donor	Days Incubated	Total Embryos	No. with Ectopic Chondrogenesis
Separate Embryos	1	24	18
	2	15	15
	4	<u>3</u>	<u>3</u>
	Total	42	Total 36 (85.7%)
Same Embryo	1	6	6
	2	2	2
	4	<u>8</u>	<u>7</u>
	Total	16	Total 15 (93.8%)
Transplant to Thigh	1	2	2

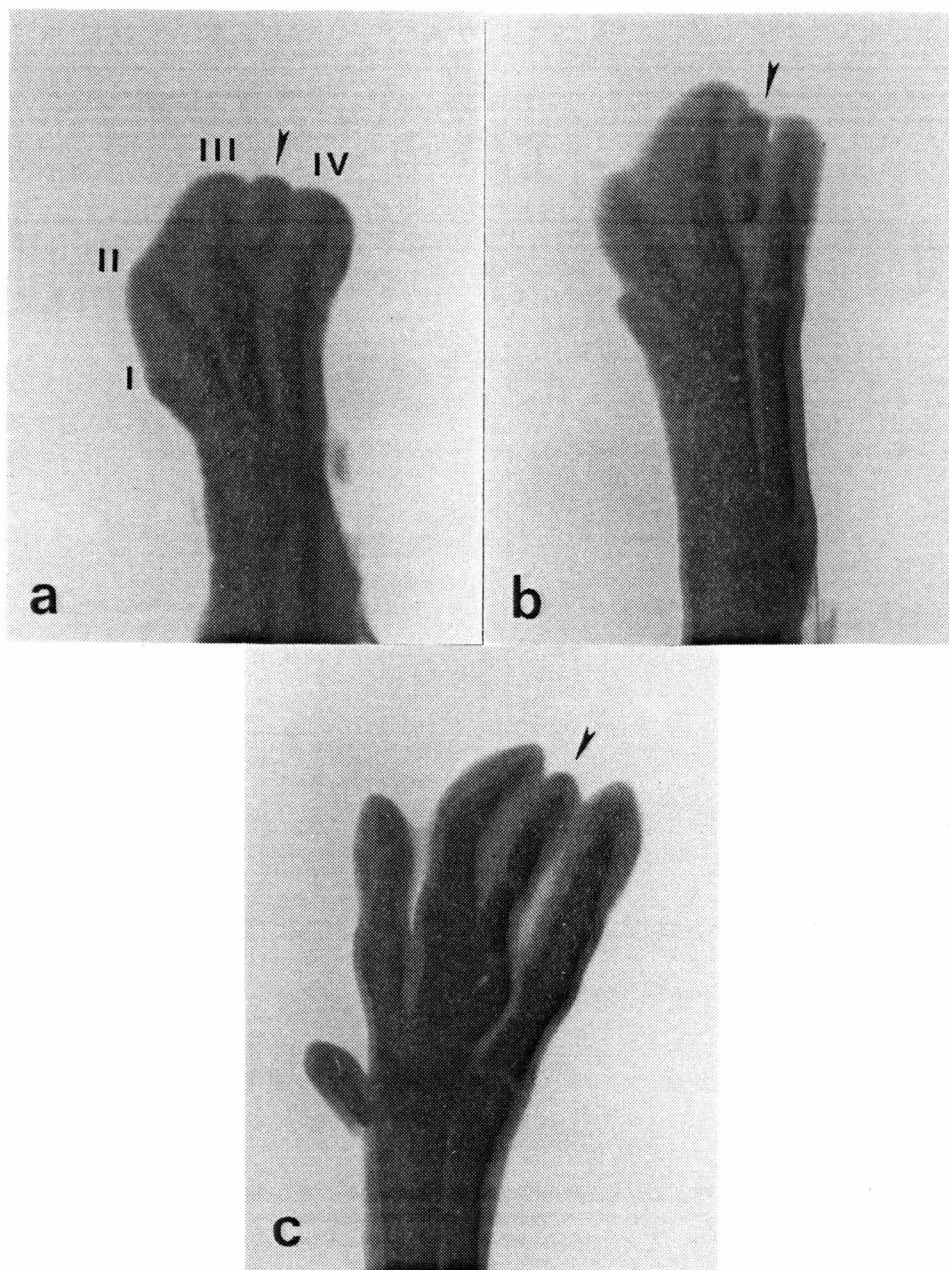


Fig. 4. Ectopic chondrogenesis after transplanting III-IV interdigital tissue; following (a) one day, (b) two days, and (c) four days of incubation. Note that in (1c) complete polydactyly has resulted from transplanting interdigital tissue. Arrows indicate ectopic cartilage. The cartilage was stained with Victoria Blue. 180X.

digit including prechondrogenic condensations, suggesting a local decrease in cell death and/or migration and thus a change in developmental fate. More proximally this area appeared to be normally regressing interdigital tissue. Several embryos exhibited patches of mesoderm devoid of ectoderm dorsally and ventrally in the proximal area of the transplant. In one embryonic foot areas of mesoderm devoid of ectoderm extended from the transplant area over portions of digit IV. Two distinct areas of mesodermal condensation were detected in this specimen; one of these may be due to incomplete removal of host tissue prior to transplantation. Along the distal margin of the experimental area one or two indentations were frequently observed where the edges of the transplant healed with the host tissue.

