

The Molecular Basis of Embryonic Wound Repair

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ABSTRACT

Embryonic wound repair is both rapid and perfect. In this thesis, I describe my studies investigating the molecular mechanisms underlying this process. I report my experiments searching for genes which are upregulated at the wound site in a mouse embryo model, and explore the hypothesis that the cellular and molecular mechanisms that underlie tissue repair are the same ones that drive natural morphogenetic tissue movements.

My studies have focussed on two animal models: the E11.5 mouse embryo and the *Drosophila* embryo. In the mouse I have used two approaches to identify “wound-induced” genes (WIGs) upregulated in response to wounding: 1. An informed guesswork approach, looking for expression of genes which seem likely candidates for upregulation at the wound site; and 2. A subtractive hybridisation approach, to identify unknown WIGs. My predictive approach reveals a dramatic upregulation of the zinc-finger transcription factors *krox-20* and *krox-24*, within minutes of wounding, following an identical timecourse of expression as *c-fos*, the first embryonic WIG ever identified. My subtractive search reveals a number of exciting WIGs, including a protective anti-oxidant, Non-selenium glutathione peroxidase, which has previously been identified in a cell-based search for wound genes.

Alongside these mouse studies, I have characterised two wound healing models in the fruitfly, *Drosophila melanogaster*: one in late stage embryos and the other in wing imaginal discs. I have shown that the tissue movements of morphogenesis, particularly dorsal closure, and wound repair are remarkably similar, both in their gross appearance and the cytoskeletal machinery they employ. I have demonstrated that *Drosophila* embryos and imaginal discs use exactly the same cytoskeletal machinery to close a wound as vertebrate embryos, assembling a contractile actin cable within minutes of wounding, suggesting that mechanisms of embryonic wound repair are remarkably well conserved. I have also shown that it is possible to investigate gene expression at the wound site in imaginal discs, and serendipitously struck upon another wound healing model in the larval epidermis, where the tissue polarity gene *Dfz2*, a homologue of which was identified in my mouse wound subtractive screen, is shown to be upregulated following wounding.

The characterisation of the *Drosophila* wound models described in this thesis will allow further genetic dissection of the molecular events governing wound repair, in a genetically tractable model. These future studies will allow us to determine whether the cellular and molecular tools facilitating tissue repair are indeed the same ones that drive natural morphogenetic tissue movements in the embryo.

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ABBREVIATIONS

AER	Apical ectodermal ridge
Aop	Anterior open protein
AP-1	Activator protein-1
ARP2	Actin-regulated protein-2
BCIP	X-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate
BLAST	Basic local alignment search tool
BMP	Bone morphogenetic protein
Ca ²⁺	Calcium ion
cDNA	Complementary deoxyribonucleic acid
CTGF	Connective-tissue growth factor
DD-PCR	Differential display polymerase chain reaction
DFz2	Drosophila Frizzled-2
DIG	Digoxigenin
DNA	Deoxyribonucleic acid
dnDRac	Dominant-negative <i>Drosophila</i> Rac
Dpp	Decapentaplegic
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
Egr-1	Early gene response-1
ERK	Externally-regulated kinase
EST	Expressed sequence tag
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
FLP	Flipase
FRT	FLP recombinase target
Fz	Frizzled
GFP	Green fluorescent protein
GTPase	Guanosine triphosphatase
HB-EGF	Heparin-binding epidermal growth factor
HMDS	Hexamethyldisilazane
IEC-6	Intestinal epithelial cell line-6
iNOS	Inducible nitric oxide synthase
JNK	Jun N-terminal kinase
KGF	Keratinocyte growth factor
MAPK	Mitogen-activated protein kinase

MEK	MAPK effector kinase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
NBT	4-Nitro blue tetrazolium chloride
NSGP	Non-selenium glutathione peroxidase
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PKC α	Protein kinase C alpha
PLAP	Phospholipase A2-activating protein
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse-transcription polymerase chain reaction
SEM	Scanning electron microscopy
SRE	Serum response element
SRF	Serum response factor
TEM	Transmission electron microscopy
TGF α	Transforming growth factor alpha
TGF β	Transforming growth factor beta
TIMP	Tissue inhibitor of Metalloproteinase
tPA	tissue-type Plasminogen activator
TRITC	Tetramethylrhodamine isothiocyanate
TSP1	Thrombospondin-1
UAS	Upstream activation sequence
uPA	urokinase-type Plasminogen activator
VEGF	Vascular endothelial growth factor
WIG	Wound induced gene

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CHAPTER ONE

Introduction

General Introduction

This thesis describes my studies investigating the molecular mechanisms underlying embryonic wound repair. Further to describing genetic activity behind the wound response in our mouse embryo model (McCluskey and Martin, 1995), it explores the hypothesis that the molecular and cellular tools facilitating tissue repair in the embryo are the same ones that drive normal morphogenetic movements such as gastrulation and neurulation.

Studying wound repair *in vivo* in embryonic models allows us to try to understand why healing of these relatively simple tissues is so efficient. Adult healing is far harder to study cleanly; the skin has an intricate architecture and there is a significant inflammatory response. Indeed, adult repair is far less efficient, and the results are not so satisfactory. Perhaps learning how healing can occur perfectly in the embryo will give clues to how adult wounds could be treated to improve their repair.

In addition to my mouse studies, I characterise wound healing in two novel *Drosophila* models; an embryonic model and an imaginal disc model. There is no better organism than the fruit fly in which to investigate the hypothesis that embryos use identical signalling and cytoskeletal machinery both for healing wounds and driving morphogenesis. Moving into the realms of *Drosophila* presents an opportunity to investigate the molecular basis of repair without the complications of genetic redundancy that plague mouse studies, and allows us access to a plethora of morphogenetic mutants.

In this introductory chapter I review both adult and embryonic vertebrate wound healing literature, before moving on to outline *Drosophila* morphogenesis. I focus particularly on papers that address the initiation and mechanics of both wound repair and morphogenesis.

Adult wound healing

The wound healing response mounted in the adult comprises several stages; the wound must be plugged, an inflammatory response initiated, closure facilitated and remodelling performed. Although the phases of closure may overlap *in vivo*, I shall address the various underlying processes separately.

- ***The fibrin clot as a first line of defence***

Adult skin comprises a protective keratinised layer of stratified epidermis overlying a thick dermal layer of connective tissue. For cells beneath the outermost layer of keratinocytes the environment is protective from the ravages of the outside world. Cutaneous wounds expose these normally cosseted cells to extreme conditions of desiccation and infection. Clot formation is the first barrier erected to prevent excessive tissue damage and to expedite wound repair. Within seconds of wounding, blood leaking from damaged capillaries begins to coagulate following a cascade of enzymatic cleavages. These result in thrombin cleaving fibrinogen to give a cross-linked fibrin mesh packed full of activated platelets (Clark, 1996). As the platelets degranulate they release myriad cytokines and growth factors which serve as the early signals to initiate the wound closure process; stimulating re-epithelialisation and connective tissue contraction, recruiting inflammatory cells to the wound site and instigating fibroplasia and angiogenesis.

- *Adult wounds invoke an inflammatory response*

Once started, the wound response gathers momentum via positive feedback loops resulting largely from cytokine and growth factor signalling. The body's scavenger cells, neutrophils and macrophages, are recruited by chemotactants such as PDGF and TGF β 1 released by degranulating platelets (Riches, 1996). Changes in selectin expression by endothelial cells of capillaries at the wound site facilitate extravasation of these cells, allowing neutrophils to kill invading bacteria and macrophages to engulf debris.

Once at the wound site both neutrophils first, and later macrophages, reinforce the pool of pro-inflammatory cytokines to activate neighbouring fibroblasts and keratinocytes (Hubner et al., 1996). If macrophage infiltration is blocked then healing is severely impaired (Leibovich and Ross, 1975). Ongoing studies in our lab are addressing wound healing in transgenic *PU-1* knockout mice which lack macrophages due to a block in the cell lineage pathway (McKercher et al., 1996). There is a close correlation between the age of onset of scarring and the age at which an inflammatory response is first invoked following wounding (Hopkinson-Woolley et al., 1994; Martin, 1997), so it will be interesting to see whether the absence of macrophages in this mouse results in a wound healing phenotype.

- *Re-epithelialisation occurs via lamellipodial crawling*

The basal keratinocyte layer of adult skin is firmly attached to its underlying basal lamina by hemidesmosomal contacts, using α 6 β 4 integrins to bind to laminin in the extracellular matrix. Before re-epithelialisation can occur these attachments have to be dissolved and new integrins upregulated to form the attachments necessary for migration. Since the basal lamina is disrupted in all but the most superficial wounds, leading edge keratinocytes have to migrate over the fibronectin/type I collagen rich granulation tissue.

Expression of $\alpha 5\beta 1$ and $\alpha v\beta 6$ fibronectin/tenascin receptors and $\alpha v\beta 5$ vitronectin receptor, coupled with the relocalisation of their $\alpha 2\beta 1$ collagen receptors allows them to gain purchase on the wound matrix (reviewed in Clark, 1996). These changes in integrin profile take some hours and account for the lag phase of up to 18 hours before epidermal migration commences.

Unlike the embryonic epithelium which migrates across the wound surface by actin pursestring contraction (Martin and Lewis, 1992), the adult epithelium moves by crawling, with leading edge cells extending a tongue-like lamellipodium, forming new attachments and then pulling themselves forwards. Motile force comes from the contraction of intracellular actinomyosin filaments, which insert into new adhesion complexes formed at the distal basal surface of the lamellipodium (Mitchison and Cramer, 1996). It is also possible that, in addition to the crawling of basal keratinocytes, suprabasal cells may expedite the process by leapfrogging over basal cells as they migrate (Garlick and Taichman, 1994).

- ***Proteases and growth factors play important roles in re-epithelialisation***

In the embryo, wound marginal epithelium advances over underlying mesenchyme unhindered, sweeping forward the small amount of cellular debris in its path. In the adult the fibrin clot provides a somewhat sterner obstacle to re-epithelialisation. To cut through, or under, the clot, migrating keratinocytes must use a cocktail of digestive enzymes, primarily fibrinolytic ones. The main one is plasmin, derived from plasminogen within the clot via proteolytic cleavage by one of two key activators, tissue-type plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA). Both of these enzymes are upregulated in migrating keratinocytes (Grondahl-Hansen et al., 1988; Romer et al., 1991 and 1994), and in transgenic mice lacking plasminogen, re-epithelialisation is almost totally blocked (Romer et al., 1996). Other enzymes secreted

by leading edge keratinocytes include several members of the matrix metalloproteinase (MMP) family, the most important in this context being MMP-9 (Gelatinase B), which cuts basal lamina and anchoring fibril collagens (type IV and type VII collagen respectively).

Until recently, interest in growth factors expressed at the wound site had focussed on members of the epidermal growth factor (EGF) family, in particular EGF, TGF α and heparin-binding EGF (HB-EGF). These all act as ligands for the EGF receptor and have been shown experimentally to exert both a motogenic and mitogenic effect on keratinocytes (Brown et al., 1986; Schultz et al., 1987; Barrandon and Green, 1987). One downstream effect of EGF signalling is activation of the small GTPase Rac, shown in fibroblast tissue culture studies to mediate lamellipodial extension and focal adhesion complex formation - key elements of the crawling phenotype (Ridley et al., 1992; Nobes and Hall, 1995).

The finding that keratinocyte growth factor (KGF, or FGF-7) is massively upregulated by dermal fibroblasts within 24 hours of wounding sparked a new wave of growth factor research (Werner et al., 1992). KGF is expressed at reduced levels in several models of impaired healing (Brauchle et al., 1995), but in transgenic KGF knockouts there is no discernible wound healing phenotype (Guo et al., 1996). This is likely to reflect a degree of genetic redundancy, since in mice expressing a dominant negative form of the FGFR2 isoform found in basal keratinocytes, both marginal cell proliferation and re-epithelialisation are delayed (Werner et al., 1994). Topical application of exogenous KGF to skin wounds results in increased expression of MMP-10 and plasminogen activators, resulting in faster re-epithelialisation and increased cell proliferation (Staiano-Coico et al., 1993; Pierce et al., 1994; Madlener et al., 1996), so although there are sure to be other growth factors involved in re-epithelialisation, KGF is clearly one of the key players.

- *Wound contraction occurs after an initial lag phase*

As with embryonic wound repair, concomitant with re-epithelialisation in the adult is contraction of the underlying connective tissue. In adult skin wounds this tissue is termed granulation tissue, due to the pink granular appearance of the invading capillaries. Following wounding there is a lag phase of 3-4 days before this contraction occurs. During this time quiescent fibroblasts surrounding the wound tissue are activated by signals from isoforms of PDGF, TGF β and activin, and begin to proliferate and migrate into the provisional wound matrix. Once there, they start laying down a collagen rich matrix to serve as a scaffold on which they can pull to generate contractile force. If the wound is rewounded at this stage there is no lag phase prior to fibroblast invasion, confirming that the delay is due to inertia on the part of quiescent fibroblasts (McClain et al., 1996). Wound fibroblasts express connective tissue growth factor (CTGF) as an immediate early gene in response to TGF β 1 signalling (Igarashi et al., 1993), which ties in nicely with our hypothesis that homologues of some signalling cascades from *Drosophila* embryogenesis are used during vertebrate wound repair, since CTGF is homologous with the product of the *Drosophila* gene *twisted gastrulation*, which is downstream of *dpp*, a TGF β family member (Martin, 1997).

Normal dermal fibroblasts are capable of initiating wound contraction (Grinnell, 1994). However, about a week after wounding, some of the newly resident fibroblasts differentiate into a specialist contractile myofibroblast cell type (Darby et al., 1990), expressing α -smooth muscle actin and generating most of the contractile force. Studies in which the borders of an adult rat excisional back wound were tattooed to allow connective tissue contraction measurement showed that, as in our embryonic model, mesenchymal contraction contributes roughly 50% of the healing effort (Abercrombie et al., 1954).

- ***Angiogenesis is triggered by two main stimuli in the adult wound***

There are two main angiogenic factors at the wound site; FGF2, which is released firstly from damaged endothelial cells and later by macrophages (Clark, 1996), and vascular endothelial growth factor (VEGF), which is expressed by wound edge keratinocytes and macrophages, possibly in response to KGF and TGF α signalling (Brown et al., 1992).

When FGF2 is depleted using a monospecific neutralising antibody, wound angiogenesis is massively decreased (Broadly et al., 1989) and in diabetic mice, where VEGF expression at the wound site is minimal, wound healing is impaired (Frank et al., 1995).

Endothelial cells respond to these angiogenic signals by upregulating $\alpha v\beta 3$ integrins at the tips of the invading capillaries. Administering blocking antibodies targeted to $\alpha v\beta 3$ stops angiogenesis and results in failure to heal (Brooks et al., 1994). Invading capillaries also show tightly regulated expression of proteases to enable them to penetrate the wound matrix (Fisher et al., 1994).

- ***Adult wound repair results in scarring***

From the moment of wounding until well after wound contraction is complete, the wound matrix undergoes extensive remodelling. It starts with the generation of a fibrin mesh during clot formation and is followed later by fibroblast invasion and its accompanying fibrolysis and collagen deposition. Once contraction is complete endothelial cells and myofibroblasts undergo programmed cell death (Compton et al., 1989; Desmouliere and Gabbiani, 1995). Fibronectin and hyaluronic acid are removed and collagen fibrils remodelled by collagenases released from macrophages, epidermal cells and fibroblasts. Tissue inhibitors of metalloproteinases (TIMPs) are also released to regulate this dynamic process, as collagen fibrils are reoriented and the ECM meshwork reconstructed (Hasty et al., 1986).

TGF β isoforms are thought to be critical triggers of fibrosis and scarring in adult tissues.

In embryonic wounds the expression of TGF β 1 is transient and relatively muted (Whitby and Ferguson, 1991; Martin et al., 1993), whereas in the adult it is expressed at high levels for a prolonged period (Frank et al., 1996). TGF β 1 is implicated in many fibrotic conditions and delivery of neutralising antibodies to TGF β 1 and 2 reduces scarring (Shah et al., 1992). Application of exogenous TGF β 3 reduces scarring by downregulating TGF β 1 and 2 (Shah et al., 1995), so it seems essential that a balance be struck between the levels of the various isoforms. The remodelling process can continue for several months after wound closure in adults and is estimated to, at best, leave scar tissue with only 70% of the strength of intact skin (Clark, 1996).

Embryonic wound healing

Embryos heal wounds both rapidly, efficiently and in a scar free fashion. Here I review key descriptive studies of healing in various models, and describe studies that have contributed to our understanding of molecular and cellular processes underlying the healing effort.

- ***A brief history of embryonic wound repair***

Long before biologists became interested in embryonic wound healing *per se* it was apparent, from classical, surgical developmental biology studies, that embryos have a remarkable ability to heal wounds rapidly and perfectly (Martin 1996). A variety of model organisms have been studied and the chick embryo has been used extensively as an excellent model, because of its amenability to experimental manipulation. Early

studies focussed on wounds to the endoderm of the area pellucida of chick embryos at the primitive streak stage (England and Cowper, 1977). These incisional wounds initially gaped, as tension in the sheet of cells was released, but subsequently closed rapidly. A more detailed study on 5 day chick embryos (Thevenet, 1981) showed that an excisional flank wound of 0.3mm^2 was able to heal in 24 hours, with the epithelial wound margin migrating with an apparently rounded leading edge with no membrane protrusions. This suggestion that the epithelial cells were not extending lamellipodia to crawl forward over the underlying mesenchyme supported similar observations by Stanisstreet and colleagues in the neurula stage embryo of the frog, *Xenopus laevis* (Stanisstreet et al., 1980). However, at later stages and when basal lamina remained intact (Radice, 1980), wound edge cells were seen to crawl, just as in adult skin healing.

Stanisstreet elegantly illustrated that *Xenopus* tadpoles failed to heal wounds when their environment was perturbed either by the addition of cytochalasin-D, which blocks actin polymerisation, or EDTA, a calcium chelating agent. This suggested that both actin polymerisation and calcium ion influx were necessary for successful wound closure (Stanisstreet, 1982) but unfortunately follow-up experiments in cultured neurulating rat embryos failed to give conclusive findings (Smedley and Stanisstreet, 1984).

