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# Epidermal Ridge Formation During Limb Regeneration in The Adult Salamander, *Ambystoma annulatum*

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## ABSTRACT

Regeneration was studied in the *Ambystoma annulatum* by the amputation of the right forearm of twenty-four adults, over a twelve month period. At the termination of the experiment the limbs were reamputated 1-2 mm proximal to the original amputation site. The regenerated portions were staged, examined at the gross morphological level, and prepared for histological examination.

Gross examination revealed a thickened, ridge-like projection along the distal edge of all regenerating forelimbs at the Early Bud through Middle Palette Stages. Histological examination confirmed the existence of this structure as early as Wound Healing and continuing through Middle Palette to a pseudo-epidermal ridge in the Late Palette Stage. This epidermal ridge is continuous with the adjacent epidermis, but the epidermal ridge extends only along the dorsal distal edge of the regenerate. The epidermis proximal to the original amputation site was, at the maximum, 4-5 cells thick, whereas the epidermal ridge proved to be 60-65 cells at its maximum thickness.

It is significant that an epidermal ridge appears on the distal tip of a regenerating adult forelimb in the stages Wound Healing through Middle Palette. A comparable structure, the apical ectodermal ridge, occurs during similar stages in embryonic chick limb development and embryonic amphibian limb development and has been considered a unique, embryonic feature.

One might postulate that the function of the epidermal ridge during forelimb regeneration in the adult salamander parallels the function of the apical ectodermal ridge of chick limb bud as an inducer for continued mesodermal outgrowth.

## INTRODUCTION

Regeneration, the process by which an organism can replace all or a portion of a missing forelimb, involves three distinct phases: degeneration-dedifferentiation, growth, and redifferentiation. The first phase, after an initial wound healing stage, involves the formation of a mesenchyme-like cell mass, the blastema, dedifferentiated from cellular elements closely associated with the amputation surface: dermis, muscle, bone, connective tissue, but excluding vascular, nervous, and epidermal tissues. During the growth phase, there is an increase of the mesenchyme-like cells, with a subsequent redifferentiation into the lost portion of the forelimb.

Studies of limb regeneration at the morphological level in newts, both larval and adult, and in larval salamanders has been reviewed extensively by Singer (1952) and Iten and Bryant (1973). However, there has been no report of limb regeneration in any species of adult salamander. During the course of the investigation, it was determined that complete regeneration does occur in the animals studied (Young and Bailey, 1978a).

The purpose of this study was to describe the events which occur during limb regeneration in the adult salamander *Ambystoma annulatum* and report one unique feature of this regeneration, the appearance of an epidermal ridge.

## METHODS AND MATERIALS

Fifty adult *Ambystoma annulatum*, endemic to Northwest Arkansas, were collected during their fall breeding migration. They were maintained in the laboratory at 23° C in a terrarium, containing a mixture of peat moss and potting soil. Earth worms were found to be their preferred food. After a six month acclimation period, twenty-five salamanders, of comparable size and weight, were selected as an experimental population. The amputation schedule is described in Young, 1977.

## RESULTS

The normal stages of limb regeneration in the adult salamander, *Ambystoma annulatum* (Young and Bailey, 1978a) are:

- I. Wound Healing (0-34 days post amputation)
- II. Early Bud Stage (32-66 days post amputation)

- III. Middle Bud Stage (60-96 days post amputation)
- IV. Late Bud Stage (90-130 days post amputation)
- V. Early Palette Stage (124-160 days post amputation)
- VI. Middle Palette Stage (156-198 days post amputation)
- VII. Late Palette Stage (190-226 days post amputation)
- VIII. Early Digit Stage (220-254 days post amputation)
- IX. Middle Digit Stage (250-292 days post amputation)
- X. Late Digit Stage (286-330 days post amputation)
- XI. Complete Regenerate Hand (324-370 days post amputation)