The amount of time for the cut edges of the transplants to heal to the host tissue varied from embryo to embryo. Frequently the mesoderms of the host and transplant healed to each other within 24 hours. In a few embryos, ectoderm, which was continuous with surface (host and/or donor) ectoderm, appeared in the interdigital mesoderm as if it had grown between the transplant and the host foot prior to healing. Occasionally the injured surfaces did not appear to heal together nor had ectoderm grown over them so that a space between the host and the transplant resulted. Extracellular matrix and cells protruded into the space from both exposed surfaces. In

those embryos sacrificed after 24 hours incubation which did not exhibit prechondrogenic condensation it cannot be determined whether the experimental interdigits were not becoming chondrogenic or were simply late in doing so. Experimental interdigital tissue, in which condensation was not detected, bulged dorso-ventrally similar to the digital areas and had one or two indentations in the distal margin. Thus an increase in interdigital tissue was common, whether or not chondrogenesis occurred.

After two days incubation most transplants had healed completely to the host tissue, but in a few cases they had not as evidenced by ruptured ectoderm. These embryos also had portions of exposed mesoderm where ectoderm was not present proximally and ectoderm which extended into the mesoderm on the other side of the transplant.

After four days of incubation, frequently a complete, although malformed, extra digit had formed in the transplant area (Fig. 4c, p. 24). The most proximal ectopic phalange fused with the first phalange of digit III. Transplantation frequently effected the development of the host digits. The distal phalanges of digit III curved laterally in all specimens and digit IV curved medially in one embryo. The developing ectopic cartilage nodules also angled laterally, the proximal tips near digit III and the distal tips towards digit IV. Digit III of the contralateral control also curved laterally. The joints

between the ectopic phalanges appeared to be aligned with those of digit III or IV such that one joint was level with one in digit III and the next was level with one in digit IV (Fig. 4c, p. 24).

Transplants were grafted to the thigh to determine if the chondrogenic capacity of the interdigital tissue was dependent on the site of transplantation. Four of six experimental embryos died due to the severity of the wounding; major blood vessels had been severed when host tissue had been removed. Both embryos which survived developed nodules of ectopic precartilage whose axes paralleled that of normal host cartilage. The mesoderm of transplanted wedges of interdigital tissue can differentiate into chondrocytes in vivo if they are inserted into the thigh demonstrating that interdigital tissue can differentiate into nondigital cartilage whose shape and orientation are determined by the environment in which the transplant is located.

An interdigital transplant which is inserted into an interdigital area can develop into an extra digit; one which is inserted into a thigh can develop as an ectopic nodule which is aligned with the normal chondrogenic axis of the thigh.

The Effect of Simple Incisions on Interdigital Differentiation

In a series of experiments, similar to the transplantation experiments, one or two incisions were cut in III-IV interdigital tissue. The embryos were allowed to develop for an additional one to four days.

Single and double incisions completely through III-IV interdigital tissue can result in the development of up to two distal phalanges (Table 4, Fig. 5, Fig. 6). The results of the single and double incision experiments are essentially the same except where noted. The results of this experiment are similar to those of the transplantation experiment, except that only a partial extra digit instead of a complete one developed.

After 24 hours of incubation the experimental interdigit was characterized by one or two indentations on the distal margin at the incision sites, bulging of cells dorso-ventrally similar to the digital areas, and condensation of mesodermal cells. In the majority of embryos the incisions appeared to have healed and were covered by a continuous layer of ectoderm. In a minority of embryos in which the incisions had not healed completely patches of proximal mesoderm were devoid of ectoderm. Embryos in which ectopic chondrogenic condensation could not be detected appeared to be slightly

Table 4: Incisions in III-IV interdigital tissue can result in ectopic chondrogenesis.

<u>Incisions</u>	<u>Days Incubated</u>	<u>Total Embryos</u>	<u>No. with Ectopic Chondrogenesis</u>
Double	1	24	14
	2	4	4
	3	8	6
	4	<u>8</u>	<u>8</u>
	Total	44	Total 32 (72.7%)
A.F. Double*	1	2	2
	4	<u>1</u>	<u>1</u>
	Total	3	Total 3 (100%)
Single	1	6	4
	2	3	1
	3	3	3
	4	<u>6</u>	<u>5</u>
	Total	18	Total 13 (72.2%)

*Amniotic fluid was replaced with sterile PBS prior to incising.