- ***What signals kick start the embryonic repair process?***

In adult wounds, there are two obvious sources of growth factors and cytokines which presumably kick start the wound healing response; degranulating platelets and macrophages. Neither of these cell types are present at a mid-gestational embryonic wound site - megakaryocytes, the precursors of platelets, have not yet differentiated and the embryo wound does not attract an inflammatory macrophage response. So what are the signals that initiate repair? One possibility is that the amniotic fluid, which contains a rich cocktail of growth factors and cytokines, acts as an alternative source of signals to trigger wound closure. This seems not to be the case, at least for mouse embryos, since

studies in our lab show that wounds heal well in roller culture, in a chemically defined serum-free medium containing no additional growth factors (McCluskey, 1995).

Cells at the embryonic wound margin become transiently leaky upon wounding (Brock et al., 1996), either directly, due to membrane damage, or indirectly, due to gaping of the wound opening stretch-activated ion channels. An influx of calcium ions into wound edge cells, and back to undamaged cells via gap-junctional links, has been mooted as a means by which the front few rows of cells might be activated for a coordinated wound healing response. In support of this speculation, Errington and Martin (unpublished data) have shown that reducing extracellular Ca^{2+} levels at the time of wounding a tissue culture monolayer blocks the Ca^{2+} wave back from the wound edge, and concomitantly prevents the wound-induced upregulation of *c-fos*.

- *A contractile actin pursestring drives re-epithelialisation of an embryonic wound*

As alluded to earlier, epithelial cells at the embryonic wound margin have a rounded morphology, implying that they do not migrate by means of lamellipodial crawling. Indeed, Martin and Lewis (1992) showed that excisional wounds made to the young chick embryo limb bud re-epithelialise by assembly and contraction of an actin pursestring in leading edge cells. These findings were also confirmed in the mid-gestational mouse embryo model (McCluskey and Martin, 1995), on which I shall be focussing in later chapters.

The actin cable is assembled within two minutes of wounding, which is too rapid for it to be dependent on *de novo* actin synthesis. Rather, assembly must be by relocalisation and polymerisation of intracellular filamentous and monomeric actin stores. The cable can be visualised clearly in fixed tissue by staining with fluorescently tagged phalloidin, a fungal toxin with a high affinity for filamentous actin. The signals controlling formation and contraction of this pursestring are not yet clearly understood but, using the chick embryo

model, Brock and colleagues (1996) have shown that activity of the small GTPase molecular switch Rho is necessary for cable assembly.

- *Embryonic wound re-epithelialisation bears analogies to embryonic morphogenesis*

It is unlikely that amniote embryos such as chick or mouse would be wounded during their normal development, so it is likely that there is no specialized machinery for embryonic wound closure. Instead, it is hypothesised that embryos simply adapt tissue movements used during normal morphogenetic events to close a wound (Martin, 1997).

In my *Drosophila* introduction I discuss the similarities between re-epithelialisation and two actinomyosin driven morphogenetic movements in the fly embryo; gastrulation and dorsal closure. There are other morphogenetic movements which occur during vertebrate development which also involve actin based tissue movements; neurulation, where the neural plate folds in on itself to form the neural tube is almost certainly facilitated by actin based contraction of cell apices. Evidence for this comes from rat embryo culture experiments, where inclusion of an actin polymerisation blocker, cytochalasin-D, in the culture medium blocks neurulation (Morriss-Kay and Tuckett, 1985).

Zebrafish epiboly is another cell migratory movement, where the embryonic epithelium migrates ventrally to envelop the yolk cell on which the fish embryo sits. As a result of recent large scale screens, many epiboly mutants are available, so ongoing wound healing studies in our lab are exploring the possibility that similar mechanisms are involved in both processes.

- *Mesenchymal contraction plays an important role in closure of an excisional embryonic wound*

The contribution of re-epithelialisation to the wound healing effort is clear to see from scanning electron microscopy, but it is harder to determine how much of the wound healing effort comes from contraction of the wound mesenchyme. Studies in the rabbit foetus reported that mesenchyme exposed by excisional wounds did not contract if uncovered (Somasunderam and Prathap, 1970) but did when covered with a silastic patch (Somasunderam and Prathap, 1972), suggesting that some factor in the amniotic fluid may be inhibiting contraction. More recent studies from our lab have shown that, in both the chick (Martin and Lewis, 1992) and the mouse (McCluskey and Martin, 1995), contraction of the embryonic wound bed mesenchyme plays a major part in the wound healing effort. Contraction can be visualised using the fluorescent lipophilic dye DiI to label discrete populations of mesenchymal cells around the wound margin and tracking the labelled cells as the wound closes. The above studies demonstrate that mesenchymal contraction plays a major role in embryonic repair - contributing roughly 50% of the closure effort. Contrary to the adult wound healing situation, embryonic fibroblasts do not differentiate into myofibroblasts at the wound site. These specialised contractile cells are always seen within the granulation tissue of an adult wound and are believed to provide the major contractile force. Instead, it seems embryonic fibroblasts can perform a sufficiently contractile role in their undifferentiated state (McCluskey and Martin, 1995). During normal, unperturbed development, embryonic fibroblasts are capable of exerting considerable contractile forces as they drive various crucial morphogenetic events (Oster et al., 1983; Stopak and Harris., 1982). It may be that, as with pursestring contraction in the epithelium, embryonic fibroblasts are simply using the cytoskeletal machinery of normal morphogenesis to heal embryonic wounds.

- *Genes involved in embryonic wound repair are upregulated within minutes of wounding*

As already discussed, cytoskeletal processes involved in wound repair are initiated almost immediately following wounding, far too soon for any gene upregulation to be involved. However, various downstream events during the tissue repair process will be under more direct genetic control. We are beginning to understand more of the genetics underlying the embryonic wound healing process. The immediate early gene *c-fos* is upregulated rapidly and transiently at the wound edge - cFos protein is detectable in the front four rows of wound margin epithelial cells and superficial mesenchyme within 30 minutes, with mRNA levels decreasing by 1 hour post-wounding (Martin and Nobes, 1992; McCluskey, 1995). C-Fos is a transcription factor of the Fos/Jun family which heterodimerises with other family members to form a complex known as Activator Protein-1 (AP-1). This complex is localized to the nucleus where it binds to AP-1 binding sites in the upstream regulatory domains of many potential target genes (Chiu et al., 1988; Rauscher et al., 1988). Intriguingly, *c-fos* has been shown to be highly calcium-inducible (Sheng et al., 1988), so would fit nicely into a model where extracellular Ca^{2+} leakage and transfer to neighbouring cells was responsible for signalling tissue injury. Immunocytochemical studies in our lab have shown other transcription factors of the fos/jun family are upregulated as part of the early wound response (McCluskey, unpublished data). However, experiments on *c-fos* knockout mouse embryos showed that, although strongly upregulated in wild types, c-Fos is not essential for wound repair, since wounds to nullizygous embryos healed perfectly normally.

- *Growth factors in the embryonic wound*

Downstream of immediate early genes come the effector genes responsible for driving wound closure. Likely target genes at this juncture are various growth factors and indeed, *TGF β 1* mRNA, which has AP-1 sites in its 5' upstream regulatory region (Kim

et al., 1990; Roberts et al., 1991), is upregulated by 1 hour after injury (Martin et al., 1993). Again, knockout studies seem to suggest genetic redundancy exists since mice null for *TGFβ1* are able to heal wounds without impairment (McCluskey, unpublished data). One aim of this thesis, therefore, is to further elucidate the pattern of gene expression following injury, in an attempt to determine what redundancy pathways exist in the mouse embryo.

Although the profile of growth factor expression at adult wound sites is well documented, the roles of many of these molecules in the embryo remain to be determined. Recent work has focussed on TGFβ1, as discussed above, as well as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) (Whitby and Ferguson, 1991), where lip wounds to foetal mice were shown to transiently upregulate PDGF but not bFGF or TGFβ1. It seems that in embryonic wounds the expression of TGFβ1 is much more tightly controlled than in the adult, where prolonged expression is thought to be one of the factors responsible for causing scarring (Shah et al., 1992 and 1994).

- *Embryonic and foetal wound repair studies show scar free healing*

Scarring is characterised by abnormal organisation of collagen fibrils into dense parallel bundles, instead of the usual meshwork that exists in unwounded skin. Levels of type I, II and IV collagens do not alter significantly during foetal repair (Whitby and Ferguson, 1991), but embryonic, foetal and adult models all show elevated type III collagen levels. Unwounded embryonic skin has far higher type III collagen levels than does adult skin (Smith et al., 1986). This tolerance to normally high concentrations of type III collagen may be a factor in enabling embryonic wounds to heal without scarring.

Foetal wounds are also rich in hyaluronic acid, a high molecular weight proteoglycan thought to help provide a stable matrix around which collagen fibres can be organised in a

normal fashion (Longaker et al., 1991). Also present in the foetal extracellular matrix at elevated levels following wounding is the glycoprotein tenascin. It is reported to play a role in facilitating cell migration by reducing cell adhesion, and the timing of its upregulation correlates well with the onset of cell movement in a mouse foetal lip wound (Whitby and Ferguson, 1991).

It is not simply the foetal environment that allows scar free healing; wounds to human foetal skin, grafted onto adult nude mice, showed perfect repair (Lorenz et al., 1992) whereas adult skin grafted into the foetal environment still showed scarring (Longaker et al., 1994). A marsupial *Monodelphis domestica*, which gives birth to young at similar developmental stage to an early mouse foetus, gives a fascinating insight into foetal healing in an adult environment without the need for grafting, and indeed shows scar-free healing in an environment outside the womb, for some days after birth (Armstrong and Ferguson, 1995).

Drosophila morphogenesis

So far we have confined ourselves to looking at vertebrate models. In this final introductory section I introduce the fruit fly as a plausible model for not only studying the molecular basis of wound repair but, more temptingly, elucidating the level of homology between molecular and cellular events governing repair and morphogenesis.

- *The fruit fly Drosophila melanogaster is an excellent model for studying the genetics of embryogenesis*

The power of *Drosophila* as a model organism stems from its offering us the potential for combining genetic and molecular approaches to questions of gene expression, cell biology and development (Greenspan, 1997). The development of the *Drosophila*

embryo has been studied in great detail both at the gross anatomical level and, since the morphogenetic mutant screening of Nusslein-Volhard and Wieschaus in the early 1980s, at the more complex genetic level. In the 1980's and early 90's much work was done characterising the laying down of the basic axial pattern in the *Drosophila* embryo. It was shown that a hierarchy of gene families (gap, pair rule and segment polarity genes) split the embryo into segments, and then the Hox gene family gave identity to these segments (reviewed in Wolpert, 1998). In recent years some of the focus has switched to understanding the various morphogenetic movements that give shape to the embryo. The *Drosophila* embryo undergoes a series of elegant morphogenetic movements during its development, each of which are amenable to genetic dissection thanks to mutants generated from the above screen.

- ***Drosophila embryos show actin dependent morphogenetic movements during gastrulation***

The fifteen hours spanning fertilization of the egg cell to completion of dorsal closure bear witness to a sequence of morphogenetic movements. The earliest of these is ventral furrow formation, the fly equivalent of vertebrate gastrulation, closely followed by posterior midgut invagination. Both of these events involve epithelial infolding to form an enclosed tube. The cytoskeletal rearrangements required for such an involution are relatively simple, with cells constricting their external face whilst maintaining the breadth of their basal face, transforming an undifferentiated columnar epithelium into one with a patch of polarised wedge shaped cells. The forces thus exerted cause this region of the epithelium to buckle and invaginate, as illustrated in the following diagram:

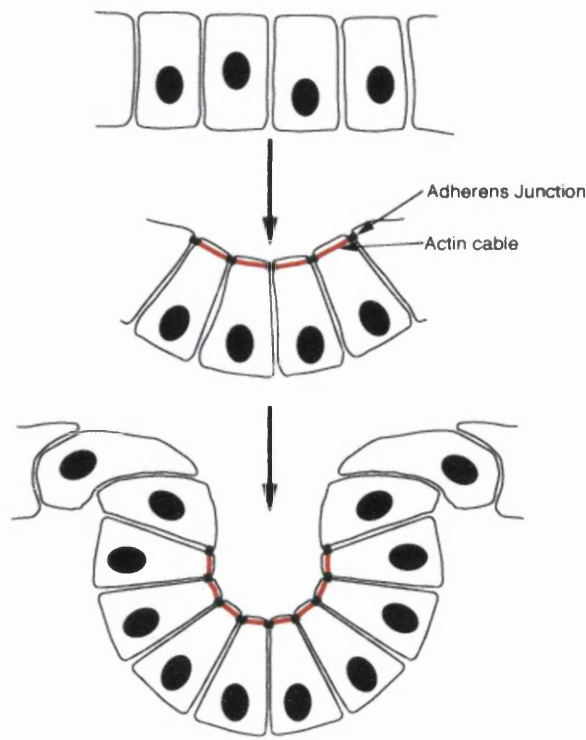


Figure 1.1 *Drosophila* ventral furrow formation

These apical constrictions are thought to be driven by actinomyosin based contraction (Young et al., 1991; Leptin, 1990), with the apical membrane buckling in order to accommodate the decrease in surface area (Leptin et al., 1990; Sweeton et al., 1991). It is known that mutating any one of many of the early patterning genes, such as the transcription factors *twist*, *snail*, *huckebein*, *tailless* and *forkhead*, blocks these invaginations (Costa et al., 1993), by disrupting the specification of cell fate, rather than specifically interfering with the cells contractile machinery. However, two genes, *concertina* (*cta*) and *folded gastrulation* (*fog*) have been identified as being specifically required for ventral furrow formation and posterior midgut invagination. Mutant analysis suggests that *cta* and *fog* facilitate the cell-cell communication that coordinates cell shape changes amongst neighbouring cells, rather than being vital for apical contraction per se (Costa et al., 1994).

cta is a maternally expressed gene encoding a G-protein alpha subunit which is expressed ubiquitously in the embryo (Parks et al., 1991). As Cta protein is found throughout the embryo, it suggests another factor must act to specify which cells are to invaginate. *folded gastrulation* is a zygotically transcribed gene encoding a putative diffusible ligand

for the G-protein coupled receptor associated with Cta. *fog* expression maps precisely to the invaginating primordia and, whilst mutant analysis suggests that Fog protein may not be necessary for initiation of the first constrictions along the ventral midline, more lateral cells are absolutely dependent on *fog* signalling to initiate their shape change (Costa et al., 1994). Embryos mutant for either *cta* or *fog* only initiate apical constrictions in a few cells around the ventral midline but the morphogenetic defect arises from the failure of these initial constrictions to trigger the wave of cell contractions that ensues in normal gastrulation.

Recent work has shown that a molecular switch in the signal transduction pathway downstream of Fog/Cta signalling may be the small GTPase DRho. Flies with either mutations in *DrhoGEF2* (required for Rho activation) or expressing dominant negative DRhoA fail in these gastrulation movements (Barrett et al., 1997; Strutt et al., 1997; Hacker and Perrimon., 1998).

- ***Drosophila* embryos show actin dependent morphogenesis during dorsal closure**

The morphogenetic movement in *Drosophila* which appears to be most similar to wound healing movements is that of dorsal closure. This event occurs towards the end of embryogenesis, about 12 hours after egg laying. At the completion of germ band retraction, the dorsal surface of the embryo is covered by a transient extraembryonic membrane, the amnioserosa. Dorsal closure is the 2 hour process by which the lateral epidermis sweeps dorsalwards from both sides of the embryo and zips together at the dorsal midline to form one continuous epithelium, thus completing embryogenesis.

This epithelial movement is accompanied by the assembly of a contractile intracellular actin pursestring at the apices of cells in the leading edge. Contraction of the cable is facilitated by non-muscle myosin II motors (Young et al., 1993), with adherens junctions at cell-cell contacts providing the links between cells to form an effectively continuous

contractile cable around the leading edge of the lateral epithelium. In this regard, the contractile machinery appears almost identical to that which assembles at the margin of a vertebrate embryo wound.

- ***Many Drosophila mutants fail to complete dorsal closure***

There were approximately 30 fly morphogenetic mutants generated in the 1984 screen by Wieschaus and colleagues (Wieschaus et al., 1984) which were unable to complete dorsal closure. They have been studied extensively over the past few years and can be broadly classified into two groups; mutants in which some component of the driving cytoskeletal machinery is defective or mutants in which some element of the signalling machinery is impaired. This group has been added to in recent years by homology cloning of genes shown to be key in actin regulation in vertebrate tissue culture cells (Barrett et al., 1997; Strutt et al., 1997; Hacker and Perrimon., 1998). *Appendix I* contains a list of some of the main cytoskeletal and signalling mutants. Most of the above mutants totally or partially fail to complete dorsal closure. One mutant, *puckered*, does achieve closure but cells along the dorsal midline where the lateral epithelia fuse do not intercalate properly to form a continuous epithelium and the result is a “puckering” of the epidermis. Severe *puc* alleles do not develop beyond this late embryonic stage (Ring et al., 1993; Martin-Blanco et al., 1998).