The surface of the non-regenerated limb and the limb stump proximal to the amputation site is covered by an epidermal layer which is three to four cells thick. In stages Wound Healing through Late Digit, most of the regenerate, except the distal surface, is covered by an apical epidermis which is three to four times the thickness of the stump epidermis, or 12-16 cells thick. However, a large, thickened, epidermal ridge persists along the dorsal distal tip of regenerating limbs from the Wound Healing Stage through the Late Palette Stage. The thickness of this epidermal ridge (ER) varies from 16-20 to 60-65 cells thick. In the Wound Healing Stage this epidermal ridge is 60-65 cells thick and appears as a mound of epidermis (Plate 1). Since it extends over one millimeter the distance along the distal tip it can be considered a ridge-like structure. Blastema cells appear to be aggregating in an area of the limb directly adjacent to the epidermal ridge, and these blastema cells appear to extend into the base of this epidermal ridge. During regeneration the thickness of this epidermal ridge varies: Wound Healing 60-65 cells thick; Early Bud 16-20 cells thick; Middle Bud 25-30 cells thick; Late Bud 50-55 cells thick; Early Palette 30-35 cells thick; Middle Palette 55-60 cells thick (Plate 2); and Late Palette with a pseudo-epidermal ridge of 25-30 cells thick. During these stages, the number and extent of coverage of the blastema cells increase from one stage to the next.

## DISCUSSION

Limb regeneration does occur in the adult salamander, *Ambystoma annulatum* (Young and Bailey, 1978a), and parallels, to some extent, limb regeneration in aquatic urodeles: newt, larval

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and adult, and larval salamander (Iten and Byrant, 1973). One significant exception is in the presence of the epidermal ridge (ER) (Plate 1 and 2). The epidermal ridge is visible throughout the first and second phases of regeneration, degeneration-dedifferentiation and growth. It is visible from the Wound Healing Stage to its termination at the Late Palette Stage. As the blastema bud elongates, the epidermal ridge is present as the most distal structure of the regenerate, with the blastema cells adjacent to the base of the ridge. Concurrently, while the distal movement of the epidermal ridge from the amputation site, the underlying blastema cells are rapidly multiplying and following the distal movement of the epidermal ridge from the amputation site, which proceeds until the Middle to Late Palette Stage. At this time there is a major overlap in the phases of regeneration. The mass of blastema cells at the base of the epidermal ridge have concluded their degeneration-dedifferentiation and growth phases and are progressing towards the redifferentiation phase. Meanwhile the blastema cells along the site of amputation initiate degeneration and dedifferentiation of cellular elements proximal to the amputation site subsequent multiplication of these cells occurs during the growth phase. After the growth phase is nearly completed these proximal blastema cells begin to redifferentiate.

Thus in adult salamander regeneration, the regenerate portion proceeds toward the adult configuration from a distal to proximal redifferentiation of the blastema cells. The presence of the epidermal ridge during the first and second phases of regeneration, degeneration-dedifferentiation and growth, may signify a two-fold purpose: 1) to direct the aggregation of the blastema, and 2) to act as an inductor for continued mesodermal outgrowth.

In regenerating limbs of amphibian larvae, the wound epithelium becomes thickened to establish the apical epidermal cap, beneath which mesenchyme cells aggregate to form the regeneration blastema. It has been proposed that the function of the apical epidermal cap (AEC) in regenerating limbs of amphibian larvae is to direct the aggregation and outgrowth of the blastema cells (Thornton, 1954).

It has been shown by Thornton (1960) that the apical epidermal cap (AEC) can be shifted to an eccentric position by the use of microsurgery. The blastema cells will aggregate beneath the eccentric cap and form a corresponding eccentric blastema which will develop into an asymmetrical regenerate. Thornton also demonstrated that if eccentric epidermal caps were induced in regressed limbs after all tissues had completely dedifferentiated, then the blastema cells would aggregate beneath the eccentric epidermal cap and produce eccentric blastema and regenerates. Thus the eccentric epidermal cap induced a corresponding eccentricity of regenerative outgrowth.