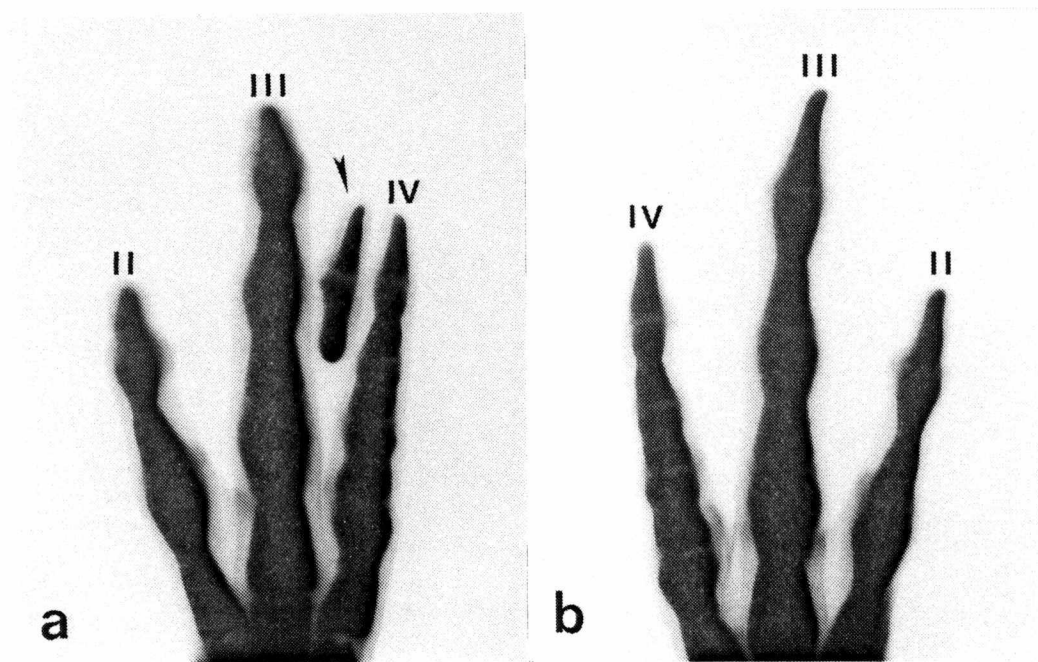


Fig. 5. Ectopic chondrogenesis following four days of incubation after two incisions were made in the III-IV interdigit. (a) Experimental foot with an extra partial digit (arrow); (b) contralateral control. Note the ectopic phalanges appear to be copies of the distal most joint of digit IV. 180X.

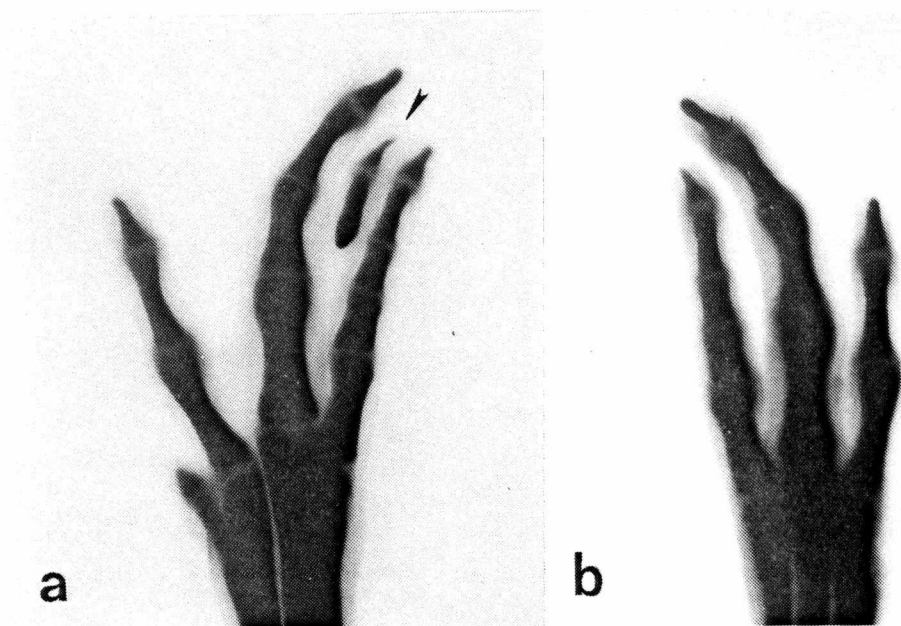


Fig. 6. A single incision in III-IV interdigital tissue can result in ectopic chondrogenesis; (a) as two phalanges (arrow), following three days of incubation; (b) contralateral control. Note that the ectopic phalanges resemble the distal most phalanges of digit IV and the retention of interdigital tissue in the experimental and contralateral interdigits. 180X.

younger than those in which condensation was detected. The incised interdigital tissue of these embryos bulged dorso-ventrally similar to the digital areas and had one or two notches in the distal margin. In two singly incised interdigits the distal margin between digits III and IV was flattened. This flattening could indicate a loss of AER function or a loss of interaction between the AER and the mesoderm.