- ***Dorsal closure is controlled by a JNK signalling cascade***

Over the past two years, the signalling cascade controlling dorsal closure has been genetically dissected, and is known to involve a Jun Kinase pathway under the control of members of the Rho family of small GTPases (reviewed in Noselli, 1998). In mammalian systems the specific functions for each of these GTPases have been partially characterised. It seems that Cdc42 regulates filopodia, Rac regulates lamellipodial extensions and Rho regulates stress fibre formation (Ridley et al., 1992a and b; Nobes

and Hall, 1995). Curiously it seems that *Drac*, *DrhoA* and *Dcdc42* may all play an important part in dorsal closure, although their specific functions are not known. Our current understanding of the basic JNK signalling pathway can be summarised in the following diagram:

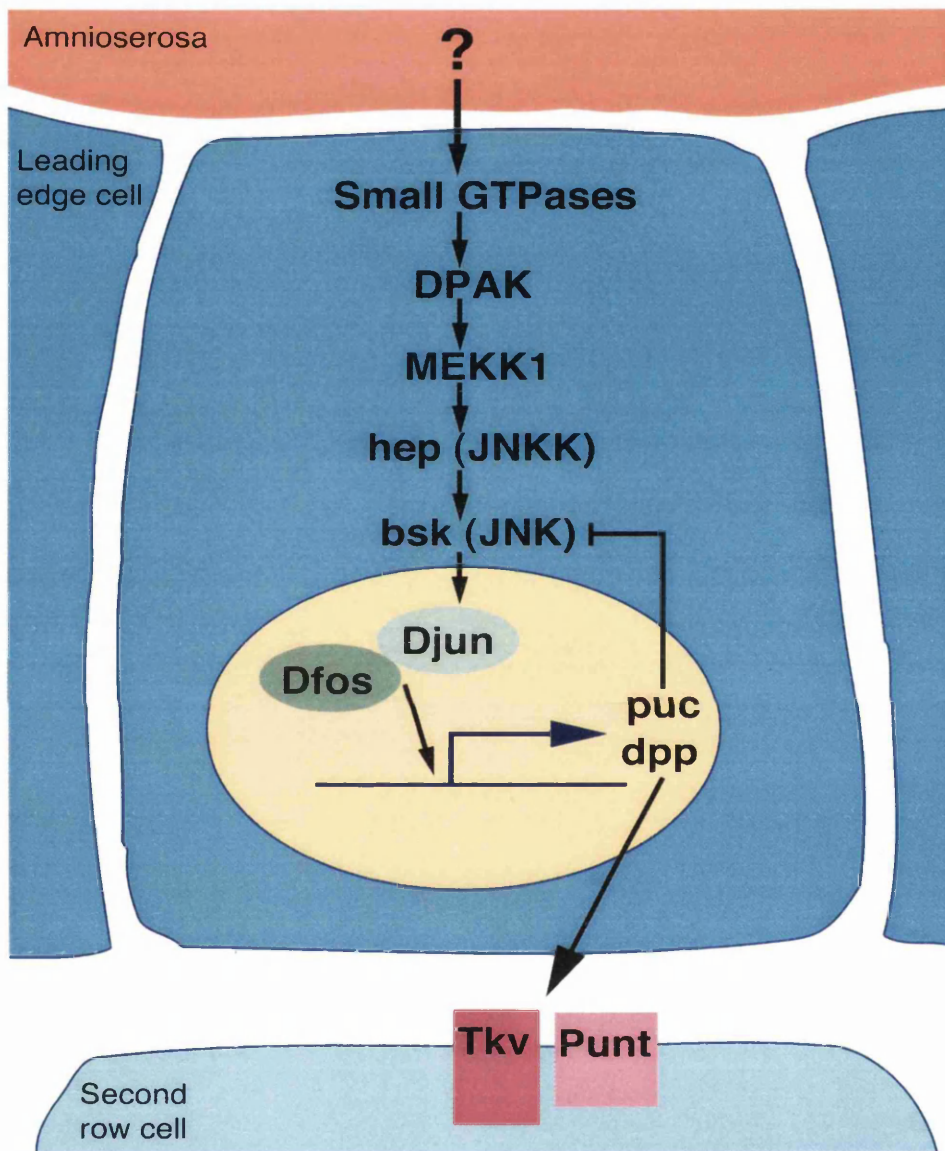


Figure 1.2 JNK signalling in dorsal closure (Goberdhan and Wilson, 1998)

The ordering of genes in this cascade has been deduced from a series of elegant experiments, the key ones of which are outlined below:

Constitutively activated DJun expressed under heat shock control can rescue *Drac*, *hep* and *bsk* dorsal hole phenotypes (Hou et al., 1997; Riesgo-Escovar et al., 1997a). *Dfos* and *Djun* have been shown to cooperate to regulate the expression of *dpp*, with Dpp

being the short range signal which causes the wave of shape change in the cells back from the front row (Riesgo-Escovar et al., 1997b). Two controlling genes have been identified downstream of *Djnk*; one encodes a phosphatase, *puckered*, which dephosphorylates activated DJNK and the other a transcriptional repressor *anterior open* (*aop/yan*) which is inactivated by activated DJNK (Riesgo-Escovar et al., 1997b).

The cells of the front row of the epithelial leading edge show specific expression of several genes, including *puc* (Fig. 1.3) and these are the first cells to elongate.

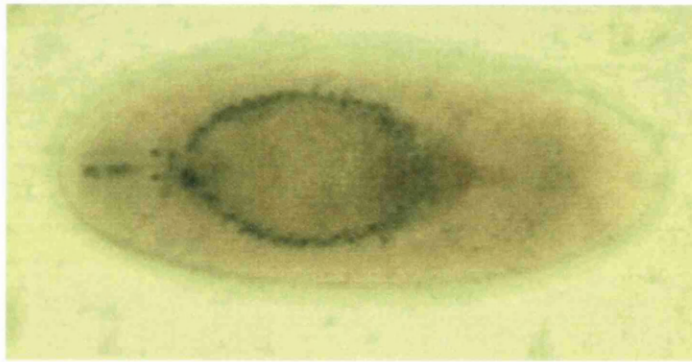


Figure 1.3 *Puckered-lacZ* expression in the leading edge cells of the lateral epithelium during dorsal closure, visualised using X-gal staining.

A wave of elongation propagates back from these cells, mediated by Dpp. Flies expressing mutated forms of the Dpp receptors *thick veins* and *punt* show contraction and elongation of cells of the leading edge but not of those cells further back, and dorsal closure fails (Riesgo-Escovar et al., 1997b).

Of the cytoskeletal mutants, the one of most immediate interest is *zipper*. *zipper* mutant embryos form a normal actin cable in their leading edge cells, presumably in response to the signalling elements outlined above, but they lack zygotic non-muscle myosin II, so have no functional myosin motors to provide the driving force for pursestring contraction (Young et al., 1993).

- *The signals initiating dorsal closure are poorly understood*

Whilst our understanding of much of the signalling cascade and cytoskeletal machinery underpinning dorsal closure has blossomed over the past couple of years there is still little known about what the signals are that initiate this movement. One hypothesis is that germ band extension/retraction generates an increased tension in the leading edge of the lateral epithelium, in much the same way that the gaping following wounding of an intact epithelium causes the cells around the wound margin to stretch. This stretch signal, perhaps mediated by ion channels or mechanotransducing receptors, could then activate JNK signalling.

Alternatively, a paracrine signal from the amnioserosa may activate the cells forming the leading edge of the lateral epithelium. Perhaps the tissue polarity gene *frizzled* (*fz*) is involved and the dorsal closure cascade is downstream of a Wnt signalling pathway. Tissue polarity genes are known to be required for correct establishment of planar epidermal structures, particularly in the developing eye (Strutt et al., 1997). Since the JNK cascade controlling dorsal closure is also known to be vital for normal eye development, it is plausible that *fz* signalling is involved in both processes.

- *The amnioserosa may play an active role during dorsal closure*

Studies into the development of the *Drosophila* heart during embryogenesis suggest that far from being a static extraembryonic membrane over which dorsal closure proceeds, the cells comprising the amnioserosa actively constrict their apices and invaginate, thus expediting dorsal closure (Rugendorff et al., 1994). Concomitant with the lateral epidermis advancing dorsalwards, cells of the squameous amnioserosa adopt a columnar shape, thus decreasing the area over which the epidermal cells must migrate. As the epithelia zip together at the dorsal midline, the amnioserosa cells detach from the epidermis and are separated from it by the lateral cardioblast precursors fusing to form the

dorsal vessel. The amnioserosa cells subsequently undergo apoptotic cell death and are engulfed by haemocytes, the fly equivalent of macrophages.

- *The fruit fly is an excellent model for studying the genetics of wound repair*

The main advantages of choosing *Drosophila* as a model to work on focus on its genetic tractability. A major perk is the lack of genetic redundancy. In vertebrates, there are many large gene families encoding closely related proteins which act to mask the phenotypes which one might otherwise predict when gene expression is disrupted in transgenic knockout experiments. In the fly, this genetic redundancy is largely absent. An example of particular interest to our lab is the immediate early gene *c-fos*. Wound healing studies in our lab have shown that *c-fos* expression is upregulated within minutes of wounding a limb bud stage mouse embryo (see earlier). The expression data is suggestive of a role for AP-1 signalling in activation of wound closure, but genetic redundancy makes experiments to test the speculation very difficult to perform in the mouse. Indeed, when homozygous transgenic *c-fos* knockout mice are wounded, they heal their wounds as efficiently as wild type littermates. In *Drosophila* only one *fos* gene (*kayak*), and one *jun* (*Djun*), have been identified, which clearly offers a way to circumvent the problem of genetic redundancy - if *fos* is required for wound repair then a *kayak* embryo should show impaired healing.

Since there already appear to be analogies to be drawn between movements in tissue repair and those of natural morphogenesis, the recent genetic analysis of dorsal closure is an invaluable resource for my *Drosophila* wound healing studies. There are fly stocks available which have mutations in almost all known genes vital to the initiation, regulation and execution of dorsal closure. As well as loss of function mutants there are many dominant negative construct-expressing flies. Wounding dorsal hole phenotype mutants should allow identification of those morphogenetically important genes which are also key components of the fly wound healing response.

There are some practical difficulties when trying to wound fly embryos; they are extremely small, less than 500µm long, and therefore only tiny wounds of a few cell diameters across can be studied. Underwood and Mahowald performed a fate mapping study on *Drosophila* embryos (Underwood et al., 1980) in which they removed discrete populations of cells from embryos, between 2.5 and 4.5 hours of development, using a glass micropipette, in order to mark various regions of the embryo. These wounds of approximately 15µm diameter had healed by the time the embryos were fixed at 9 hours of development, although wound closure *per se* was not examined. This study shows that a *Drosophila* embryo is capable of surviving a small wound and so paves the way for us to further investigate the genetics of tissue repair in this organism.

- ***Excisional wounds to wing imaginal discs show complete pattern regeneration***

An alternative model offering potentially the same powerful genetics but in a more experimentally flexible fashion is the *Drosophila* wing imaginal disc, derived from 3rd instar larvae (see Fig. 1.4):

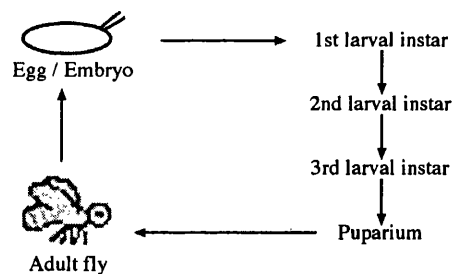


Figure 1.4 *Drosophila* life cycle

Imaginal discs are the larval precursors of adult tissues. They are present in pairs in the larval stages and develop into adult structures during pupariation. They can be readily isolated from 3rd instar larvae, with the two wing discs being the largest. Several studies have looked at pattern regeneration following excisional wounding of imaginal discs, showing that discs are capable of healing large wounds and regenerating parts which were removed by the excision (Bryant, 1975; Dale and Bownes, 1985). Although these

studies elegantly describe regeneration they do not cover the cytoskeletal or signalling machinery that is effecting wound closure. My studies, therefore will try to address the more basic question of how an imaginal disc, effectively just a flattened balloon of epithelial tissue, heals an incisional wound in culture.

CHAPTER TWO

Investigating the molecular basis of wound repair in the E11.5 mouse embryo

Introduction

Previous studies from our lab have characterised excisional (Martin and Lewis, 1992) and incisional (Brock et al., 1996) wounds in the chick embryo, and excisional limb amputation wounds in the mouse embryo (McCluskey and Martin, 1995). Such wounds have been shown to heal both rapidly and perfectly, and the mechanics underlying the tissue movements comprising the healing responses are relatively well understood. In this chapter I describe my studies of the molecular basis of mouse embryo wound repair.

I will first discuss literature related to these studies, moving on to outline the available strategies one can adopt when searching for genes upregulated in response to wounding. I will highlight the advantages and disadvantages of each, and explain why I chose to adopt a subtractive hybridisation approach. Since knowledge of gene expression patterns alone is of limited value unless we have the means to study gene function, in the last part of this introduction I will review the methods available to us for perturbing target gene expression and protein function.

- *What is known about the genetics of the embryonic repair process?*

As discussed in the general introduction, the environment at a wound site in an E11.5 mouse is considerably less complex than in the adult; there is no platelet degranulation, no inflammatory response and the skin architecture is limited to a simple bilayered epithelium. However, far more is known about the molecular basis of wound repair in the adult compared to the embryo. There is no clear evidence regarding what signals trigger the wound healing response in our embryo model, but studies from our laboratory have suggested that transient leakiness in cells at the wound margin, caused by mechanical damage, may allow a Ca^{2+} ion influx to serve as an early signal initiating repair (Brock et al., 1996; Errington and Martin, unpublished data). Molecular studies have described both *c-fos* (Martin and Nobes, 1992) and *TGF β* (Martin et al., 1993) as genes which are transiently upregulated soon after wounding. Functional investigations of these genes have been performed using transgenic knockout mice, null for c-Fos and TGF β 1 (J. McCluskey, DPhil. Thesis Univ. Oxford, 1995). In each of these experiments, E11.5 litters from heterozygous parent crosses were wounded by hindlimb amputation and cultured for up to 24 hours. Scanning electron microscopy of fixed embryos allowed the extent of wound closure to be measured before genotyping results were known, so that the study was performed blind. Since the ratios of knockout:heterozygote:wild type embryos were Mendelian (1:2:1) in both transgenic lines, wound repair in embryos lacking functional c-Fos or TGF β 1 protein could be compared directly to wild type and heterozygote littermate controls.

- *c-fos is upregulated immediately following wounding*

The immediate early gene *c-fos* is upregulated rapidly and transiently at the wound edge in the mouse embryo model; c-Fos protein is detectable in the front four or five rows of wound margin epithelial cells and also in the superficial mesenchyme within 30 minutes

of wounding (Martin and Nobes, 1992; J. McCluskey, DPhil. Thesis Univ. Oxford, 1995). c-Fos is a transcription factor of the Fos/Jun family which heterodimerises with other family members to form a complex known as Activator Protein-1 (AP-1). This complex becomes localized to the nucleus where it binds to AP-1 binding sites in the upstream regulatory domains of many potential target genes (Chiu et al., 1988; Rauscher et al., 1988). Intriguingly, *c-fos* has been shown to be highly calcium-inducible (Sheng et al., 1988), so would fit nicely into a model where extracellular Ca^{2+} leakage and transfer of this signal to neighbouring cells was responsible for coordinating a response to tissue injury amongst adjacent cells at the wound edge. Immunocytochemical studies in our lab have shown other transcription factors of the Fos/Jun family, specifically *c-jun* and *fosB*, are upregulated as part of the early wound response (McCluskey, unpublished data). However, wounding experiments on *c-fos* knockout mouse embryos (Wang et al., 1992) showed that, although strongly upregulated in wild types, c-Fos is not essential for embryonic wound repair, since wounds to nullizygous embryos healed perfectly normally (J. McCluskey, DPhil. Thesis Univ. Oxford 1995).

- *Some growth factors are upregulated in the embryonic wound*

Downstream of immediate early genes come the effector genes responsible for driving wound closure. Likely target genes at this juncture are various growth factors and, indeed, *TGFβ1* mRNA, which has AP-1 sites in its 5' upstream regulatory region (Kim et al., 1990; Roberts et al., 1991), is upregulated within 1 hour of wounding the embryo (Martin et al., 1993). Again, transgenic knockout studies seem to suggest genetic redundancy of wound signalling systems, since mouse embryos null for *TGFβ1* (Kulkarni et al., 1993) are able to heal wounds just as well as their heterozygote and wild type siblings (McCluskey, unpublished data). One aim of this chapter, therefore, is to uncover gene upregulations following injury, in an attempt to determine what other wound-activated signalling pathways exist in the mouse embryo.

Although the profile of growth factor expression at adult wound sites is well documented, the roles of many of these molecules during embryo wound healing remains to be elucidated. Recent work has focussed on TGF β 1, as discussed above, as well as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) (Whitby and Ferguson, 1991). Lip wounds to foetal mice were shown to transiently upregulate PDGF but not bFGF or TGF β 1. It seems that in embryonic wounds the expression of TGF β 1 is much more tightly controlled than in the adult. In fact, it is now believed that the prolonged expression of TGF β 1 during adult repair may be one of the key factors responsible for causing scarring. Reducing levels to those approaching the transient pulse seen in the embryo, by injection of neutralising antibodies into the adult wound bed, appears to significantly reduce scarring (Shah et al., 1992 and 1994).

- *Several methods are available for gene expression studies*

Fundamental to our understanding of the molecular basis of any given biological process is the capability to define gene expression profiles. The ability to compare patterns of gene expression in different tissues and developmental stages, in normal and disease states, in differing *in vitro* cell conditions or in wounded and unwounded tissues, allows the elucidation of signalling pathways and their downstream effectors. Gene expression can be studied using a variety of methods; Northern blot analysis, RT-PCR, RNase protection assays or *in situ* hybridisation, but these methods focus on only a few genes at a time and will not allow us to identify novel genes. If we are to identify unpredicted or novel genes then we must turn to techniques such as differential display, subtractive hybridisation or high-throughput arraying. In the following section I describe each of the above techniques, consider their advantages and disadvantages, and detail the chosen approaches of some key studies.

1. Northern blotting

In Northern blotting, total RNA is first isolated from cell or tissue samples and then electrophoresed on a denaturing agarose gel. RNA molecules separate according to size and are then transferred to a nylon membrane where they are bound irreversibly by UV crosslinking. These blots can subsequently be probed with radioactively-labelled single-stranded DNA molecules, and mRNA levels visualised by autoradiography.

The advantages of this technique are that it is relatively cheap and easy and that it also allows us to quantify expression levels. These are largely outweighed by the disadvantages, which are that radioactivity must be used and that it takes a relatively long time, so it has been largely replaced by RNase protection and reverse transcription polymerase chain reaction (RT-PCR).

