Cell. Mol. Life Sci. 65 (2008) 73 – 79
1420-682X/08/010073-7
DOI 10.1007/s00018-007-7433-z

Cellular and Molecular Life Sciences

Wound epidermis formation and function in urodele amphibian limb regeneration

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Online First 20 November 2007

Abstract. Upon amputation of the urodele limb, the epidermal cells surrounding the amputation plane migrate to heal the wound. The resulting wound epidermis (WE) induces the regeneration process, resulting in blastema formation, cell division, and ultimately repatterning into a new limb. Despite its central role in the initiation of limb regeneration, little is known about how the WE forms. Here we discuss various models of WE formation and the experimental data in support of each. (Part of a Multi-author Review)

Keywords. Limb regeneration, cell migration, wound healing, wound epidermis, positional information, blastema, dedifferentiation.

Introduction

In order to reproduce both a morphologically correct and functional limb, urodele limb regeneration requires that each tissue participate in the process. However, dissecting the roles of individual tissues has been a challenge given the interconnection of the tissues involved. Fortunately, significant progress has been made in the elucidation of blastema formation. In addition, grafting experiments have determined the influence of individual tissues on limb patterning. Despite these insights, it is still unclear which tissues are responsible for the initiation of regeneration. Here we focus on the role of one tissue, the epidermis, and how its response to amputation leads to a structure, the wound epithelium (WE), that has an essential function in the initiation of regeneration.

Histologically, the sequence of events leading to regeneration following amputation of the adult urodele limb have been well described. Immediately after amputation, epidermal cells from the circumference of the limb migrate to cover the wound surface. This wound-healing phase is achieved strictly through cell movement without cell division [1, 2] and is completed within 24 h after amputation [3]. Over the next few days, this thin layer of epithelial cells thickens into the multilayered WE. The thickening of the epidermis is accompanied by histolysis of stump tissues such as bone and muscle, from which emerges dedifferentiated cells that accumulate directly beneath the thickened WE. These cells re-enter the cell cycle and give rise to the blastema, an accumulation of mesenchymally derived cells that are believed to largely, if not completely, originate from dedifferentiation of previously differentiated cells [4]. At early bud stage, the blastema is visible only as a small bump; however, continued cell division results in enlargement into a cone-stage blastema. As the blastema continues to expand, the dedifferentiated cells redifferentiate into limb tissues following many of the same patterning programs that were originally employed during embryonic limb development.

The process of limb regeneration in the urodele amphibian thus has three roughly distinguishable phases: wound healing, blastema formation and

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repatterning. Since blastema formation and limb repatterning clearly cannot occur unless the amputation wound has healed, it is reasonable to suppose that the wound-healing phase plays an important role in the initiation of the regenerative process. Therefore research focusing on WE formation can potentially answer the question of why urodele amphibians can regenerate while mammals cannot.

WE formation and maturation

The first critical events in regeneration occur within hours after amputation as the amputation plane is covered by a thin sheet of migrating, non-proliferating epithelium [1, 2]. Migration begins as the basal layer of epidermal cells around the circumference of the wound change shape. These cells lose hemidesmosomes, detach from the basal lamina [5] and migrate across the wound surface as sheets of long, flattened cells which extend across the fibrin network that results from the injury blood clot [3]. Once the wound is covered, the epidermal cells begin to proliferate, creating the multi-layered WE structure. The structure consists of up to 15 cell layers, whereas normal epidermis is only 3–4 layers [6]. The thickened WE structure is occasionally referred to as an apical epithelial cap (AEC), and its thickening correlates with the height of histolysis [7, 8]. It is widely accepted that this thickened WE, in its final form, is required for the regenerative process.