After two days of incubation the feet from only two singly incised embryos did not exhibit ectopic chondrogenesis, although one had a lump of tissue on the medial side of digit IV near the distal margin of the regressing interdigital tissue.

After 3-4 days of incubation ectopic cartilage appeared as a nodule or as two distal phalanges of an extra digit attached to digit III and/or IV by soft tissue (Fig. 5, p. 30; Fig 6, p. 31). Interdigital tissue proximal to the ectopic piece of digit was still present and regressing more slowly than normal. The orientation of the ectopic phalanges often paralleled that of digit III or IV with the joint between the ectopic phalanges aligned with the most distal joint of one of the two neighboring digits. Ectopic tendons appeared ventral and less often dorsal to the ectopic cartilage nodules.

In two doubly incised interdigits the ectopic cartilage fused with a phalange of digit III or IV (Fig.

7). Three doubly incised interdigits developed two distinct areas of chondrogenesis (Fig. 8).

In two doubly incised embryos which did not develop ectopic cartilage the III-IV incised interdigital tissue regressed more slowly than normal, but was not thicker dorso-ventrally (appeared to be normal interdigital tissue). Development of digits III and IV were occasionally effected by the incisions as evidenced by their curving at their distal ends either laterally or medially.

The Effect of Amniotic Fluid on Interdigital Differentiation

The in vivo and in vitro AF experiments were performed to determine whether or not AF was involved in the induction of ectopic chondrogenesis when III-IV interdigital tissue was wounded. In one series of experiments AF was removed and replaced with sterile PBS prior to incision. All three doubly incised interdigital tissues developed ectopic cartilage attached by soft tissue to neighboring digits (Table 4, p. 29; Fig. 9) indicating that AF probably does not effect ectopic chondrogenesis in vivo.

In a second series of experiments wedges of III-IV interdigital tissue were cultured with MEM plus one of the

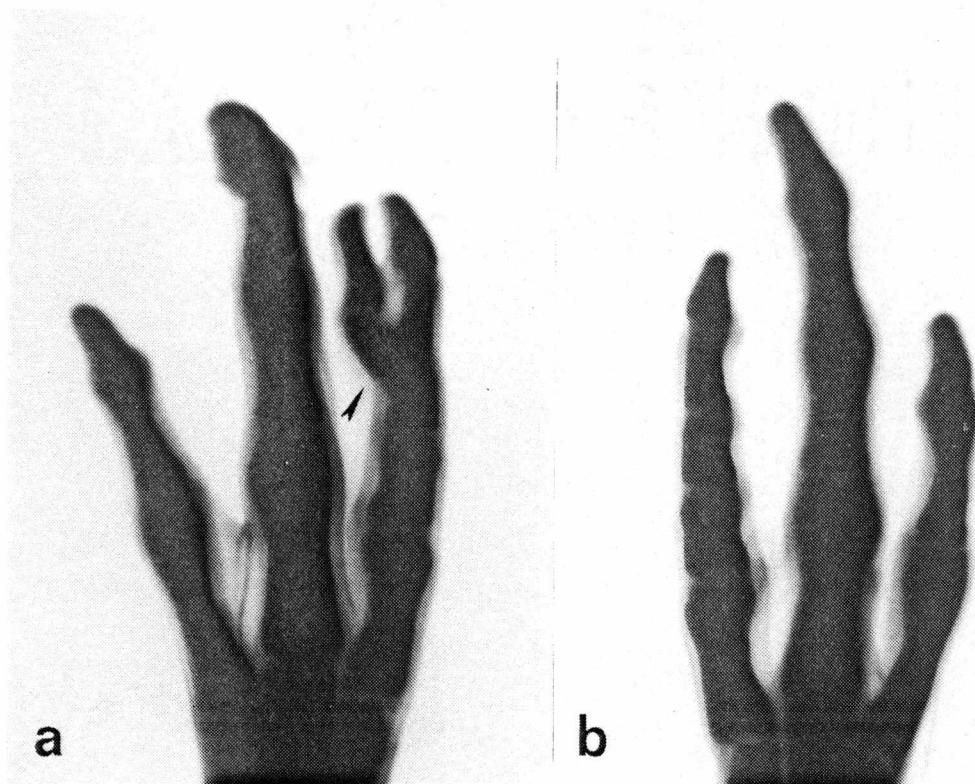


Fig. 7. An ectopic phalanx fused with the third and fourth phalanges of digit IV after the III-IV interdigital tissue was doubly incised; (a) following four days of incubation; (b) contralateral control. Arrow indicates ectopic phalanx fused with digit IV phalanges. 210X.