2. RT-PCR

RT-PCR is a sensitive technique for determining whether a gene is expressed by any given tissue sample. RNA is isolated from the sample and complementary DNA (cDNA) generated using reverse transcriptase and an oligo dT primer, which binds to the 3' untranslated tail of all mRNA molecules. Gene specific primers can then be used to amplify target sequence by conventional PCR, so that gene expression can be visualised by electrophoresing PCR products on an agarose gel containing ethidium bromide.

A classic wound healing study in which this approach was used to great effect involved determining which growth factors were expressed by small numbers of glass adherent macrophages isolated from mouse wound fluid using subepidermal glass cylinders (Rappolee et al., 1988). Through reverse transcription of mRNA isolated from macrophages and subsequent cDNA amplification in a polymerase chain reaction primed with growth factor sequence-specific primers, several mRNA species could be assayed

simultaneously. Rappolee and colleagues were able to demonstrate that macrophages isolated from a wound site, and not exposed to cell culture conditions, expressed mRNA transcripts for TGF α , TGF β , platelet-derived growth factor A-chain, and insulin-like growth factor-1. One limitation of this kind of approach is that one can only study expression of those genes for which primers are available, so one can only reveal whether or not predicted genes are upregulated and cannot identify novel genes.

Although this technique is non-radioactive and good for detecting very low levels of transcript it does not always allow reliable quantitation of expression, due to the exponential amplification rates of PCR. A fair idea of expression levels can be obtained by including competitive target sequence of varying concentration in the PCR mix, in quantitative RT-PCR, but for a truer representation RNase protection is more often used.

3. RNase protection

This is currently the most common method of quantitating gene expression levels, allowing levels of target sequence to be directly compared to levels of housekeeping gene expression. Total RNA is again isolated from sample tissue and allowed to hybridise with radioactively-labelled RNA probe specific to the gene of interest. Hybridised samples are then digested with an RNase cocktail that degrades all single stranded ribonucleotide molecules. Double stranded RNA is not degraded, thus the target sequence is “protected” by binding to the probe. Samples can then be electrophoresed and autoradiographed to give an accurate picture of gene expression levels.

This technique has been used in a wound healing context in several studies; allowing quantitation of expression of TGF β isoforms and their receptors during adult rat wound healing (Frank et al., 1996). RNase protection assays revealed a large induction of all three TGF β isoforms and also of TGF β types I and II receptors following wounding.

Similar studies from the Werner lab have shown nitric oxide synthase (iNOS) upregulation after wounding (Frank et al., 1998) and described differential regulation of many cytokines in normal and impaired wound healing models (Hubner et al., 1996).

Despite being the method of choice for tissue culture studies and many *in vivo* experiments, RNase protection does not allow one to visualise gene expression patterns in tissues or to determine which cell type is expressing the gene in question. For analysis of this kind we must look at intact samples using *in situ* hybridisation.

4. *In situ* hybridisation

This method is described in detail in the Materials and Methods section of this chapter. Briefly, it allows one to take either sections of tissue or whole embryos and visualise gene expression *in situ*, such that expression can be resolved to the cellular level. Probes can be labelled either radioactively or using digoxigenin and hybridised to mRNAs that have been fixed in place in the sample. *In situ* hybridisation studies have been used to study expression patterns of numerous genes at the wound site. An early example was KGF which had been shown by RNase protection to be upregulated 160-fold following wounding (Werner et al., 1992), but only *in situ* showed that it was actually dermal cells effecting the upregulation. Similarly, in the previously mentioned expression study of TGF β isoforms and their receptors following wounding (Frank et al., 1996), *in situ* hybridisation was used to complement RNase protection data, revealing that each TGF β isoform had distinct sites of expression in the wound.

In situ hybridisation provides invaluable insight into the tissue and temporal specificity of gene expression and, although quite labour intensive and vulnerable to problems with RNase contamination, was the method I chose for analysing wound induced gene expression patterns.

5. *Differential display PCR*

All of the above methods allow us to look at the expression of already known genes. However, to search for expression of novel or unpredicted genes we need to use slightly different techniques. Differential display PCR (DD-PCR) has been widely used in expression studies, both in tissue culture and animal models. For this and related techniques, mRNAs must first be isolated from samples, and the quality of these mRNAs is crucial to all of the following steps.

DD-PCR uses a combination of primer sets; one of which is an oligo dT sequence targetted to the 3' poly-A tail of all mRNAs, and the other which comprises a pool of random primer sequences. When paired with the oligo dT sequence in a PCR reaction, these random primers yield PCR products corresponding to many of the genes being expressed in the sample tissue. The theory is that this primer cocktail generates a reproducible number of PCR products for any given tissue sample which, if radioactively labelled, can be visualised by autoradiography following electrophoresis. The pattern of PCR fragments for control and experimental tissue can then be compared, and any bands which vary in intensity should correspond to differentially expressed genes. Bands of interest can be isolated and the PCR products cloned and sequenced to identify the genes they represent.

The theory is relatively straightforward, but in practice results are often difficult to reproduce, with many false positive clones being obtained. Clearly this technique has yielded good results over recent years in varied fields of research and specifically in wound healing. For example, the Werner lab has recently used this approach to identify genes upregulated by KGF treatment of cultured keratinocytes. One such gene is the human homologue of bovine non-selenium glutathione peroxidase (Frank et al., 1997), a free radical scavenger upregulated both in the above screen and subsequently shown to be induced by wound edge keratinocytes *in vivo*, following incisional wounding of an adult

rat model (Munz et al., 1997). These studies demonstrate that there are useful links to be made between *in vitro* and *in vivo* models.

6. *Subtractive hybridisation*

As with ddPCR, subtractive hybridisation allows the comparison of two populations of mRNAs, and isolation of clones of genes upregulated in one population relative to the other. Details of the form of subtractive hybridisation I elected to use are in the following Materials and Methods section. Where this technique differs from ddPCR is that it allows direct biochemical comparison of mRNA populations, rather than post-PCR visual comparison of band patterns. It therefore removes a subjective step and generates a more reliable outcome. There are many examples of successful subtractive hybridisation screens using a variety of protocols but, since I opted for the Clontech PCR-Select™ approach, it is a developmental study using this method that I shall discuss.

In a search for genes regulated by the transcription factor encoded by *brachyury* (*xbra*) in *Xenopus*, Jim Smith's group developed a screen based on the PCR-Select™ system (Tada et al., 1998). They engineered a steroid inducible *xbra* construct and compared mRNA populations isolated from animal caps treated +/- dexamethasone. Cycloheximide pretreatment of the system blocked protein synthesis so that only genes directly downstream of *xbra* were identified. They describe several genes, including *xwnt11* and some *bix* genes, which they subsequently showed by *in situ* hybridisation to be expressed in similar domains to *xbra*, in a stripe around the developing frog embryo corresponding to mesodermal tissue.

This system has also proved successful in a wound healing model in the Werner group, who now also use this technique in preference to differential display. In their keratinocyte stimulation tissue culture model they have identified several key genes as being

upregulated, including an actin polymerisation regulating protein, ARP2 (Marcus Gassmann, ETH Zurich, pers. comm.).

7. Nucleic acid arraying

Recent marriage of robotic microprinting technology with high throughput sequence analysis has led to the development of cDNA microchips which allow high throughput gene expression analysis. cDNAs coding for already known genes and for expressed sequence tags (ESTs) are dotted onto filters in a precise pattern and denatured to fix them in place. Current technology allows around 10000 cDNAs to be printed on a standard microscope slide so, where it was previously possible to tailor an array of only several hundred sequences to target particular batteries of genes; e.g. growth factors, transcription factors, cytokines etc., one can now hybridise entire cDNA populations, to give a much more thorough readout. Differential gene expression patterns are identified by separately hybridising two identical arrays with fluorescently labelled cDNAs generated from mRNA populations of interest and comparing the relative levels of signal from each cDNA spot on the microarray.

In a study published early this year, Iyer and colleagues applied microarraying technology to a cell culture system which turns out to bear striking similarities to wound healing physiology (Iyer et al. 1999). They looked at the response of quiescent fibroblasts to serum stimulation; starving cells of serum for 48 hours before adding back 10% fetal calf serum. They isolated mRNA from cells at 16 timepoints over the next 24 hours and hybridised fluorescently tagged cDNAs, generated from each of these populations, to identical microarrays, in the presence of differently tagged cDNAs derived from quiescent fibroblasts. The analysis is relatively simple; control cDNAs are tagged with Cy3 (green) and experimental cDNAs are tagged with Cy5 (red). Therefore, following hybridisation of both populations to the same array, genes upregulated upon serum stimulation appear as green spots, those downregulated appear red, and those with no significant variation in expression pattern are yellow.

Their results show differential expression of hundreds of genes following serum stimulation, and cluster analysis allows them to group them into discrete populations showing similar expression dynamics. The grouping of cDNAs with similar profiles is a useful tool in the assignation of putative function to novel ESTs which show interesting expression patterns. In their discussion section they remark that, following the serum stimulus, one of the most striking features of the unfolding transcriptional program is the appearance of numerous genes with known roles in processes relevant to the physiology of wound healing. Almost all of the currently demonstrated wound-induced immediate early genes (WIGs) are represented and over 170 more besides. Several of this number will no doubt turn out to be upregulated at *in vivo* wound edges. This may not be too surprising, since the only time that cells would be exposed to serum *in vivo* would be at a wound site, following blood clotting. This link may be tenuous and serum stimulation of fibroblasts is clearly not a very robust model of wound healing, but this study clearly demonstrates the powers of microarraying as a tool for gene expression analysis.

- *Several methods allow us to test gene function*

1. Neutralising antibodies

Antibodies are routinely used in immunohistochemical studies, where gene expression in a sample can be studied by labelling specific proteins with antibodies that recognise a specific epitope on the encoded protein. In addition to studies on fixed tissues, antibodies can also be applied to live cellular tissue; in cases where an antibody acts to ablate the protein's function it can be described as a neutralising, or blocking, antibody. These types of molecule have been used extensively to test the function of various growth factors in wound repair processes, so I will highlight just a few of the studies.

TGF β is one of the most keenly studied molecules in wound healing and has been the subject of several neutralising antibody studies, most famously by Shah and colleagues (Shah et al., 1994 and 1995) who showed that neutralising antibodies to TGF β 1 and 2 had an anti-scarring effect when applied to cutaneous wounds. Similar findings were made in a rabbit corneal wound model (Moller-Pedersen et al., 1998). Pretreatment with TGF β 1 blocking antibodies has also been shown to block myofibroblast differentiation in cultured fibroblasts (Desmouliere et al., 1993).

Another well studied wound repair molecule, Fibroblast growth factor-2 (FGF-2), has been successfully blocked using neutralising antibodies. One such study showed that FGF-2 is important in the healing of skin wounds (Broadley et al., 1989). Sponges containing neutralising antibodies to FGF-2 implanted subcutaneously in adult rats showed a significant reduction in granulation tissue infiltration, with reductions in DNA, protein, and collagen content, when compared to control sponges containing either pre-immune IgG or nothing. More recently, a similar approach has shown FGF-2 to be functionally important in the repair of cortical lesions in the brain (Rowntree and Kolb, 1997). FGF-7 (KGF), another important wound healing molecule, has also been subject to blocking antibody studies, with competitive binding experiments using blocking antibodies targetted to specific regions of the KGF protein allowing prediction of both its heparin and KGF receptor binding sites (Kim et al., 1998).

There are clearly many examples of neutralising antibody studies which give useful information about possible protein function but there are disadvantages to this approach also. Antibody production is both time-consuming and expensive, and there is no guarantee of efficacy or specificity, so other approaches are often adopted.

2. Pharmaceuticals

From non-healing leg ulcers to hypertrophic scarring and fibrosis there are many wound healing disease states to generate interest in the pharmaceutical industry. The generation of therapeutic agents which could improve tissue repair in any of these conditions is mainly focussed on small molecules which might modulate the function of key wound signalling proteins. These small molecule drugs can be generated reproducibly and relatively cheaply at the basic research stage, and are often easier to administer than other treatments described in this section and generally overcome the problems of eliciting an immune response.

Most drug-based studies fail to get published due to the intense competition and secrecy in the pharmaceutical industry, so although high throughput screening of chemistry-led small molecule drugs has yielded results, such as the collagenase blocker UK-221316 (Pfizer Ltd) described later, few of these data are present in the literature. Several studies have used drugs in a non-specific manner, such as using steroid treatment to model impaired wound healing scenarios, including a seminal Leibovich and Ross (1975) study of macrophage depletion in the guinea pig, but most relevant to this introduction is the use of drugs that inhibit specific target proteins.

Drug based approaches have proved especially fruitful in the cytoskeletal field, where agents such as cytochalasin D, a fungal metabolite which blocks actin polymerisation (Flanagan and Lin, 1980), and colchicine, an alkaloid isolated from crocus plants which blocks microtubule assembly (Malawista and Bensch, 1967), have facilitated studies of cytoskeletal dynamics. In addition to these two widely used drugs, several exist that have been of particular value to actin pursestring studies in our lab: C3-transferase, an exoenzyme isolated from the bacterial pathogen *Clostridium botulinum* that ADP-ribosylates the small GTPase molecular switch Rho (Rubin et al., 1988), has been shown to specifically block actin cable formation in wound edge cells (Brock et al., 1996); BDM, a myosin ATPase inhibitor (Zhao et al., 1995), blocks myosin driven contraction of the

actin pursestring in the same cells (J. Brock PhD Thesis UCL, 1997); and also Y-27632, a chemical blocker of the Rho-associated protein kinase p160ROCK (Uehata et al., 1997) is currently under investigation.

3. *Antisense oligonucleotides*

Antisense oligonucleotides are short DNA molecules, usually around 20 nucleotides long, that are complementary to part of a target gene sequence. They can be used to ablate gene expression by hybridising specifically to transcribed mRNA and acting as a substrate for RNase H, an endogenous RNase which recognises and digests DNA-RNA hybrid molecules (Cazenave et al., 1987). One problem can be delivery of these oligonucleotides into target cells *in vivo*, and in this regard wound healing is an ideal process to study since the target cells are easily accessible. Another problem is controlling for toxicity, especially where modifications have been made to the phosphodiester DNA backbone to enhance stability (Woolf et al., 1990), and for this reason carefully chosen sense or switch controls are required. A sense control involves simply adding an identical concentration of sense oligonucleotide rather than antisense, whereas a switch control changes the position of just 3 or 4 nucleotides, without altering the overall base composition.

Several studies have used antisense oligonucleotide approaches to study gene function in the wound healing context. DiPietro and colleagues (1996) used antisense oligonucleotides to ablate Thrombospondin 1 (TSP1) in a rat cutaneous wound healing model. TSP1 is a glycoprotein extracellular matrix molecule produced by many cell types that are involved in wound repair and treating wounds with TSP1 antisense oligos resulted in less macrophage recruitment to the wound site and a marked delay in repair. This delay included a decreased rate of re-epithelialisation as well as a delay in dermal reorganization, suggesting that TSP1 production by macrophages facilitates the repair process and provides evidence that TSP1 production is an important component of wound healing.

Other studies include using PKC α antisense in a rabbit eye wound model, where corneas cultured in the presence of antisense oligos showed an inhibition of wound closure, being more than half as efficient as sense-treated controls (Chandrasekher et al., 1998). TGF β has also been targetted using an antisense approach, with Choi and coworkers suggesting that antisense TGF β 1 oligonucleotides could be used for ameliorating scar formation during wound healing in a mouse model, though quantitating scarring of a tissue is difficult (Choi et al., 1996).

If efficient delivery and specific activity can be demonstrated, then antisense oligonucleotides can be valuable tools in experimental models for which receptor antagonists or selective inhibitors of intracellular components are currently unavailable.

4. Transgenic mice

A key reason for choosing an embryonic mouse model for my studies, rather than the well characterised chick wound healing model, was the greater genetic tractability of the mouse. Although the chick is physically an easily accessible model for wounding, there are far fewer genes cloned in chick than in mouse, and there is no current capacity to efficiently generate transgenic chickens. Transgenic approaches are frequently used to study gene function in mice and despite the difficulties that genetic redundancy can pose, they can offer huge advances in the understanding of gene function, in a league above those approaches listed previously. I have described studies from our lab, earlier in this introduction, which investigated wound healing in both *c-fos* and *TGF β 1* knockout mice, and in neither instance was a significant difference in repair capacity observed between wildtype embryos and their heterozygous and nullizygous littermates (J. McCluskey, DPhil. Thesis Univ. Oxford, 1995). The table in *Appendix III* summarises the main studies to have used transgenic mice to assess specific gene function in various wound healing models to date.

Despite the clear limitations in terms of genetic redundancy, transgenic approaches offer a powerful tool for the identification of key players in wound repair processes. Clear cut examples of where knockout mice have shown impaired wound healing phenotypes include the *fgf-2* knockout (Ortega et al., 1998) and the *plasminogen* knockout (Romer et al., 1996). Mice null for *fgf-2* are phenotypically indistinguishable from wild type littermates, but show delayed healing of excisional skin wounds, whilst Plasminogen null mice, lacking the main fibrinolytic enzyme, have epidermal wound fronts which remain blunt edged and fail to bore a pathway through the fibrin matrix.

Whilst we see many transgenic studies which fail to deliver the predicted outcome, with strategic planning and luck it is possible to get clear results. An example of this has already been described in the wound healing literature; in 1994 Werner and colleagues reported that mice with a dominant-negative KGF receptor targeted to basal epidermal cells, are severely impaired when challenged to heal a wound. However, KGF $-/-$ mice reported by Guo and co-workers (1996) apparently have no wound healing phenotype. The explanation for this striking disparity must be that some related growth factor is compensating for missing KGF in the KGF null mice, and that this compensatory signal is also blocked in the dominant negative receptor mouse. More recently KGF-2 (FGF-10) has been identified, which also binds to the KGF receptor, and could well be compensating for the lack of functional KGF in KGF null mice (Beer et al., 1997).