This distal epithelial structure is clearly specialized and distinct relative to normal epidermis. Once the epidermal cells migrate across the fibrin clot, they begin to synthesize their own extracellular matrix proteins, including laminin, collagen type IV and collagen type XII [9, 10]. Matrix metalloproteinases (MMPs) are also produced and may play an important role in dedifferentiation through matrix degradation [11]. MMP3/10b is detected in the thickened WE of regenerating limbs [12], and MMP 9 is detected in the WE as soon as healing is finished [13]. Complement factors, which are expected to play an important role in regeneration, also distinguish the WE from normal epidermis. Complement component 5 (C5) expression is absent in the unamputated limb but is expressed in the WE [14]. Rather than having an immunological role, complement C5 may be involved in proliferation of the blastema tissue.

The necessity of the WE was attributed, for a short time, to the nerve supply that invades the structure. Innervation of the blastema is known to be required for regeneration [15], and since the epithelium covering the wound also becomes highly innervated [16], the activity of the WE was believed to be due to this nerve supply [6]. However, it is now known that limbs can successfully regenerate when nerve fiber invasion of the WE is inhibited [17]. Although successful regeneration under non-aneurogenic conditions requires blastemal innervation, the role of the WE does not rely on nerve fiber invasion.

Markers of the WE

It has been established that the WE is a specialized structure with properties that distinguish it from normal epidermis. Besides extracellular matrix proteins and MMPs, many other factors are produced by this structure, several of which have been employed as markers in regeneration research (see Table 1). One of the earliest identified markers of the WE was the antigen of the WE3 antibody, a monoclonal antibody generated through immunizations of mice with mid- and late-bud stage blastemas from newt forelimbs [18]. In addition to its strong staining with the thickening of the wound epithelium, WE3 also reacts with a few cells in normal skin that have been identified as skin glands, suggesting that the WE3 antigen plays an important role in secretion. Further studies suggest that the antigen is an actin-binding protein [19]. Another marker, the WE6 antibody, displays reactivity similar to WE3. It was generated by immunizing mice with WE from regenerating newt limbs [20]. Contrary to the late reactivity of WE3, the WE6 antigen is expressed as early as 1 day after amputation. WE6 also reacts against the normal skin epidermis near the plane of amputation. The WE6 antigen is hypothesized to be a keratin with molecular weight of 39 kDa [21].

Dlx3, a homeobox-containing transcription factor, also serves as a marker for the WE. This gene was cloned from a newt cDNA (complementary DNA) library and characterized with expression in developing and regenerating limbs [22]. Through *in situ* hybridization, *Dlx3* expression in the WE has been determined to correlate with the accumulation of dedifferentiated mesenchymal cells [23]. This relates with the function of the *Drosophila* homolog *Distal-less*, which is required for distal outgrowth of the limb [24]. Another homeobox-containing transcription factor, *Msx2*, has also been detected in the apical epidermis of the regenerating limb [25, 26]. While its expression level is very low in uninjured limb tissues, higher levels are detected in the epidermis as early as 1 h after amputation. However, this expression is also detected in lateral skin wounds [25], suggesting that *Msx2* may be more directly involved in wound healing than in progression through the regeneration process. Other genes that have been detected in the

Table 1. Markers for the wound epidermis during urodele limb regeneration.

Molecule	Detection Method	Expression Profile	Refs.
Laminin	polyclonal Ab	WE at early bud; WE basal layer at mid to late bud	[9]
Collagen type IV	polyclonal Ab	WE at early bud; WE basal layer at mid to late bud	[9]
Collagen type XII	monoclonal Ab MT2 and riboprobe	WE basal layer at early dedifferentiation; basement membrane and blastema at bud stages	[10]
MMP3/10-b	riboprobe	early to mid bud	[12]
MMP9	riboprobe	wound healing and early dedifferentiation	[13]
Complement C5	riboprobe	early to late bud	[14]
Actin-binding glycoprotein WE3	monoclonal Ab WE3	early bud to early digit	[18,19]
Keratin WE6	monoclonal Ab WE6	early dedifferentiation through bud; glandular tissues throughout body	[20,21]
Dlx3	riboprobe	early bud through early digit	[22,23]
Msx2	riboprobe	wound healing through early digit; lateral wound healing	[25,26]
Id2, Id3, HES1	riboprobes	early to late bud; some expression in blastema tissues	[27]
FGF1	extraction and binding assays	WE and blastema tissues	[28]
FGF2	monoclonal Ab	WE and nerves of medium bud	[23]
Fgf8	riboprobe	WE basal layer and most distal blastema from mid bud to late bud	[34,35]
Fgf10	riboprobe	early bud to mid bud; majority of expression in blastema	[34]