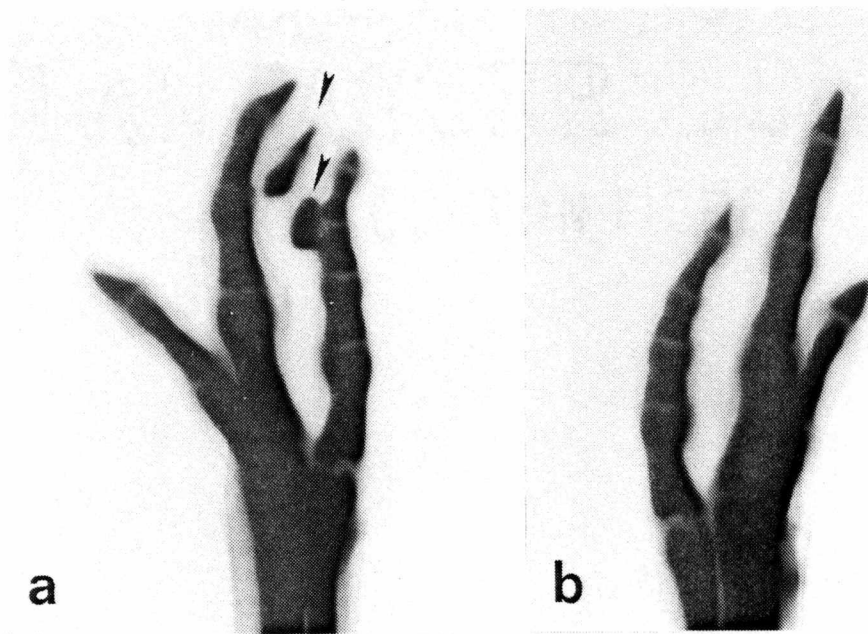


Fig. 8. Two separate chondrogenic areas (arrows) developed as a result of double incisions in III-IV interdigital tissue; (a) after three days of incubation; (b) contralateral control. Note one of the chondrogenic areas has developed into two phalanges.

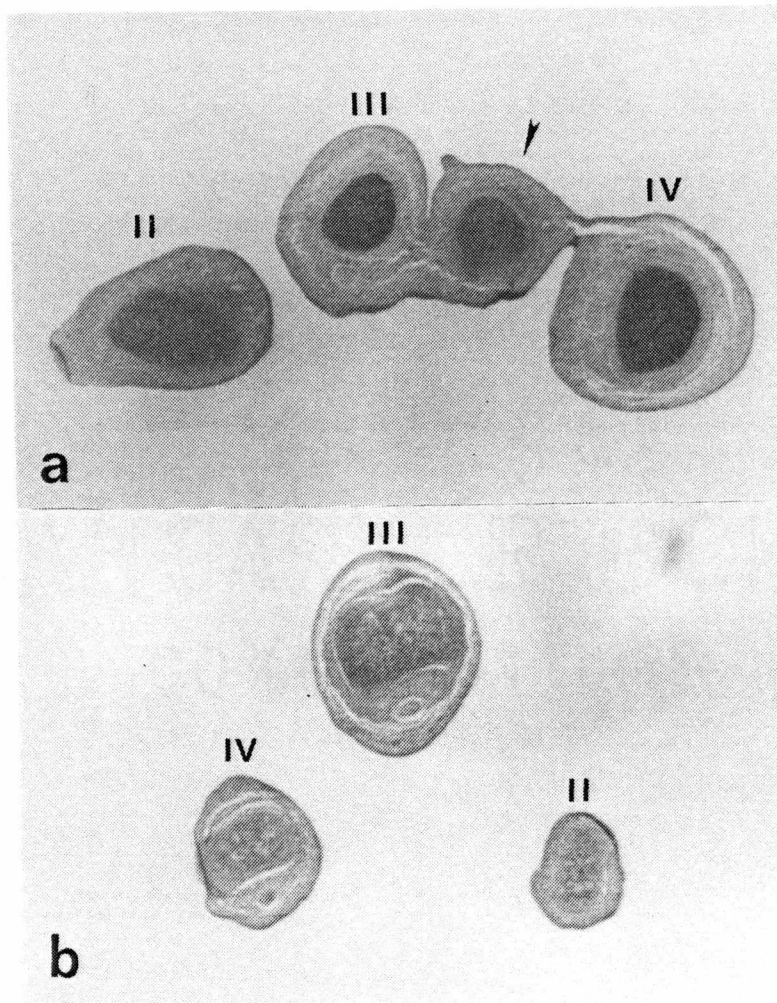


Fig. 9. Replacement of 1.0 ml AF with sterile PBS prior to doubly incising III-IV interdigital tissue does not inhibit ectopic chondrogenesis; following four days of incubation (a) experimental foot; (b) contralateral control. Arrow indicates ectopic phalange. 438X.

following: 1) 10% CS, 2) 10% CS + 10% AF, 3) 10% AF, or 4) 20% AF. Slight differences in results were observed between the tissues supplemented with 10% CS with or without 10% AF and the 10% or 20% AF.