Although transgenic approaches do not always yield the anticipated results, there is no doubt that with the development of tissue-specific and inducible constructs they are becoming increasingly potent as a means of investigating protein function.

In this chapter I report my subtractive hybridisation screen for genes upregulated at 1 hour post-wounding and also my studies of several immediate early genes predicted to be involved in the initial wound response.

Materials and methods

- ***Delivery, wounding and culturing of E11.5 mouse embryos***

Timed matings were performed between female albino mice (CD1 strain) of age 2-4 months and stud males of the same strain. Adult female mice come into oestrus every 4-5 days, as judged by the pink, swollen appearance of the vagina. Single receptive females were introduced to stud males in the early evening and plug-checked the following morning. Mating success was established by the presence of a hard, white seminal plug. At noon on the day of plug-checking, embryos were considered to be at embryonic day 0.5 (E0.5). The time-mated pregnant females were sacrificed by cervical dislocation and the abdomen washed with 70% alcohol before being opened. The peritoneum was then cut to expose the two horns of the uterus and the uterus removed by cutting at the end of each horn and through the cervix. After being rinsed in phosphate buffered saline (PBS), the uterus was placed in a petri dish containing fresh PBS and the muscular uterine wall cut open using iridectomy scissors in a single incision along the anti-mesenteric border, to expose the litter of embryos. The embryos were then teased away from the uterus using forceps, leaving each embryo in its own yolk sac, encased within Reichert's membrane and the decidua (Fig 2.1a). The decidua and Reichert's membrane were carefully torn away from the yolk sac using watchmaker's forceps and then trimmed to the level of the placenta with iridectomy scissors, taking care not to cut into the highly vascularised placenta. Embryos were transferred into a petri dish containing explant saline (see *Appendix IV* for recipe) using a smooth glass transfer pipette and the yolk sac cut around 80% of its base where it abuts the placenta, avoiding large blood vessels and leaving enough of the membrane attached to prevent rotation problems during subsequent roller culture. At this stage only the thin, transparent amniotic membrane remains intact. This lies very close to the embryo and can be grabbed with forceps and pulled to tease the embryo from the yolk sac. The amniotic membrane can then be torn with forceps

delivering the embryo, still attached to both yolk sac and placenta by the umbilical vessels, out from the yolk sac. Using forceps to position the embryo with its left side uppermost, with its tail over the top of the umbilical vessels, the embryo is both ready to culture and accessible for wounding (Fig 2.8B) (Cockroft et al., 1990).

Standard wounding was performed by amputating the left hindlimb at its base using iridectomy scissors (McCluskey et al., 1995). Other wounding techniques were also employed to generate slash wounds to the flank or limbs. Slash and stab wounds were made using an electrolytically sharpened tungsten needle to generate superficial point or incisional wounds of approx 500µm length. The embryo was then transferred into a 50ml Falcon tube containing 4ml culture medium (3ml culture saline - see *Appendix IV*, plus 1ml normal rat serum). The tube was then gassed for 1 minute with a mixture of 95% Oxygen:5% Carbon dioxide, a mixture in which embryos of this stage can survive for up to 48 hours, provided they are re-gassed every 12 hours. The rim of the tube was lubricated with silicone grease (Dow Corning) to create an air-tight seal and the embryo, in its new home, placed on inclined rollers in a roller incubator (BTC Engineering, Cambridge, UK.) and rolled at 30 revolutions per minute (rpm) at 37°C.

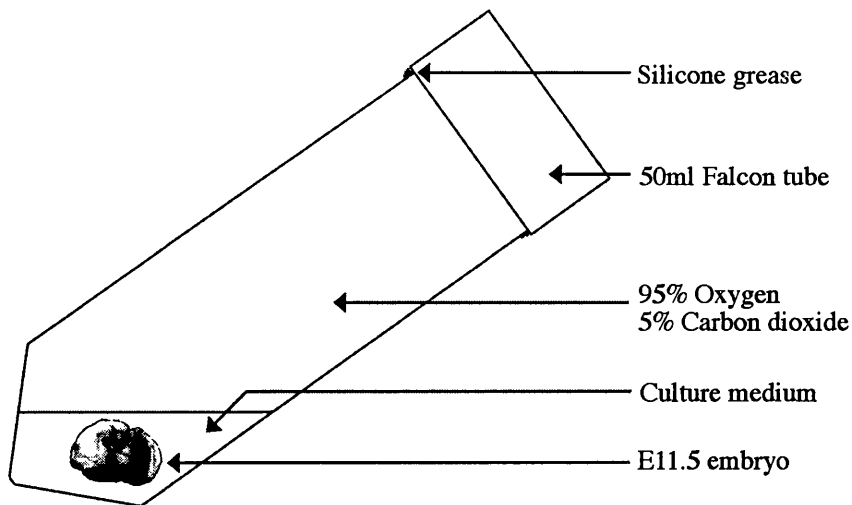


Figure 2.1 Mouse embryo roller culture

- *Harvesting of embryos and processing for scanning electron microscopy (SEM)*

After culturing for the desired time, embryos were removed from the roller incubator and transferred to a glass vial containing freshly prepared, ice-cold 1/2 strength Karnovsky's fixative (Karnovsky, 1965 - see *Appendix IV* for recipe). Specimens were fixed overnight at 4°C. After fixation the embryos were transferred to a petri dish, still in 1/2 strength Karnovsky fixative, and the umbilical vessels cut close to the body, to detach the placenta and yolk sac. Embryos were then returned to glass vials using a glass transfer pipette and washed twice for 15 minutes in 0.1M sodium cacodylate solution, before being post-fixed for 60 minutes in a solution of 1% osmium tetroxide / 0.1M sodium cacodylate at 4°C in the dark. Specimens were then rinsed twice in 0.1M sodium cacodylate solution before being dehydrated through a series of alcohols; 30%, 50%, 70%, 90%, 95% and 100%, for 10 minutes in each, with two final rinses in 100% ethanol. Embryos were then washed sequentially for 15 minutes each time in the following ethanol:acetone mixtures (3:1, 1:1, 1:3), followed by 100% acetone. Specimens were then dried at critical point in carbon dioxide. Once dry, embryos were mounted, wound side uppermost, on SEM stubs using silver DAG (Agar Scientific). After the DAG had dried, embryos were sputter-coated with approximately 30nm gold and viewed on a Jeol 5410LV scanning electron microscope.

- *Wholemount in situ hybridisation on E11.5 mouse embryos*

NB: All steps were at room temperature for 5 minutes with gentle rocking unless otherwise stated.

Embryos were wounded and cultured for the desired length of time as described earlier, before being fixed in 4% paraformaldehyde in PBS overnight, at 4°C. Extraembryonic tissue was removed and embryos washed twice in PBT (PBS + 0.1% Tween-20).

Specimens were dehydrated by washing in increasing concentrations of methanol:PBT (1:3, 1:1, 3:1, absolute methanol) to prevent gas bubbles forming during the subsequent bleaching step.

NB: Embryos can also be stored at -20°C in 100% methanol for several months.

Specimens were rehydrated by washing in decreasing concentrations of methanol:PBT and then bleached with 6% hydrogen peroxide in PBT for 1 hour. After 3 washes with PBT, embryos were treated with 10µg/ml proteinase K in PBT for 20 minutes at 37°C.

NB: Embryos become very fragile after this step until they are post-fixed.

Following two washes with PBT, specimens were post-fixed in 0.2% glutaraldehyde : 4% paraformaldehyde in PBT for 20 minutes. After another 3 PBT washes, embryos were rinsed once with hybridisation solution (see *Appendix IV*), then pre-hybridised in hybridisation solution at 70°C for at least 1 hour. Hybridisation solution was replaced with fresh hybridisation solution, pre-warmed to 70°C, containing approximately 1µg/ml DIG-labelled RNA probe, and specimens incubated at 70°C overnight.

The following morning the hybridisation solution was removed and specimens washed twice with solution 1 (50% formamide, 5x SSC, 1% SDS) for 30 minutes at 70°C, then washed twice with solution 2 (50% formamide, 2x SSC) for 30 minutes at 70°C. Specimens were then washed 3 times with TBST (see *Appendix IV*) at room temperature (RT) and once with TBST at 70°C for 30-40 minutes. Prior to adding anti-DIG antibody, embryos were pre-blocked with TBST + 10% goat serum for 60-90 minutes at RT. While specimens were blocking, 3mg embryo powder (see *Appendix IV*) was added to an eppendorf and 0.5ml TBST added. The tube was vortexed and incubated for 30 minutes at 70°C, then vortexed again and left for 10 minutes at RT. After cooling on ice, 5µl goat serum was added along with 1µl anti-DIG:AP conjugated antibody (Boehringer

Mannheim Cat No. 1093274). The antibody was presorbed by shaking gently for at least 1 hour at 4°C, then spun in a microfuge for 10 minutes at 13000g. The resulting supernatant was diluted to 2ml with 1% goat serum in cold TBST and added to the embryos, after removal of the 10% serum blocking solution, and rocked overnight at 4°C.

The next day embryos were washed 3 times with TBST for 15 minutes, given hourly changes of TBST through the day, then left washing in TBST overnight at RT.

The next day embryos were washed 3 times with NTMT (100mM NaCl, 100mM Tris-HCl pH 9.5, 50mM MgCl₂, 0.1% Tween-20, 2mM Levamisole) for 15 minutes, and incubated with NTMT containing 4.5µl/ml NBT and 3.5µl/ml BCIP (Boehringer Mannheim Cat. Nos. 1383213 and 1383221, respectively). They were wrapped in foil and rocked at RT until desired reaction product intensity was achieved, then the reaction was stopped by washing thoroughly in PBT + 20mM EDTA. Specimens were then photographed on a Leica dissecting scope, or Leitz DiaPlan microscope.

- *Vibratome sectioning of gelatin-albumen embedded E11.5 mice*

Embryos were coated with gelatin-albumen embedding mix for 5 minutes then embedded between two layers of freshly prepared mix containing glutaraldehyde. Blocks containing embryos were stuck to a vibratome chuck with superglue and 50µm sections cut into PBS using a Vibratome. Sections were collected using a paintbrush and viewed on a Leitz DiaPlan microscope after mounting with 70% glycerol in PBS.

- *X-gal staining of krox-24/lacZ E11.5 mice*

E11.5 mice were dissected from their yolk sac (which was saved at -20°C for genotyping) and placenta, and transferred to fixative (see *Appendix IV*) for 30 minutes at 4°C. They

were then washed twice in PBS for 20 minutes at room temperature before being transferred to staining solution (see *Appendix IV*), where they were left in the dark at room temperature overnight for staining to develop. When staining had developed, embryos were photographed and processed for resin sectioning.

- ***PCR genotyping *krox-24/lacZ* E11.5 mice***

Yolk sacs dissected from embryos at the time of fixing in individual 1.5ml Eppendorf tubes were digested for 2 hours in 50µl DNA extraction buffer (see *Appendix IV*) containing 25µg/ml Proteinase K, then placed in a boiling waterbath for 10 minutes. After adding 100µl of PCR quality water the samples were cleaned up by phenol/chloroform extraction, and 2µl from each sample used in subsequent PCR reactions. PCR amplification was performed using the GeneAmp PCR kit (Perkin Elmer) with hot start WaxGem beads (Perkin Elmer), in a reaction volume of 40µl. Three primers were included in each reaction; Krox-24 sense, Krox-24 antisense and LacZ antisense. These primers were designed so that the wildtype Krox-24 allele was amplified by Krox-24 sense and Krox-24 antisense primers to yield a 350 bp fragment and the transgenic allele was amplified by Krox-24 sense and LacZ antisense primers to yield a 500 bp. The LacZ insertion was between the two Krox-24 primer sites and thus prevented amplification of the wildtype Krox-24 band. Samples were subject to the following amplification cycle:

- Mix dNTPs, reaction buffer and primers.
- 78°C, 8 minutes to melt WaxGem, then add sample DNA and AmpliTaq DNA polymerase.
- 94°C, 1 minute ⇒ (94°C, 30 seconds; 60°C, 45 seconds; 72°C, 45 seconds) x 30 ⇒ 72°C, 5 minutes ⇒ 4°C.

- Electrophoresis samples on 2% Agarose gel containing Ethidium bromide.

In addition to genotyping embryos, DNA was genotyped from the uterus of each mother to confirm that she was of heterozygous *krox-24/lacZ* genotype. A sample gel is illustrated below:

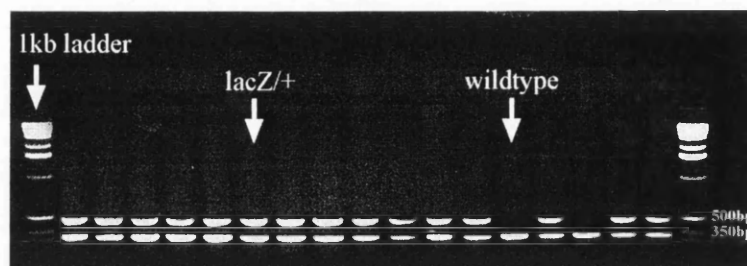


Figure 2.2 PCR-genotyping of *krox-24/lacZ* mouse embryos

- *Wounding and isolating mRNA from E11.5 limb buds*

E11.5 mice were dissected as described earlier such that their hindlimbs were accessible for amputation. Limbs were amputated and either immediately snap frozen by placing in a 1.5ml eppendorf tube floating in liquid nitrogen, or pipetted into a 60mm petri dish containing culture medium and then wounded. Wounds were made using tungsten needles, giving multiple slashes to generate as many wound edges as possible. Wounded limbs were cultured in 1.5ml eppendorf tubes in embryo culture medium, containing 25% rat serum, for 60 minutes at 37°C. The tubes were then spun for 20 seconds at 13000 rpm in a microfuge to pellet the limbs, and supernatant aspirated, before the tissue was snap frozen in liquid nitrogen. Wounded and unwounded limbs were stored at -80°C until sufficient numbers were obtained to begin mRNA isolation.

Messenger RNAs were isolated from the two sets of limbs using the Stratagene mRNA isolation kit (Cat. No. 20034). After using guanidinium thiocyanate to lyse and denature tissue, polyadenylated mRNA was then annealed to oligo(dT)-cellulose and the oligo(dT)/mRNA complex thoroughly washed before transfer to an RNase-free column, where the mRNA was eluted with 10mM Tris/HCl. Approximately 40 limbs were used

for control, unwounded mRNA isolation and 20 for obtaining wound mRNAs. These mRNAs were stored at -80°C prior to reverse transcription in the first step of the subtraction protocol.

- *Identifying genes upregulated in response to wounding*

The use of relatively small amounts of tissue meant that the yield of polyadenylated mRNA was quite poor, with only around 0.5µg of mRNA being obtained from the wounded limbs (as determined by spectrophotometric analysis on a Camspec M330 spectrophotometer). This shortage of starting material precluded the use of many commercially available protocols but was just sufficient for the Clontech PCR-Select cDNA subtraction kit. This kit allows the selective amplification of differentially expressed mRNAs using a protocol based on suppression PCR. The process can be divided into two parts, illustrated below. The diagram overleaf (Fig. 2.3) spans the steps from limb amputation to generation of tagged cDNAs:

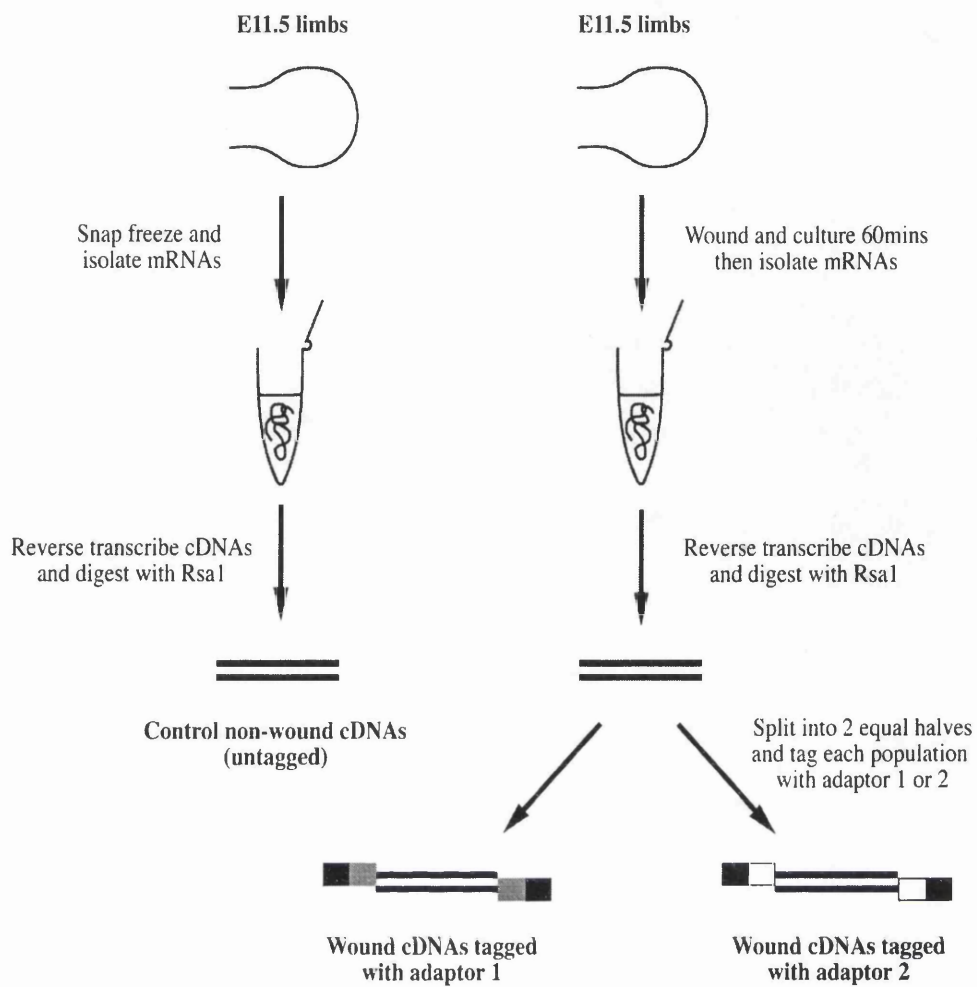


Figure 2.3 Generating wound and control cDNAs

I have already discussed the steps up to mRNA isolation. What follows is a basic description of the tagging and suppression PCR processes but for a more detailed description see the Clontech PCR-Select™ cDNA Subtraction Kit User Manual.