The thickened epidermal ridge (ER), maximum of 60-65 cells thick, seen in *Ambystoma annulatum* is not present in aquatic urodele regeneration, larval or adult newt. A comparable ridge-like structure has been described and studied in the chick embryo (Saun-

ders, 1972); amphibian embryo (Tarin and Sturdee, 1971); and human embryo (O'Rahilly, Gardner, and Gray, 1956).

In the development of the chick limb bud, mesoderm induces the ectoderm, overlying its ventrodorsal border, to thicken. This thickening is the beginning of the apical ectodermal ridge (AER), which acts as an inducer for continued mesodermal outgrowth. As the mesoderm responds to the apical ectodermal ridge (AER), it exercises a reciprocal influence for the maintenance of the ridge. This mesodermal property, called "apical ectodermal maintenance factor" by Zwilling (1956), is distinct from that which initiates apical ectodermal ridge formation. Its action is asymmetric with respect to the distal-proximal axis of the limb bud so that the greatest thickness of the apical ectoderm occurs distal-ventrally. The presence of the apical ectodermal ridge in the chick embryo is required until the wing or foot plate begins to expand. That the apical ectodermal ridge in the chick embryo is inductive, cannot be doubted: after removal of the ridge, limb outgrowth ceases (Saunders, 1948); when an extra ridge is added microsurgically (Saunders and Gasseling, 1968 and Swilling, 1956) or genetically (Goetink, 1964), two limb outgrowths form from the same mesodermal limb bud.

The epidermal ridge (ER) is present in the adult *Ambystoma annulatum* during the first and second phases of regeneration, degeneration-dedifferentiation and growth; and then disappears when the third phase, redifferentiation, occurs, after the autopodium (fused cartilaginous hand and digit bones) is well formed. This time sequence for the epidermal ridge of adult salamander regeneration parallels similar time sequences for the apical ectodermal ridge (AER) during chick embryonic limb development (Saunders, 1972) and the apical epidermal cap (AEC) during limb regeneration in the larval amphibian (Thornton, 1960). These similarities of developmental processes may indicate similar functions for the epidermal ridge (ER) in the adult salamander, that of an inductor and of a director to aggregate the blastema for continued blastemal outgrowth.

Although the arguments for the function of the epidermal ridge in the adult salamander are logical, additional experimentation is needed to prove or disprove the above postulates.

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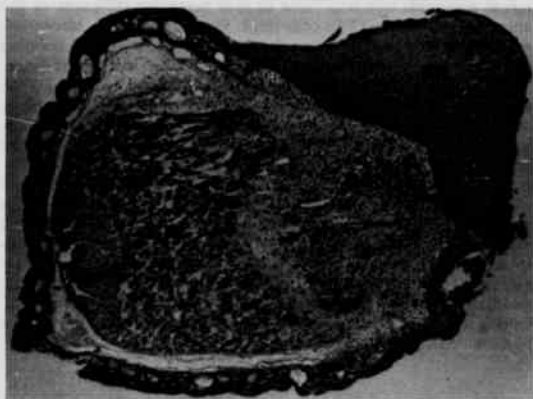


Plate 1. Wound Healing ER-Epidermal Ridge; BS-Blastema; SM-Skeletal Muscle.

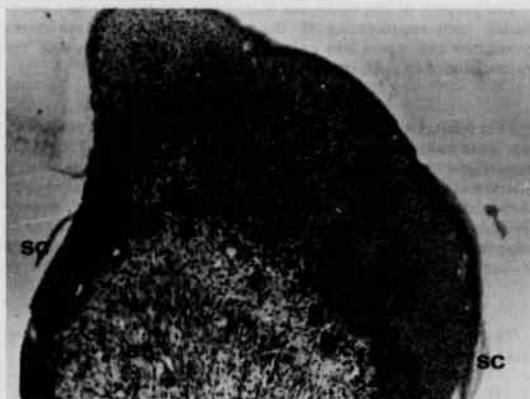


Plate 2. Middle Palette Stage ER-Epidermal Ridge; BS-Blastema; SC-Stratum Corneum.

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