Tissues cultured with 10% CS with or without AF attached within 24 hours incubation, and mesodermal cells became fibroblastic and migrated. Macrophages were observed in the explants. After three days of incubation mesodermal cells in the center of the original explants which had not migrated, developed chondrogenic condensations. Ectodermal "vesicles" of varying size and number also appeared after three days of incubation. During the last three days of the incubation period fibroblasts continued to migrate away from the explant, more mesodermal cells which had not migrated differentiated into chondrocytes.

Explanted interdigital tissue cultured in MEM + AF (without CS) developed slower than those cultured with CS (+/- AF). The tissues attached, but not very well; changing medium daily seemed to dislodge the tissues. Chondrogenic condensation of the non-migrating mesoderm was observed after three days incubation. Fewer mesodermal cells migrated away from the explants than those in cultures supplemented with CS. Those which did were squarish-to-fibroblastic in shape. Cells from tissues in 20% AF appeared healthier and more cells migrated than

those in the 10% AF cultures. Macrophages were observed in these cultures as they were in the CS cultures. All of the 20% AF cultures exhibited chondrogenesis while only a few of the 10% AF cultures showed condensation after three days incubation. After four days all 10% AF cultures contained cells which had differentiated into chondrocytes. No "vesicles" appeared in any of the AF cultures, suggesting that the "vesicles" resulted from the influence of CS in the medium. Explants cultured in MEM + AF tended to retain their original shape longer than those in MEM which had been supplemented with CS.

IV. DISCUSSION

The effect of JGB on the developing foot has been extensively studied in vivo (Hurle et al., 1986; Fernandez-Teran and Hurle, 1984; Pautou, 1978; Kieny et al., 1976; Fallon, 1972a & b; Stein et al., 1969). More recently the processes by which syndactyly and hypophalangy are produced have been under scrutiny. Both may be caused by an apparent interference of JGB with the interaction between ectoderm and mesoderm. The effect on digits is an interruption of outgrowth and thus hypophalangy. The effect on interdigital tissue however is an inhibition of cell death thus contributing to syndactyly (Hurle et al., 1986; Fernandez-Teran and Hurle, 1984; Pautou, 1978; Kieny et al., 1976). Kelley (1970) has suggested that the migration of cells from interdigit to digit also aids elimination of interdigital tissue. The effects of JGB on interdigital cell movement has not been tested previously.

The in vitro experiments described in this study examine the effect of JGB on interdigital cell migration. JGB was shown to inhibit cell migration from a block of explanted mesoderm. The intracellular mechanism by which JGB deleteriously effects in vitro cell migration is presumably its ability to disrupt oxidative phosphorylation in the mitochondria of cells (Lazarow and Cooperstein, 1953; Braun, 1954, 1966). This effectively slows down

cellular respiration (Braun, 1966; Lazarow and Cooperstein, 1953) and the energy-dependent activities the cells usually perform (Braun, 1954; Mayerson and Fallon, 1984; Fernandez-Teran and Hurle, 1984). It is presumably this decrease in cellular respiration that results in syndactyly and hypophalangy in the embryonic chick foot (Hurle et al., 1986; Fernandez-Teran and Hurle, 1984; Pautou, 1978; Kieny et al., 1976), the disruption of feather germ formation (Mayerson and Fallon, 1984), and the inhibition of cell migration in vitro. The altered shape of cells treated with JGB suggests that energy production in the mitochondria has decreased so little energy is available to construct a cytoskeleton characteristic of fibroblasts and for cell migration.

The possibility that interdigital mesodermal cells migrate in vivo has been suggested as one of the mechanisms which contributes to the formation of the digits (Kelley, 1970). An attempt to study this hypothesis in vivo by transplanting marked interdigital tissue resulted in ectopic cartilage formation in the interdigit. Because of this unexpected result it was clear that transplanted tissue does not behave normally and thus is not useful for examining cell migration in vivo. However, the change of fate from death, and possibly from migration, to chondrogenesis was quite interesting and thus became part of the focus of this thesis.

Transplantation of interdigital tissue and simple incisions in interdigital tissue produced similar results. Both have shown that wounding interdigital tissue can result in ectopic cartilage formation. The more severe the wounding, the greater the amount of ectopic chondrogenesis, so that one or two incisions can result in the formation of up to two distal phalanges of an ectopic digit and transplantation can result in the formation of an entire extra digit.