Following mRNA isolation, cDNAs from both wound and control mRNAs were reverse transcribed and digested with the restriction enzyme RsaI. This enzyme has a four base recognition sequence and therefore cuts on average every 4⁴ (256) bases, leaving blunt ends. Control cDNAs are left alone after cutting but wound cDNAs are split into two halves and each subpopulation tagged with either adaptor 1 or 2. The ends of these adaptors do not have phosphate groups, so can only bind to the phosphorylated 5' ends of cDNA fragments. Both adaptors share an identical sequence to which the PCR primer

can anneal, but they also have distinct domains which become important in the suppression PCR step. Once the adaptors have been ligated to the blunt ended wound cDNA fragments the hybridisation steps can begin. The following diagram (Fig. 2.4) summarises the steps that allow progression from tagged cDNA populations to wound specific sequences:

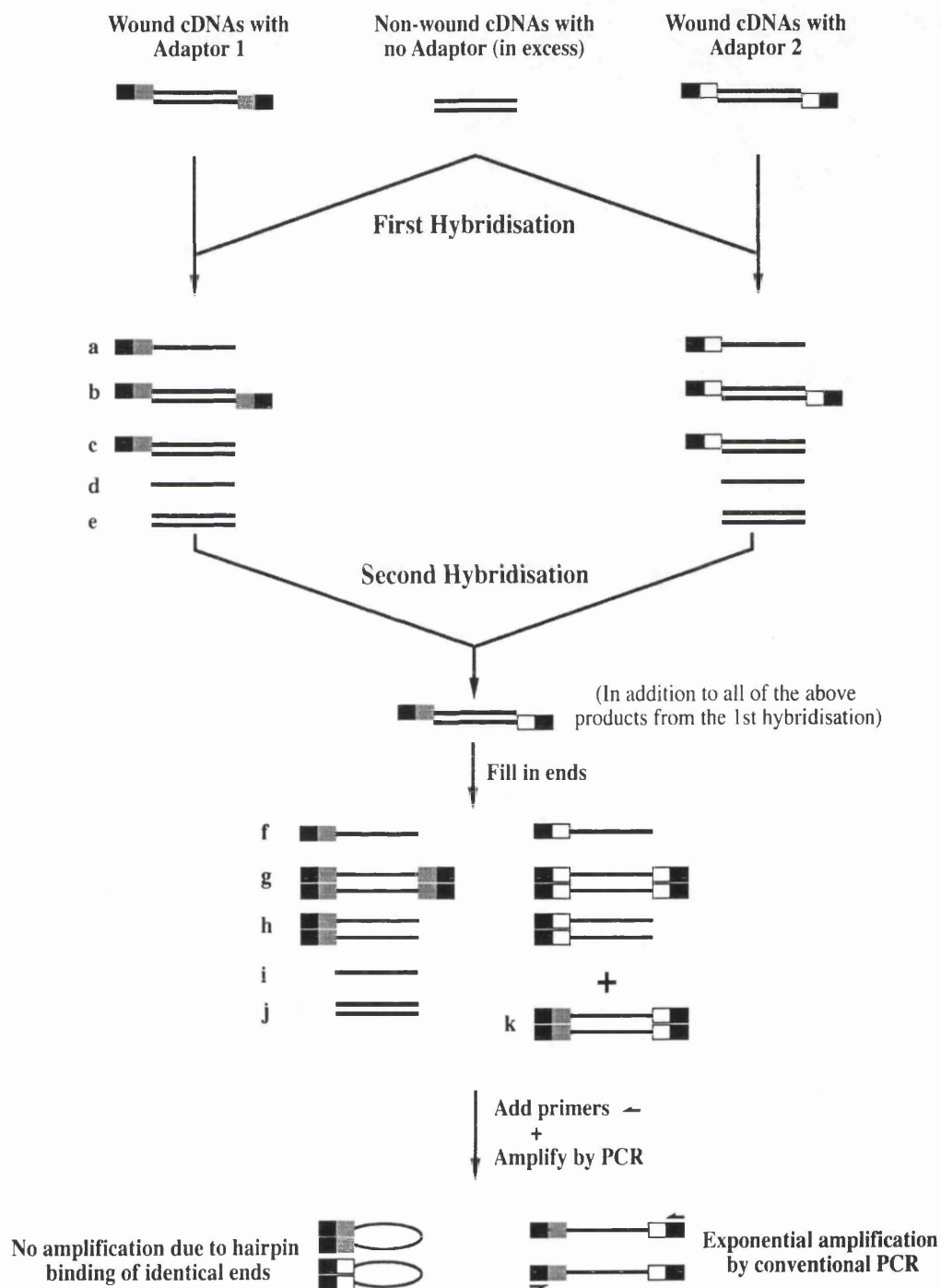


Figure 2.4 Model for PCR-Select Suppression PCR

In the first hybridisation, both populations of tagged cDNAs are heat denatured to give single stranded DNA (ssDNA) and, separately, mixed with excess control ssDNA and allowed to anneal for a limited time. This step results in sequences common to both wound and control populations (i.e. housekeeping genes) binding to complementary tagged or untagged sequences (b and c). The concentration of high- and low- abundance sequences is also equalised due to the kinetics of hybridisation being much faster for abundant sequences. Products (d) and (e) result from the excess of control untagged cDNA, but the only products of value are the single stranded tagged molecules (a), which represent sequences more abundant in the wound populations. This first hybridisation step is time-limited, to prevent these sequences of interest from hybridising, and then the two separate populations are immediately mixed, without denaturing, and a complete, overnight hybridisation performed.

Just one additional type of molecule results from this second hybridisation step, as illustrated above, and it is this type of new hybrid that will go on to give rise to PCR products during the subsequent PCR reaction. The fill-in reaction that follows the second hybridisation ensures that all hybridised molecules are totally double-stranded, and a variety of products are obtained ($a \Rightarrow f$, $b \Rightarrow g$, $c \Rightarrow h$, $d \Rightarrow i$, $e \Rightarrow j$). However, it is only molecules of type (k), with a different adaptor at each end that will be exponentially amplified by PCR, due to molecules with the same adaptor at both ends forming hairpin loops as shown which suppress amplification (hence suppression PCR). Having performed the PCR reaction the products were cloned into the T-trap vector pCR 2.1 using the Invitrogen TA Cloning Kit (Cat. No. K2000-01). This process exploits the fact that Taq polymerase based PCR amplification results in a 3' A overhang which allows PCR products to be cloned directly into a linearised vector with 3' T overhangs, as illustrated overleaf:

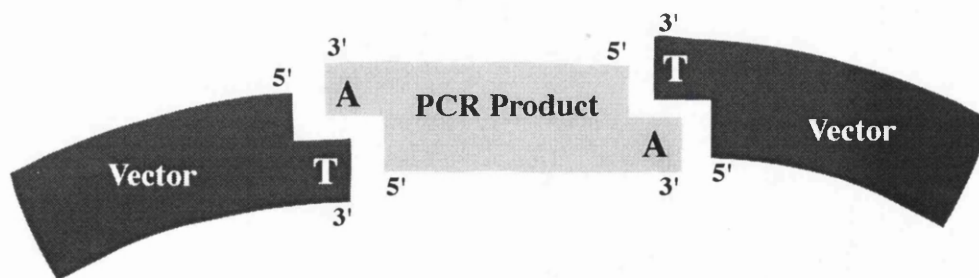


Figure 2.5 T-trap cloning of PCR products

Following ligation, One-shot™ competent *E.coli* were transformed with the ligation mixture, and plated overnight at 37°C on agar plates containing 40µg/ml ampicillin. The following morning 480 colonies were picked into five 96-well plates containing 200µl medium (Luria broth + 40µg/ml ampicillin + 15% glycerol) per well and these grown up by shaking at 225 rpm overnight at 37°C. These plates were then stored at -80°C and individual clones expanded by miniprep culturing; scraping a sterile pipette tip across the frozen surface and using this to inoculate 5ml of Luria broth (containing 40µg/ml ampicillin), which was grown up by shaking at 225 rpm overnight at 37°C. To identify clones containing PCR inserts, minipreps were made using a QiaPrep kit (Cat. No. 27106) and 1µg of DNA from each clone digested with EcoRI for 2 hours at 37°C. Digestion products were then run out on a 1.2% agarose/TBE gel containing 0.01µg/ml ethidium bromide and photographed on a UV transilluminator. An example gel is shown below:

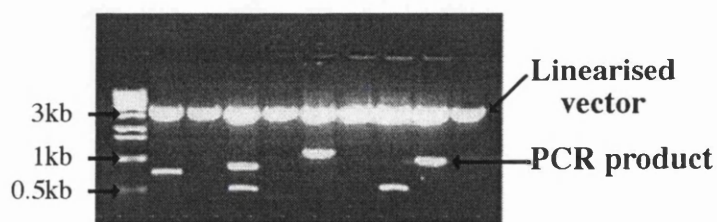


Figure 2.6 Electrophoresis of PCR products

Those clones containing an insert (see Fig. 2.6) were sequenced using a Perkin Elmer ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Cat. No. P/N 402078) and products analysed on an ABI PRISM 377 DNA sequencer (Pfizer Central Research, Sandwich, U.K.). The resulting sequences were then BLASTed against the GenBank DNA sequence database, to determine homologies to known sequences. An illustration of a typical cloned sequence is given below, showing that EcoRI sites were present each side of the cloning site, to enable detection of an insert, although obviously the inserts were in random orientation.

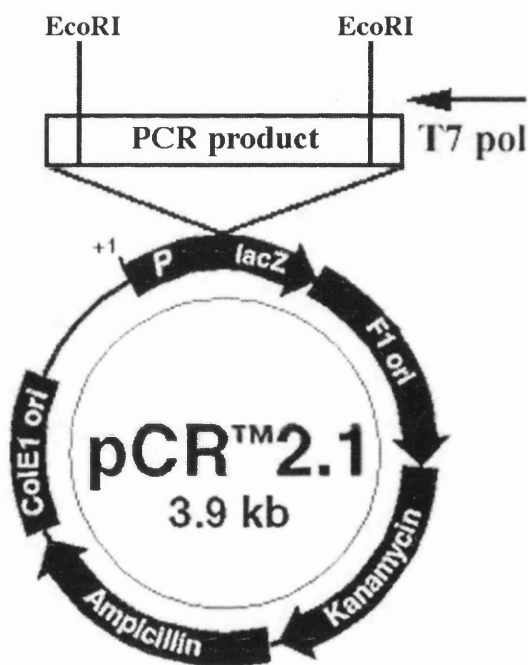


Figure 2.7 Cloned PCR construct map

Interesting clones were then analysed by in situ analysis, as described earlier, with digoxigenin-labelled mRNA probes being generated from linearised templates by run-off transcription using the Boehringer Mannheim DIG RNA-labelling kit (Cat. No. 1175 025).

Results

- ***Excisional and incisional wounds heal perfectly, but with different timecourse, in the cultured mouse embryo***

Two types of wound were made during this study; the already characterised left hindlimb amputation and a slash wound of around 500µm in length (Fig. 2.8C, D). The excisional amputation model is excellent for comparing relative re-epithelialisation and mesenchymal contraction rates in transgenic mice but, for photography of wholemount antibody or *in situ* expression studies, it is easier to get the epithelial margin of an incisional slash wound all in the same focal plane than it is that of an amputation wound. I found that to look at message upregulation within 1 or 2 hours of wounding, a slash wound was best. Tungsten needle stab wounds were also made to the flanks of some embryos, particularly when they were to be used for *in situ* studies on a message where background limb expression was high. Deep slash wounds heal perfectly in 24 hours, sometimes leaving a wound pimple (Fig. 2.9F). Shallow incisional wounds to either the forelimb or hindlimb heal within 6 hours, as reported previously for similar wounds in the chick embryo (Brock et al., 1996).

- ***A contractile actin cable is assembled within minutes of wounding***

Immediately following wounding, the epithelium around the wound margin gapes open to reveal the underlying mesenchyme of the wound bed (Fig. 2.9B). Scanning electron microscopy (SEM) reveals the leading edge of the marginal epithelial cells to be ragged and uneven (Fig. 2.9B). Mesenchymal cells, consisting mainly of undifferentiated fibroblasts, can be seen nestling in the exposed matrix meshwork of collagen fibrils, along with erythrocytes that have leaked from damaged blood vessels (Fig. 2.9C).

Within minutes of wounding, confocal laser scanning microscopy of FITC-phalloidin stained specimens reveals the accumulation of a thick, intracellular filamentous actin cable in leading edge basal epithelial cells extending around the full perimeter of the wound (Fig. 2.9E), just as previously reported for chick slash wounds and excisional mouse embryo wounds (Martin and Lewis, 1992; McCluskey and Martin, 1995; Brock et al., 1996). Contraction of this cable provides the driving force for the re-epithelialisation effort and continues until wound closure is complete. Continued contraction of the perimeter is facilitated by cells dropping back from the leading edge once they have constricted their apices (Brock et al., 1996).

High magnification SEM allows us to visualise a sprinkling of tiny microvilli (<1µm length) which rapidly assemble on the surface of superficial periderm cells adjacent to the wound edge. These microvilli appear particularly abundant at cell boundaries (Fig. 2.9C, G). Within an hour of wounding, SEM reveals that the epithelial cells at the immediate wound margin and up to three rows back appear more “hairy” (Fig. 2.9G). These periderm cells have assembled relatively large numbers of microvilli on their exposed surfaces. This increase in the number of surface villi decreases as one gets further from the wound edge. This pattern of cells at the wound margin that assemble microvilli correlates well with those cells in which expression of immediate early genes like *c-fos* has been induced in response to wounding (Fig. 2.10C, D).

- ***Wound genes can be identified in two ways: informed guesswork and systematic screening***

As described earlier, there are several possible strategies to look for gene upregulations at the wound site and I chose to use two of them in my mouse embryo wound model. The first approach simply involved informed guesswork; I looked for expression of genes which seemed likely candidates for upregulation at the wound site. My second approach

was more systematic and utilised subtractive hybridisation to identify unknown wound genes.

Since the tissues around the wound site, particularly the free epithelial margin, are relatively fragile and easily damaged by rocking during the *in situ* protocol, I used a Fibroblast Growth Factor-8 (FGF-8) probe as a standard positive control for all my *in situ* studies. FGF-8 is expressed in several clear domains in the E11.5 mouse including the mandibular and maxillar borders, the midbrain/hindbrain border, the olfactory pits and, most strikingly, in the apical ectodermal ridge (AER) (Mahmood et al., 1996; Fig. 2.10A). The AER staining proved to be a particularly useful assay of “rough handling” because it too is fragile and easily damaged by the *in situ* process. FGF-8, however, is not upregulated by epidermal cells at the wound margin.

- ***Informed guesswork revealed several interesting genes to be induced at the wound site***

Only a small number of genes have so far been studied in our embryo wound model, and none of these at the mRNA level, so a battery of candidate genes, with a likely role in the wound closure process were screened by *in situ* hybridisation analysis. As described in the Introduction, c-Fos immunostaining has previously shown that c-Fos protein is translocated to the nucleus in the front 4 or 5 rows of epithelial cells at the wound margin within 20 minutes of wounding. My wholemount *in situ* preparations show no background expression of *c-fos* mRNA in the E11.5 limb (Fig. 2.10F), but 15 minutes after wounding there is clear staining for *c-fos* message in exactly the same cells as we see positive staining for c-Fos protein (Fig. 2.10B, C, E). *C-fos* message levels peak at about 30 minutes post-wounding and the staining is cytoplasmic rather than nuclear, since the mRNA is exported from the nucleus. By 60 minutes post-wounding my *in situs* show that mRNA levels have decreased dramatically (Fig. 2.10G), confirming the idea that c-Fos is acting as a transitory early signal at the wound edge, and suggesting that it

might indeed be a key kick start signal for the wound repair process (Martin and Nobes, 1992).

- *The immediate early gene *krox-24* is upregulated rapidly following wounding*

The *krox-24* gene (*Egr-1*) encodes a zinc finger transcription factor which binds to target DNA sequence at GC rich motifs to regulate downstream gene expression. It is closely related to *krox-20*, which binds the same target sequence, and was first identified as a serum response gene with a similar pattern of regulation in activated tissue culture fibroblasts as the proto-oncogene *c-fos* (Janssen-Timmen et al., 1989). For these reasons I guessed that the *krox* genes might be induced by wounding in the embryo, and indeed both are upregulated but to differing degrees.

I have taken two approaches to studying *krox-24* expression during wound repair in our E11.5 mouse model: First, I have used *in situ* hybridisation with a DIG-labelled antisense riboprobe, to ascertain the temporal expression profile of mRNA following wounding, and second, I have utilised a *krox-24/lacZ* reporter mouse to give a clearer indication of the spatial pattern of expression.

Krox-24 message is not detectable in 0 hour wounds to the forelimb, hindlimb or flank of an E11.5 mouse (Fig. 2.11A) but within 30 minutes of wounding it is massively upregulated by both epithelial cells at the wound margin and by mesenchymal cells in the wound bed of incisional (Fig. 2.11B, D), puncture (Fig. 2.11B, E) and amputation (Fig. 2.11B, F) wounds. Transverse sections through these wounds allow us to see that *krox-24* message is upregulated by cells up to 5 rows distant from the wound edge in both the mesenchyme and epithelium (Fig. 2.11G, H). As with *c-fos*, this upregulation, although rapid, is also transient, with mRNA levels dropping to almost zero by one hour after wounding (Fig. 2.11C).