WE include *Id2*, *Id3* and *HES1* [27]. These transcription factors are all basic helix-loop-helix-type negative regulators, which act by repressing tissue-specific gene expression. These genes are expressed at low levels in unamputated limbs and are upregulated in the WE in relation to increased proliferation of the blastema.

Secreted mitogenic signals, such as members of the fibroblast growth factor (FGF) family, are abundant in the WE. FGF1 is expressed in both the WE and in blastema cells [28], and FGF2 has been localized to the WE and nerves of the regenerating limb [23]. Their receptor, FGFR1, is expressed in blastema cells, suggesting that these FGFs could be acting on blastemal tissues to promote mitotic activity [29]. Further support for their importance is underscored by studies showing that exogenous FGF, applied either *in vivo* or *in vitro*, induces blastema cell proliferation in the absence of the WE [30, 31]. The expression patterns of other FGF family members, *Fgf4*, *Fgf8* and *Fgf10*, are also of particular interest because of their roles in vertebrate limb development (for review see [32, 33]). All three genes are expressed at low levels in the uninjured limb [34]. While *Fgf4* is not detected in the regenerate, *Fgf8* and *Fgf10* transcript levels are upregulated in the blastema and are also present in the WE. Further studies have shown that *Fgf8* is localized to the basal layer of the WE [35].

Historical perspectives of the WE

The initial phase of wound healing and formation of the WE was quickly recognized to be important for regeneration through experiments that prevented WE formation. In one study, urodele limbs were inserted into the body wall immediately after amputation [36], resulting in the failure of a WE to form and regeneration to occur. It was also discovered that, when placed over the amputation plane of a freshly wounded limb, a graft of full thickness skin (i.e. with epidermal and dermal layers) prevented regeneration [37, 38]. This work specifically emphasizes the need for a direct interaction between the epidermis and the mesenchymal tissues of the stump. Further research demonstrates that the WE is active even before it matures into a thickened structure. This was shown experimentally by allowing the closure of the amputation plane by epidermal migration but preventing the maturation of this epithelial monolayer into a mature WE [39]. Following amputation of newt limbs, the WE was removed every 24 h. Two urodele amphibian species were used in the experiment, one of which failed to regenerate after repeated WE removal and another that regenerated at approximately half the rate of normal regeneration. Between the two species, the time required for healing was different. The species that did not regenerate required

longer than 24 h for complete healing. Conversely, the species that did regenerate completed WE formation in approximately 12 h. Therefore, the WE was in contact with the mesenchyme for 12 h a day – half that of normal regeneration, indicating that the WE must elicit a regenerative response from the mesenchymal tissues of the stump shortly after its formation.