Experiments performed in recent years have attempted to elucidate mechanisms which account for digit formation. Their results indicate that the ectoderm, the interaction between the AER and the mesoderm, and the extracellular matrix may be involved as well as interdigital cell death (Hinchliffe and Ede, 1973; Kelley, 1973; Hinchliffe and Thorogood, 1974; Kosher et al., 1979; Hinchliffe, 1981; Hurle and Colvee, 1982; Rowe and Fallon, 1982; Hurle and Fernandez-Teran, 1983; Hurle et al., 1986; Hurle and Ganan, 1986, 1987; Hurle and Climent, 1987). Several conditions which have been observed in the digital areas during normal digit formation may have been duplicated in the interdigital tissue when it was surgically wounded, thus resulting in the development of extra partial or complete digits. These include regression of the blood vessels prior to differentiation of the digits (Hurle et al., 1985) and reciprocal maintenance of the AER and mesoderm during

differentiation of the digits (Saunders and Gasseling, 1968).

In normal differentiation of the digits the vasculature regresses prior to chondrogenesis, while in interdigital tissue regression mesodermal cell death precedes vascular regression (Hurle et al., 1985). Surgical wounding, as described earlier, may induce digital development by decreasing blood flow to the interdigit long enough to change the fate of the cells from death to chondrogenesis. When interdigital tissue was transplanted all blood vessels into that tissue were severed, so potentially the entire interdigit could be induced to develop similarly to prospective digital tissue. When one or two incisions were made some of the blood vessels to that area remained intact. The cells whose fate was less effected were the proximal interdigital cells whose vasculature was incompletely disrupted resulting in a partial digit distally. The vascular interruption could act similar to JGB, by decreasing oxygen availability and thus energy production. Inhibiting blood flow to a tissue results in a deprivation of oxygen and nutrients which decreases energy production in that tissue. A depletion of vascularization has been shown to occur during normal chondrogenesis and, in addition, low oxygen tension promotes chondrogenesis in vitro (Caplan and Koutroupas, 1973). A decreased oxygen supply in vivo due to wounding

could then result in ectopic chondrogenesis as it did when JGB caused a decrease in energy production (Fernandez-Teran and Hurle, 1984).

It has been hypothesized that during normal outgrowth of the developing limb the mesoderm subjacent to the AER produces a maintenance factor (MF) which prevents the AER from degenerating. At the time of regression the mesoderm ceases to maintain the AER and thus it (AER) no longer induces mesodermal outgrowth in that area (Saunders and Gasseling, 1968; Hinchliffe and Ede, 1973; Kelley, 1973). Interdigital tissue regression does not occur on schedule when that tissue has been transplanted or incised. Theoretically these cells could continue to produce MF and the interdigital AER would be maintained. The retained interdigital AER could then direct outgrowth of an ectopic digit in much the same way that digital AER directs outgrowth of the digits. In several contralateral controls in the simple incisions experiment the III-IV interdigital tissue also did not regress on schedule yet did not develop ectopic cartilage.

An alternate hypothesis is that the healing process rather than the actual wounding process may be the primary determinant of ectopic chondrogenesis. Wounding results in a local tissue contraction as cells round up, a local increase in cell density, a decrease in cell division, and growth of ectoderm over the wound (Summerbell, 1977). A

cessation of mitosis and a high cell density are two conditions required for mesodermal differentiation. The healing of transplants to host tissue and the healing of incisions could fulfill these two conditions resulting in the differentiation of interdigital tissue and thus ectopic chondrogenesis. The growth of ectoderm over the wounded interdigital tissue extended from the surface deep into the mesoderm in several of the experimental feet and occasionally extended completely through the interdigit. The cut edges of the tissues may have pulled away from each other allowing ectoderm to grow between them. The extension of ectoderm into the mesoderm and the development of a space between two injured surfaces could have resulted in an environment similar to that of the prospective digit IV area such that ectopic chondrogenesis was induced.

Hinchliffe (1981) hypothesized that the number of digits which forms is regulated by the limb necrotic zones (ANZ, PNZ, and INZs). The ta^3 chick mutant (Hinchliffe and Thorogood, 1974) and ectopic chondrogenesis in JGB-treated chick interdigital tissue (Fernandez-Teran and Hurle, 1984) support this hypothesis as do my results which show that retention of interdigital tissue can result in ectopic digit formation.

Various experimental procedures have resulted in ectopic cartilage formation (Iten and Murphy, 1980; Hurle and Ganan, 1986, 1987). Limb mesoderm which does not

contribute to the skeletal structure during normal development may thus be induced to become chondrogenic (Hurle and Ganan, 1986; Kosher et al., 1979). Removal of small portions of dorsal ectoderm from marginal interdigital tissue resulted in the development of small ectopic cartilage nodules (Hurle and Ganan, 1986). Removal of a small portion of interdigital AER also resulted in ectopic cartilage formation and in many embryos developed as two phalanges separated by a joint which were attached to neighboring digits by soft tissue (Hurle and Ganan, 1986, 1987). From their results Hurle and Ganan (1987) seem to believe that not only are interdigital mesodermal cells capable of becoming chondrogenic if diverted from death, but that cell death may prevent these cells from fulfilling a chondrogenic potential.