- *The *krox-24/lacZ* transgenic mouse should allow us to test the function, if any, of *krox-24* at the wound site*

As mentioned in the Introduction to this chapter, a major advantage of the mouse model is the availability of transgenics to investigate gene function. The *krox-24/lacZ* mouse (Topilko et al., 1997) offers the opportunity to visualise *krox-24* expression by staining for activity of the reporter *lacZ* gene product β -galactosidase in heterozygous embryos, but also presents a chance to see how homozygous knockout embryos heal wounds in the absence of functional Krox-24. Unfortunately, I was unable to wound any knockout embryos since PCR genotyping revealed that of 40 embryos wounded and cultured, 8 were wild types, 32 were heterozygotes and there were no nulls.

However, I was able to follow *krox-24* expression in the heterozygotes by X-Gal staining, and this confirmed my *in situ* findings that *krox-24* is upregulated immediately following wounding and that this expression is localised to epithelial and mesenchymal cells within 4 or 5 rows of the wound margin (Fig. 2.12G). X-Gal staining showed a hint of β -Galactosidase activity at the wound site within 30 minutes of wounding (Fig. 2.12D), increasing by 60 minutes (Fig. 2.12E) and maximal at around 2 hours following wounding (Fig. 2.12F). Resin sectioning of these 2 hour wounds showed punctate X-Gal staining in the exposed wound bed mesenchyme (Fig. 2.12G) and in the advancing epithelium (Fig. 2.12H).

- *The invading hindlimb nerve trunk fails to upregulate several WIGs*

Consistently, *in situs* for *krox-24* expression in a 30 minute amputated hindlimb wound show there to be a region in the centre of the mesenchyme where expression is absent (Fig. 2.11F). Resin sections through an identical 2 hour wound in an X-Gal stained *krox-24/lacZ* heterozygous embryo show the same absence of staining (Fig. 2.12G), and

reveal that this area is occupied by the main mixed nerve trunk which has just begun to invade the hindlimb in an E11.5 embryo (Martin, 1990). This same tissue appears not to upregulate *c-fos* either (Fig. 2.10B).

- ***The immediate early gene *krox-20* is also identified as a WIG***

In addition to screening for *krox-24* upregulation via my informed guesswork approach, I also investigated the expression of its close relative *krox-20* by *in situ* hybridisation. As with *krox-24*, *krox-20* message is not detectable in 0 hour wounds to the forelimb, hindlimb or flank of an E11.5 mouse (Fig. 2.13A) but it shows rapid and transient upregulation following culture. This upregulation differs slightly from *krox-24* in that *krox-20* seems to be expressed firstly just in the epithelium around the wound margin, being clearly detectable within 15 minutes (Fig. 2.13B). By 30 minutes post-wounding it is strongly expressed by both epithelial cells at the wound margin and by mesenchymal cells in the wound bed (Fig. 2.13C), with mRNA levels dropping dramatically over the next 15 minutes (Fig. 2.13D) and to almost zero an hour after wounding (Fig. 2.13E). The *in situs* show a clear internal positive control of boundary cap Schwann cell staining at the dorsal and ventral entry zones to the spinal cord (Fig. 2.13F), but again *krox-20* expression is noticeably absent from the site where the mixed nerve exits the limb stump (Fig. 2.13C).

- ***TGF β 1 is expressed in an unpredicted domain following wounding***

I see *TGF β 1* to be upregulated by one hour following wounding in my E11.5 wounds (Fig. 2.14A-C). In contrast to immunocytochemical detection of TGF β 1 following E11.5 mouse hindlimb amputation, which shows TGF β 1 protein staining throughout the wound mesenchyme (Martin et al., 1993), my *in situ* data shows mRNA to be localised to

a small region in the centre of the wound bed (Fig. 2.14D). Sectioning through this region suggests that this expression is associated with a central blood vessel (Fig. 2.14F). A similar situation is seen in forelimb slash wounds, where $TGF\beta 1$ expression seems to be restricted to the region where the marginal blood vessel adjacent to the AER is damaged (Fig. 2.14E).

The four genes discussed above; *c-fos*, *krox-24*, *krox-20* and *TGF β 1*, represent my most successful investigations into gene expression following wounding using an informed guesswork approach. I will now present findings from my search for novel WIGs using a subtractive hybridisation approach.

- *Over 200 sequences were yielded from a subtractive hybridisation screen for messages upregulated at one hour post-wounding*

Using the Clontech PCR-select system to search for genes upregulated within an hour of wounding, I obtained over 200 clones containing a suppression PCR fragment. These inserts ranged from less than 50 base pairs up to 700 base pairs in length, as determined by restriction analysis (see Materials and Methods for details). Every clone containing a PCR product was sequenced, in both forward and reverse direction, using ABI automated sequencing technology at Pfizer Central Research, Sandwich, U.K. Sequence data were subjected to Basic Local Alignment Search Tool (BLAST) analysis. BLAST is a sequence comparison program, with heuristic inbuilt knowledge of how sequences evolve, used to obtain fast and accurate comparisons of query sequences with already published sequence data contained in the GenBank database. Of over 200 sequences analysed, around 100 were identified as coding for known genes and the others contained either vector sequence or sequence with no significant homology to sequences in the GenBank database. I decided to follow up those known genes for which we could see possible functions in a wound scenario. No further action was taken to study expression

of the unknown sequences at this point, although thorough screening of this subtractive library could still be performed.

A list of genes identified as potentially upregulated by this screen appears in *Appendix I* - I have gone on to further analyse three of these in more detail.

- *Three positive clones are confirmed by in situ analysis*

In situ analysis is the best method for looking at gene expression patterns *in vivo*, so I screened the following genes in my E11.5 mouse wound models to confirm the validity of my subtraction protocol. Of course a positive result does not necessarily mean that these genes will be translated to the protein level, but immunocytochemistry does not provide a viable alternative for primary screening.

- *Non-selenium glutathione peroxidase, a gene upregulated during adult wound repair, is identified in my embryonic screen*

The first evidence that my screen might be fruitful came when I found that one of the cloned sequences corresponded to a 700 bp fragment of the antioxidant enzyme non-selenium glutathione peroxidase (NSGP). This gene has previously been identified in a differential display screen for genes upregulated in cultured keratinocytes in response to KGF treatment (Munz et al., 1997). My *in situs* show that NSGP is expressed at relatively high background levels throughout the E11.5 mouse, and particularly so in the limb (Fig. 2.15A, B). My *in situs* for this gene did not reveal the dramatic inductions observed with the immediate early genes described earlier, but NSGP message does appear to be modestly upregulated around the margin of a 1 hour hindlimb amputation (Fig. 2.15B). Unfortunately, I failed to detect upregulation in flank wounds, which I had hoped would provide a more clear cut result, since NSGP expression is weaker in flank tissue.

- *Cyclophilin D is upregulated following wounding*

Another potential WIG identified by my screen was the protein-folding mediator *cyclophilin D*, thought to play an important role in the regulation of T-lymphocyte activation and proliferation (Handschumacher et al., 1984). *In situ* hybridisation showed that this gene is expressed throughout the E11.5 mouse, but again it was possible also to see a clear upregulation at the margin of a forelimb slash wound 1 hour after wounding (Fig. 2.15C). Interestingly, one can also see “pepper and salt” expression in distinct cells, particularly in the flank (Fig. 2.15D). The distribution of these cells suggests they may be tissue macrophages.

- *Type IV collagenase is also picked up by my screen*

Another gene identified in the screen and also known to be expressed at the adult wound site, was *type IV collagenase* (*MMP-9* or *Gelatinase B*). Of over 100 clones containing published sequence I got 4 hits for *type IV collagenase*, but was unable to get any expression data by *in situ* hybridisation. However, I was able to conduct a preliminary investigation into the function of this gene using the collagenase-blocking drug UK-221316 (Pfizer Ltd.). Twelve embryos were wounded and subsequently cultured for 24 hours in the presence of UK-221316. Treated embryos showed a dose dependent amelioration of wound healing, with 50 μ M UK-221316 wounds showing smaller wound pimples (Fig. 2.16E, F) relative to controls or lower concentrations (5 μ M) of drug (Fig. 2.16A-D). These results are not statistically significant as only 4 embryos were used for each condition, but they do suggest that blocking these collagenases may improve embryonic repair.

- *Several potential WIGs show no positive evidence by in situ of wound upregulation*

Radical fringe

In situ hybridisation studies using probe from a clone from my screen identified as coding for *radical fringe* by BLAST analysis does hint at expression in the wound but only at the tip of a forelimb slash wound (Fig. 2.15E). However, since my probe does not highlight the neural expression previously reported for this gene (Johnston et al., 1997), any wound induction results should be considered preliminary.

Frizzled, Supervillin, PLA₂, APC-binding protein and α 4-integrin

Sequences from all of the above genes were found during the sequencing of my subtractive library but *in situs* on 1 hour wounded embryos failed to give conclusive results due to either high background or no staining. Though some of these genes are well studied, there were no published *in situ* data for expression during mouse embryogenesis to serve as positive controls, to see whether my probes were highlighting previously reported expression domains in unwounded regions of the embryo.

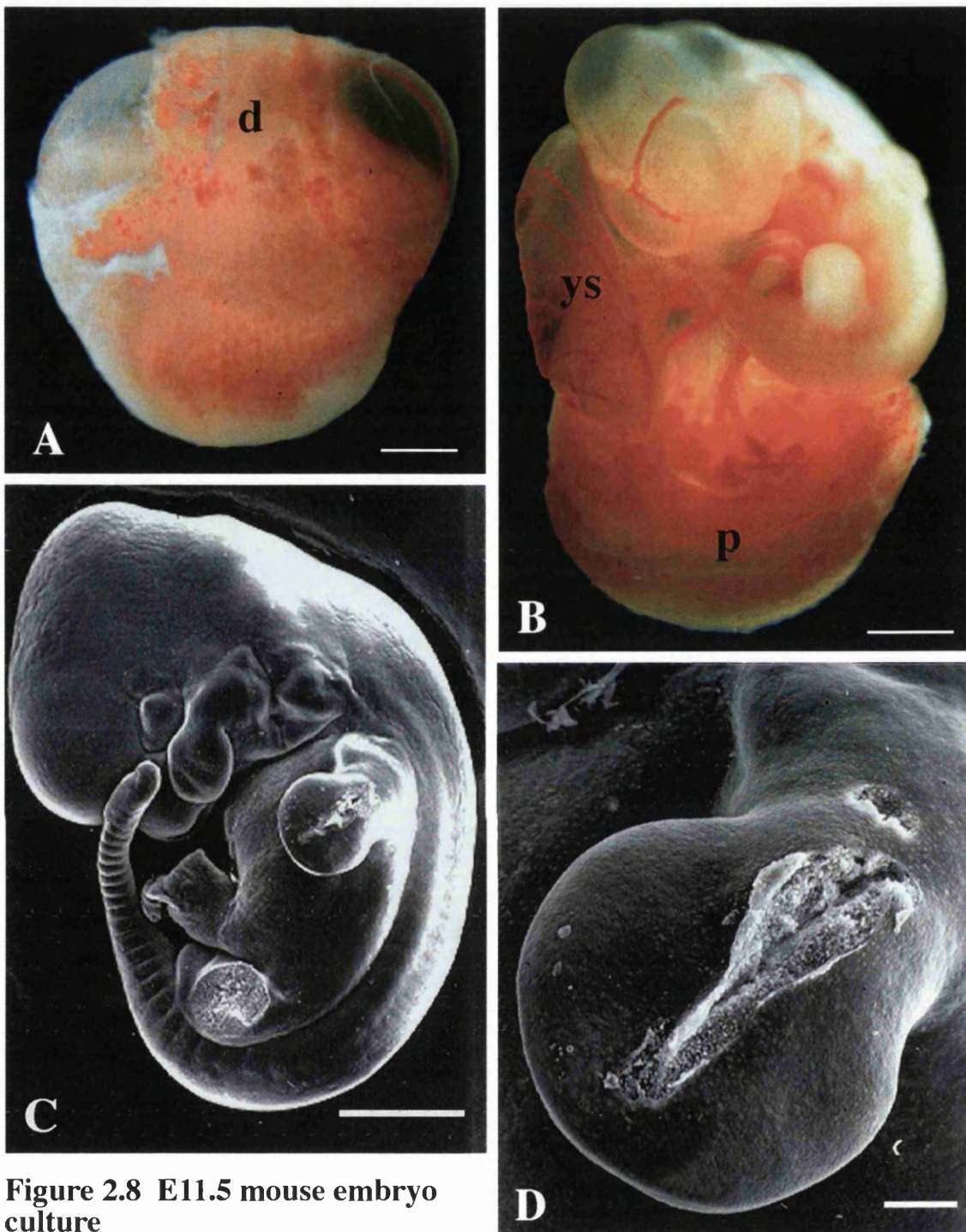


Figure 2.8 E11.5 mouse embryo culture

- A. E11.5 mouse embryo as dissected from the uterus, complete with overlying decidua (d).
- B. Dissected embryo of same stage, with yolk sac (ys) and placenta (p) shown.
- C. SEM of similar staged embryo, with a slash wound to the forelimb and hindlimb amputation. The embryo was fixed immediately after wounding and the yolk sac and placenta removed.
- D. Zoom in of 0 hour forelimb slash wound.

Scale bars: (A, B) 1mm; (C) 500 μ m; (D) 50 μ m.

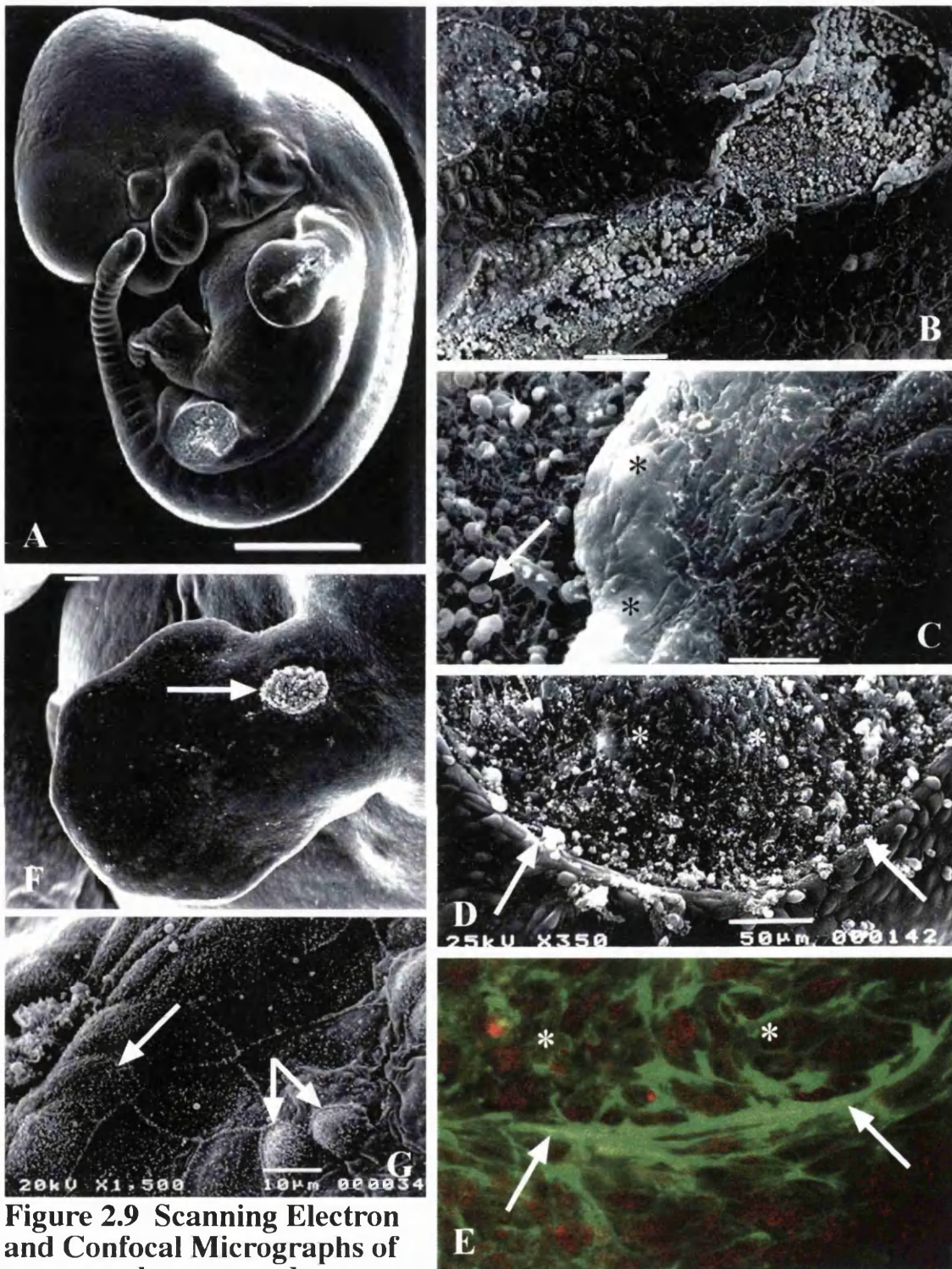


Figure 2.9 Scanning Electron and Confocal Micrographs of mouse embryo wounds

(A) An E11.5 mouse with forelimb slash wound and hindlimb amputation immediately post-wounding. (B & C) 0 hour forelimb slash wound (B) with zoom in (C) showing wound edge and exposed mesenchyme, with red blood cell marked (arrow). There is a clear lack of microvilli on the apical surface of periderm cells (asterisks). (D) The epithelial wound margin of a hindlimb amputation after 6 hours in culture, showing taught epithelial edge (arrows). (E) Confocal micrograph of the basal epithelium and actin cable in wound margin epithelium, with the actin cable (arrows) revealed by FITC-phalloidin (green) and nuclei stained red with propidium iodide, sweeping forwards over the mesenchyme (asterisks). (F) 24 hours post-wounding, a deep incisional cut has repaired leaving a healed forelimb wound with wound pimple (arrow). (G) Detail of a 3 hour forelimb incisional wound showing microvilli on periderm cells both at the wound margin and in those cells further back but undergoing cytokinesis (arrows). Scale bars: (A) 500µm; (B, D) 50µm; (E) 100µm; (C, F) 10µm