The nature of this interaction is unclear; however, the literature indicates that MMP9, in particular, is upregulated very early in the wound-healing phase and could be an important factor produced by the WE that initiates the dedifferentiation of the mesenchymal tissues. As indicated above, MMP9 expression is restricted to the WE and appears when epidermal migration is complete [13]. Furthermore, MMPs have been implicated in the process of dedifferentiation during regeneration [11]. Despite the evidence that the WE acts as a trigger for initiation of regeneration, it is believed that the epidermis plays only a passive role in this process. In contrast, it is thought that the positional information in the dermal and muscle tissues directs the regenerative response. Support for this hypothesis comes from experiments in which tissues were rotated 180° around the limb [40]. Results from these studies showed that amputations through rotated dermis and muscle tissues induced the formation of multiple digits. However, rotated epidermis and bone did not produce similar results, suggesting that the positional identity of these tissues has little influence in initiating regeneration. In addition, it is known that the positional information for directing the proximal-distal axis of the limb is encoded in the mesenchymal tissues. Application of retinoic acid to a distal limb blastema induces respecification to a more proximal fate resulting in a regenerate with a duplication of proximal limb structures [41], presumably due to the presence and activity of retinoic acid receptors localized to the blastema mesenchyme [42–44]. Furthermore, the newt homolog of CD59, Prod1, is expressed in the mesenchymal tissues of the blastema and has been implicated as a marker of positional identity along the proximal-distal axis [45]. From these data it is clear that positional identity is an essential component for regeneration. The data specifically indicate that positional identity in the mesenchyme significantly influences the process. However, the data do not preclude a role for epidermal positional identity. In the grafting experiment described above [40], in which various limb tissues were rotated prior to amputation, a WE still formed properly in each case. Despite the many different experimental permutations tested in the study, the one constant was the fact that the epidermis covering the amputation plane migrated from each radial position of the limb: dorsal, anterior, ventral and posterior. Thus, one could interpret from these experimental results that there are two separate processes at work: 1) initial formation of a WE directing the underlying mesenchyme to dedifferentiate and proliferate, which occurred in each experimental case, and 2) subsequent morphological patterning mechanisms to respecify the limb, which was disrupted upon dermal and mesenchymal tissue rotation. According to this interpretation, the positional information encoded by the mesenchyme is important for tissue patterning, whereas the contact of migrating epidermis with different positional identities upon healing of the amputation plane results in a functionally active WE (Fig. 1a).

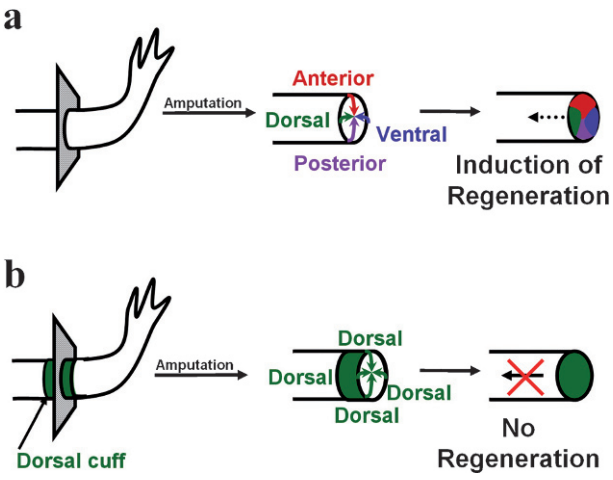

Figure 1. Positional discontinuity model of wound epidermis (WE) formation. (*a*) Diagram illustrating the hypothesis that induction of urodele limb regeneration results when epidermis with different positional identities makes contact and forms the wound epidermis. (*b*) Regeneration does not occur if the wound epidermis is only composed of cells with a single positional identity (i.e. dorsal) [46].

In contrast, a WE formed from migrating epidermis of a single positional identity would presumably be functionally inactive and fail to initiate regeneration. To test this, limb regeneration was inhibited by X-irradiation of axolotl forelimbs, and a dorsal strip of skin from a non-irradiated axolotl was then grafted onto the irradiated forelimb as a cuff [46]. Amputation through this dorsal cuff created an amputation plane surrounded by epidermis of completely dorsal origin (Fig. 1b). Four out of the 5 experimental limbs did not regenerate, while 14 out of 15 control limbs did (the control was a grafted cuff of skin representing the entire circumference of the limb). The 1 limb that did regenerate in the experimental group is reported to have done so at 10 months after amputation, indicating that non-irradiated epidermis migrating into the graft from the body of the animal may have played a

role. In accord with the interpretation described above, the results suggest that more than just the dorsal positional identity of the skin needs to migrate over the amputation plane to create a functional WE. Therefore a 'positional discontinuity model' is proposed, which states that the WE functions to initiate regeneration as a result of its formation from the contact of migrating epidermis from different positional identities.