The ectopic chondrogenesis described by Hurle and Ganan (1986, 1987) as a result of removing small pieces of interdigital AER or dorsal ectoderm and the ectopic chondrogenesis described in this thesis as a result of incising interdigital tissue are very similar. At a very basic level the two procedures involve wounding and healing of interdigital tissue; perhaps one of these or a combination of the two are involved in ectopic chondrogenesis. More severe wounding seems to result in the development of structures which more closely resemble digits anatomically; that is, the structures may have

tendons and/or a complete set of phalanges as in the development of ectopic partial/complete digits associated with incisions/transplantations in interdigital tissue. The mechanism by which various types of wounding can elicit ectopic chondrogenesis is likely to be more complicated than simply the healing of those wounds.

The previously mentioned hypotheses about the development of ectopic chondrogenesis do not account for the shape of the extra cartilage. The zone of polarizing activity (ZPA) may be responsible for setting up a gradient which at a certain level could result in a digit IV-like distal piece of digit and below that level could result in a digit III-like piece of digit as has resulted from incising interdigital tissue (Fig. 5, p. 30). The ZPA however, is found only in earlier stages of limb development and in addition, it does not explain the proximo-distal arrangement of joints in an ectopic digit, in which one joint is aligned with a joint in digit III and the next is aligned with one in digit IV.

A more plausible hypothesis to explain both the polydactyly due to transplantation and the duplicated distal phalanges due to incising interdigital tissue is that cells which would normally contribute to digits III and/or IV are diverted to form the ectopic partial or complete digit. Thus, cells which would normally develop as part of digit III would form part of the ectopic digit

in the injured interdigital tissue, and since these cells would have positional information with respect to digit III, the part of the ectopic digit they formed would be similar to that which they would have formed in digit III. Cells which would normally contribute to digit IV would also behave as described above resulting in portions of the ectopic digit resembling digit IV. A composite extra digit with joints aligned with those of digits III and/or IV could result depending on whether more cells at a particular proximo-distal level originated from digit III or digit IV cells. The extra pieces of digits which resulted from incising interdigital tissue often appeared to be duplications of the distal ends of either digit III or IV; the majority of cells which contributed to these could have been diverted from developing as part of digit III or IV. These cells which would otherwise contribute to the normal digits could originate from the prospective digital areas or could be cells which would normally migrate from the interdigital to the digital areas.

To determine whether a factor in the AF caused the induction of ectopic chondrogenesis in wounded interdigital tissue explanted III-IV interdigital tissue wedges were cultured with CS with or without AF, and with AF only. Although there were differences between cultures in CS or AF (see results for details) both allowed chondrogenesis as determined by the presence of chondrogenic condensations,

suggesting that the ability to induce chondrogenesis is not related to the accessibility of mesoderm to AF.

In a related experiment sterile PBS was substituted for AF prior to incising III-IV interdigital tissue. Removal of AF did not prevent ectopic chondrogenesis in the injured interdigit, suggesting again that the formation of ectopic cartilage after wounding is not related to the exposure of mesoderm to the amniotic environment. These experiments thus support the contention that ectopic digit formation is due to alterations within the interdigit, such as blood flow and thus oxygen and nutrient availability.

Simple wounding of embryonic tissue can induce complex events which can result in an alteration of fate of that tissue (Hurle and Ganan, 1986, 1987). Some of the consequences of simple wounding are: (1) Localized deprivation of oxygen and nutrients due to a decreased blood flow when blood vessels are severed; (2) the possibility of restructuring of the tissue layers (as when ectoderm grew into the wounded mesoderm in these experiments); (3) the possibility of changing the composition of the extracellular matrix; or (4) an increased mortality rate when the amnion is punctured (the cause of death for many of the embryos in these experiments seems to be related to the inability of the amnion to seal after wounding). Any of these or a combination of these events may have induced the ectopic digit formation

observed in these experiments. These events may also be implicated in the physical causes of birth defects, such as in amniocentesis when the embryo could be pierced by the needle or the amnion may not close properly after the procedure is performed.

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VITA

Eleanor Gallo was born in Teaneck, New Jersey on June 19, 1963. She attended elementary school in Clark, New Jersey and graduated as valedictorian from Arthur L. Johnson Regional High School in June, 1981. The following August she entered The University of Notre Dame and in May, 1985 she received a Bachelor of Science degree in Biology. At the end of that summer she accepted a teaching assistantship at The University of Tennessee, Knoxville and began study toward a Master's degree. This degree was awarded in May 1989.