WE formation, revisited

This positional discontinuity model of regeneration initiation by the WE is derived from Meinhardt's 'boundary model' of pattern formation. According to Meinhardt, the interaction between differently determined cells produces a border that can act as an organizing center to set up a secondary field [47]. This suggests that, in the case of limb regeneration, the contact of dorsal epidermal cells with ventral epidermal cells and anterior epidermal cells with posterior epidermal cells at the plane of amputation creates a border that determines the proximal-distal axis that must be regenerated. We further hypothesize that the epidermis is positionally defined so that the interaction of cells with different positional identities initiates the regeneration response immediately upon their contact with each other in WE formation.

A homologous example is formation of the apical ectodermal ridge (AER) during limb development. The AER is a thickened structure at the junction of the dorsal and ventral ectoderm of the limb, much like the WE results from the intersection of migrating dorsal, ventral, anterior and posterior epithelial sheets. Similar to the WE, the role of the AER is to act as an organizing center for outgrowth along the proximal-distal axis of the limb. In accordance to Meinhardt's boundary model, AER formation relies on the interaction between dorsally specified ectoderm that expresses *Radical fringe* (*Rfng*) and ventrally specified ectoderm that does not [48, 49]. The border between *Rfng* expressing and non-expressing cells is also believed to act in an analogous way in *Drosophila* wing formation; *fringe* on dorsal cells modifies the *Notch* ligands Serrate and Delta so that the dorsoventral margin is set up, thus serving as an organizing center for wing outgrowth [50]. Furthermore, planarian regeneration also requires the contact of epidermis of different positional identities. As in urodele limb regeneration, the first step in planarian regeneration is epidermal closure of the wound. More specifically, wound healing results from the stretching of epidermis over the wound until dorsal and ventral tissues come together [51]. Grafting experiments have indicated that the dorsal-ventral interaction is specifically important, since a blastema-like region forms at every interaction of dorsal and ventral epidermis [52].

Experimental support for the positional discontinuity model in urodele limb regeneration demonstrates that a wound surrounded by skin from different positional identities can induce an ectopic limb in the urodele amphibian [53]. In this experiment a nerve was deviated to a wound site on the anterior side of an upper arm of an axolotl. A graft of skin from the posterior side of the contralateral limb accompanied the nerve and wound. Three-fourths of the grafting cases resulted in ectopic limb formation. It was reported that all three components – wound, nerve and contralateral skin graft – are required for ectopic limb formation since wound plus nerve produced a bump that regressed and wound plus skin graft produced no response. However, recent studies along the same lines suggest that an ectopic bump can form in the presence of wound and skin grafts without innervation [R. E. Peterson, L. J. Campbell and C. M. Crews, unpublished observations]. In this case the wound is surrounded by multiple skin grafts representing several positional identities from the circumference of the limb, hence imitating an amputation plane on the side of the arm. The bump forms approximately 2 weeks after grafting, grows for 6 weeks and then regresses, presumably due to lack of a nerve supply. Again, it appears that migration and contact of skin from multiple positional identities is sufficient to induce a functional WE.

In conclusion, the wound-healing phase clearly initiates the regenerative process after urodele amphibian limb amputation. WE formation is particularly crucial because without it, regeneration fails. More specifically, it is necessary that the WE forms through the contact of migrating epidermis originating from different positional identities. This positional discontinuity model posits an active role for the epidermis in initiation of regeneration, and furthermore it assumes that the epidermis is defined by positional identity, an unanticipated concept for a tissue previously thought to be passive during regeneration. These postulations do not directly address why urodele amphibians can regenerate limbs while mammals, especially humans, cannot; however, they do provide a framework for further investigation into the cellular interactions that are necessary for WE formation and subsequent initiation of regeneration.

Acknowledgements. We thank Jeremy Brockes, Elly Tanaka and members of the Crews lab for their helpful discussions.

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