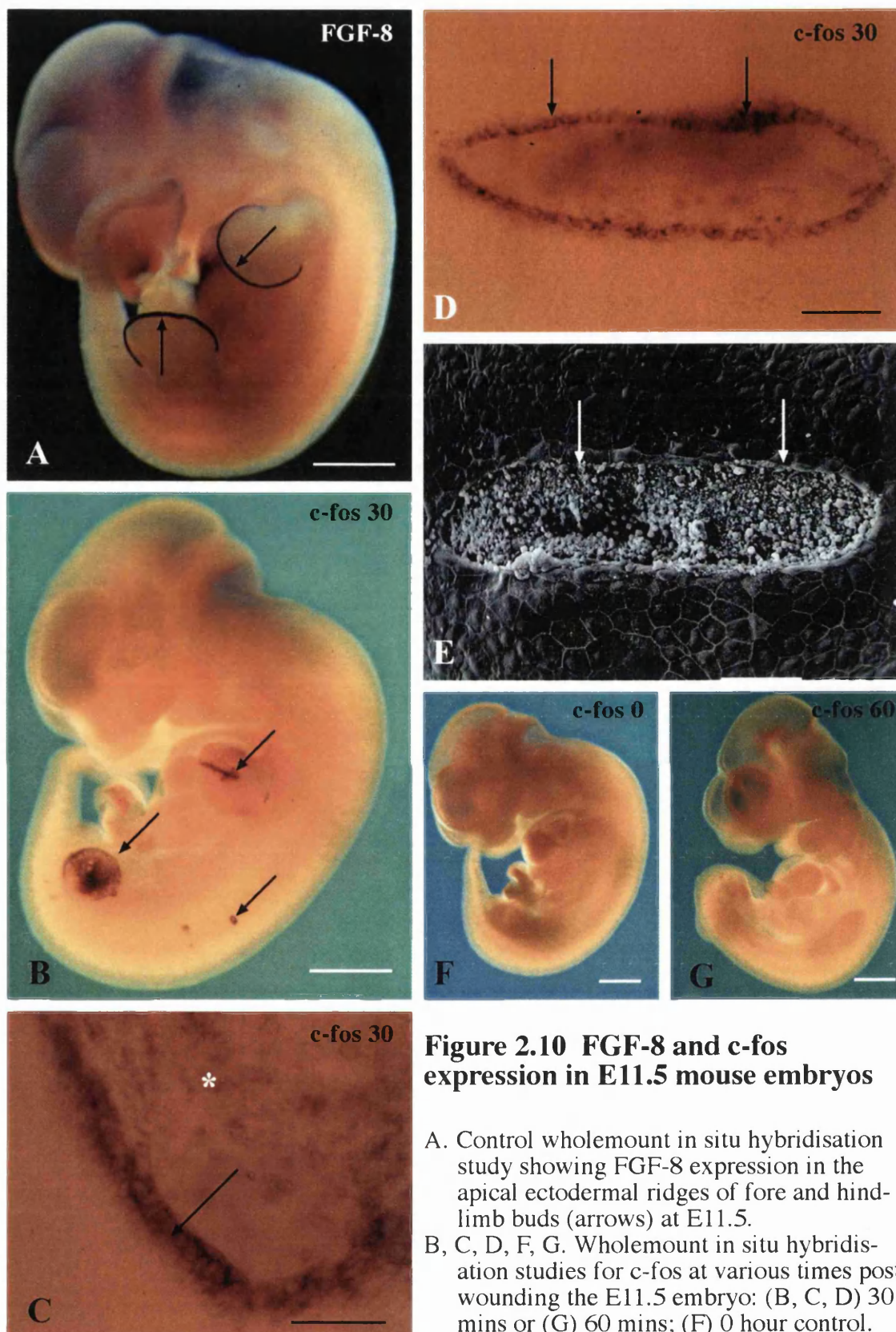


Figure 2.10 FGF-8 and c-fos expression in E11.5 mouse embryos

- A. Control wholemount in situ hybridisation study showing FGF-8 expression in the apical ectodermal ridges of fore and hind-limb buds (arrows) at E11.5.
- B, C, D, F, G. Wholemount in situ hybridisation studies for c-fos at various times post-wounding the E11.5 embryo: (B, C, D) 30 mins or (G) 60 mins; (F) 0 hour control.
- C. Detail of c-fos in situ study in a 30 min flank incisional wound, showing expression in the epithelium (arrow) and also in the exposed mesenchyme (asterisk).
- D. High magnification view of c-fos expression in a 30 min forelimb incisional wound (as in C) showing expression in the front few rows of the epithelium at the wound margin (arrows).
- E. Scanning electron micrograph of a 3 hour forelimb incisional wound, with arrows indicating the epithelial leading edge.
- Scale bars: (A, B, F, G) 500µm; (D, E) 50µm; (C) 30µm.

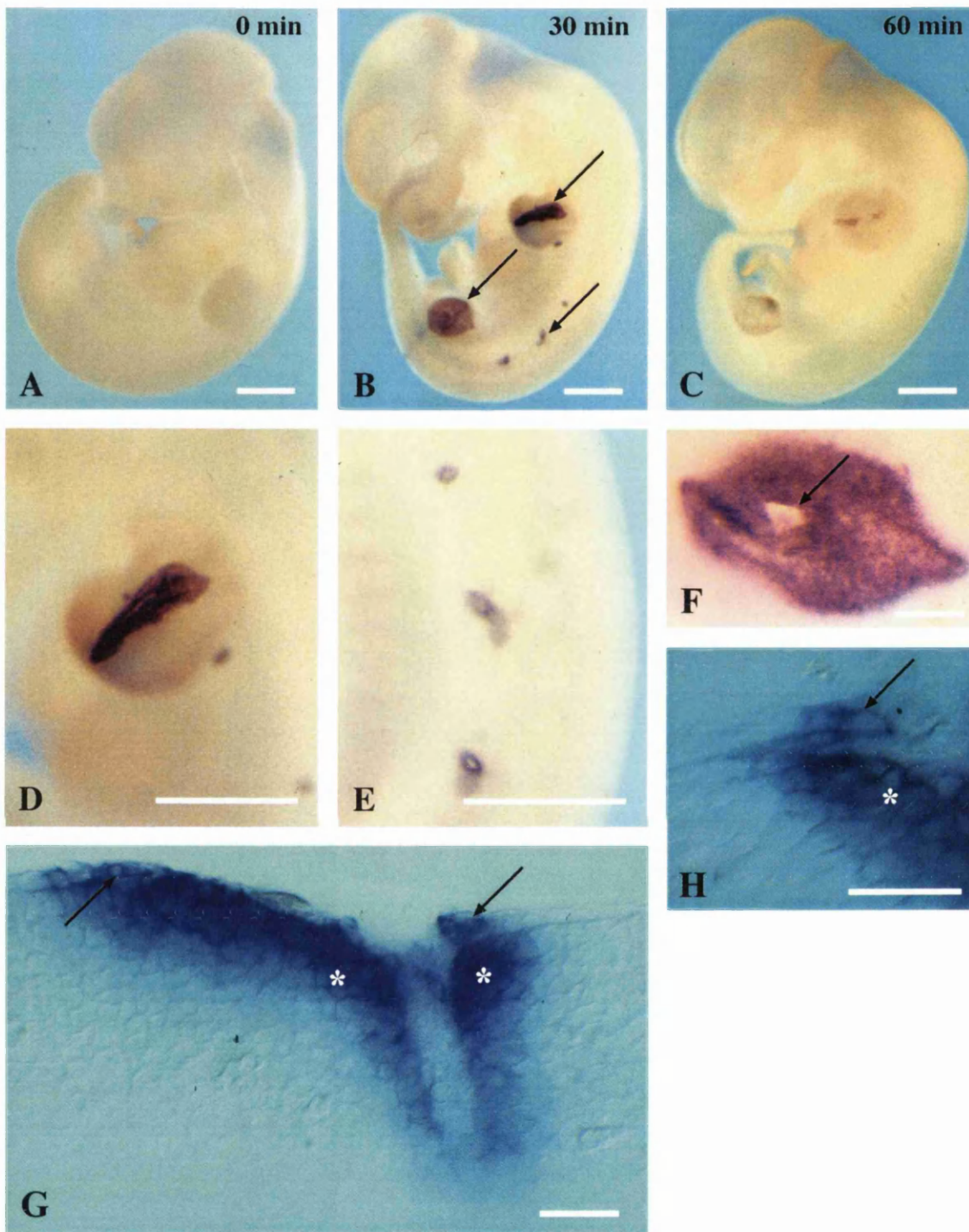


Figure 2.11 Krox-24 expression at the wound site

A-C. Krox-24 in situ hybridisation studies in E11.5 mouse embryos wounded on the forelimb, flank and hindlimb (arrows in B) and then cultured for 0 min (A), 30 min (B) and 30 min (C).

D-F. Details of 30 min wounds to the forelimb (D), flank (E) and hindlimb (F).

There is clear lack of krox-24 expression in the transected nerve trunk (arrow in F).

G. Transverse resin section through 30 min forelimb wound (from D). Krox-24 is expressed in both the epithelium (arrows) and mesenchyme (asterisks).

H. Transverse resin section through 30 min flank wound (from E). Again, krox-24 is expressed in both the epithelium (arrow) and mesenchyme (asterisk)

Scale bars: (A-E) 500µm; (F) 100µm; (G, H) 20µm.

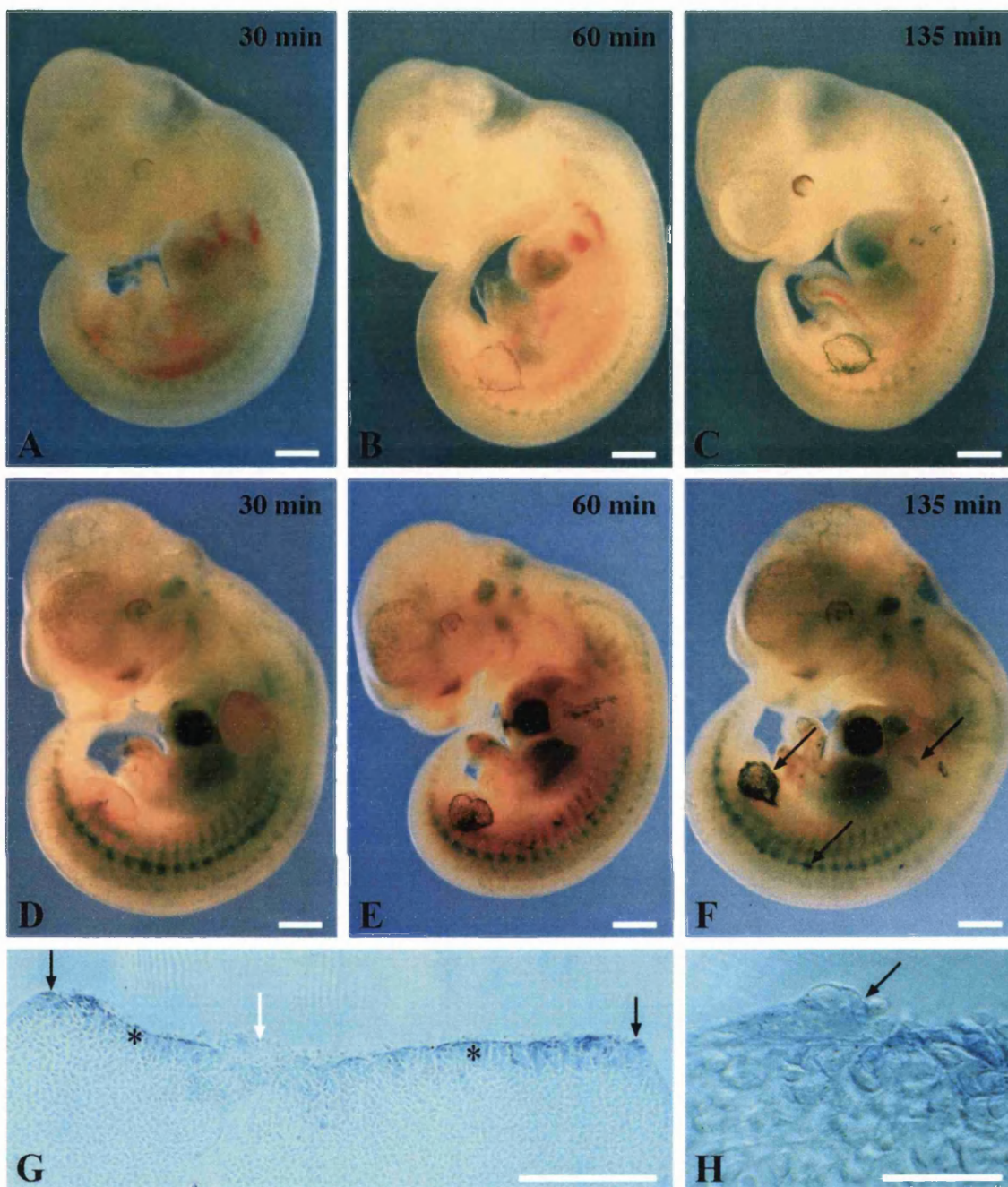


Figure 2.12 Krox-24/lacZ expression post-wounding

A-C. Embryos after 3 hours in X-gal stain. All have received forelimb, flank and hindlimb wounds and were cultured for 30 min (A), 60 min (B) and 135 min (C) prior to fixation.

D-F. Embryos wounded and cultured as for A-C but incubated in X-gal for 24 hours to reveal full Beta-galactosidase expression.

G. Resin section of a hindlimb wound after 135 min culture, showing epithelial leading edges (black arrows), exposed wound mesenchyme (asterisks) and nerve trunk (white arrow).

H. High magnification detail of free epithelial leading edge in G, showing punctate X-gal staining in both the epithelium (arrow) and wound mesenchyme.

Scale bars: (A-F) 500 μ m; (G) 100 μ m; (H) 20 μ m.

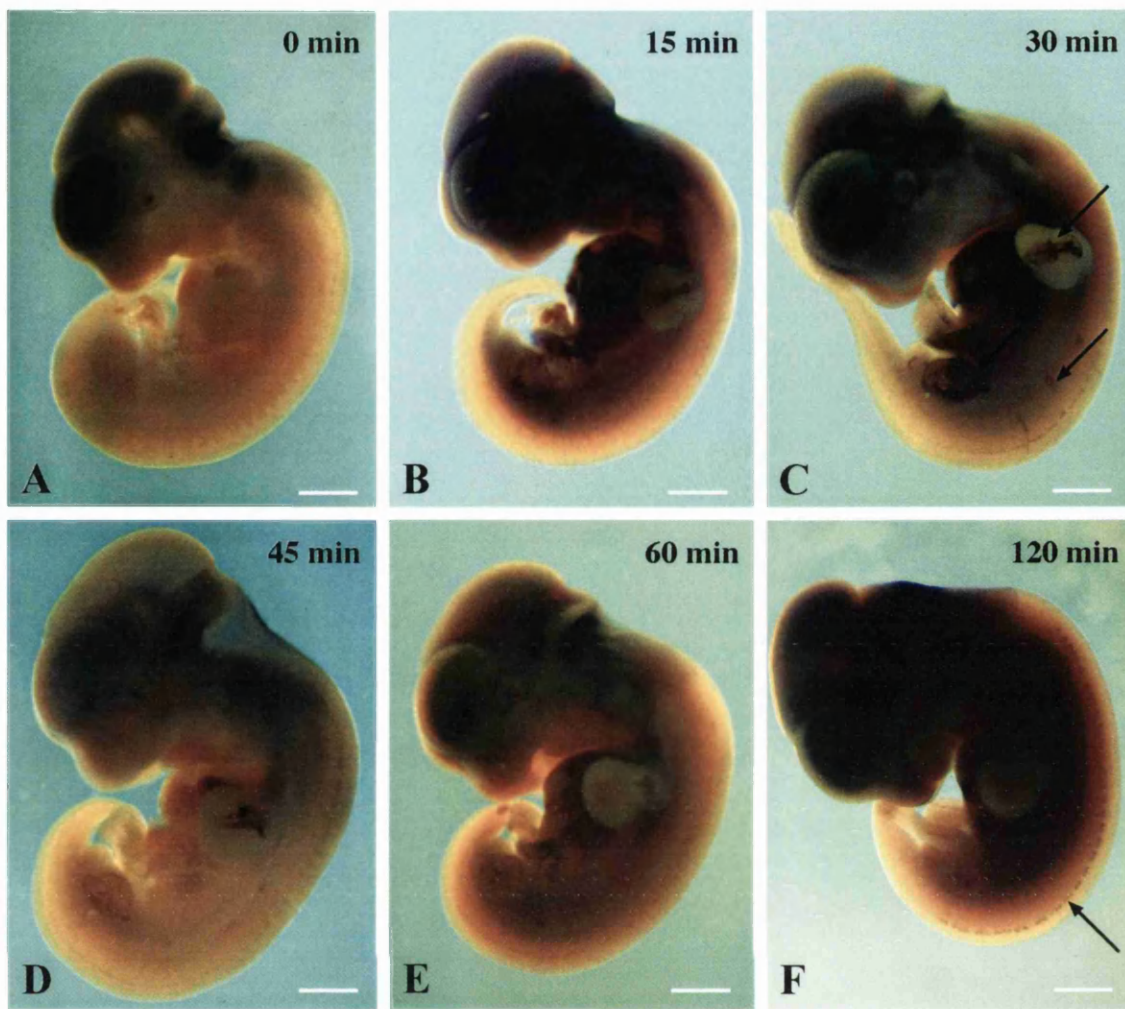


Figure 2.13 Krox-20 expression at the wound site

A-F. Krox-20 in situ hybridisation studies in E11.5 mouse embryos wounded on their forelimb, flank and hindlimb (arrows in C) and subsequently cultured for 0 min (A), 15 min (B), 30 min (C), 45 min (D), 60 min (E) and 120 min (F). In addition to expression at sites of wounding, there is clear segmental expression in the boundary cap Schwann cells at the dorsal and ventral entry zones to the spinal cord (arrow in F).

Scale bars: (A-F) 500µm.

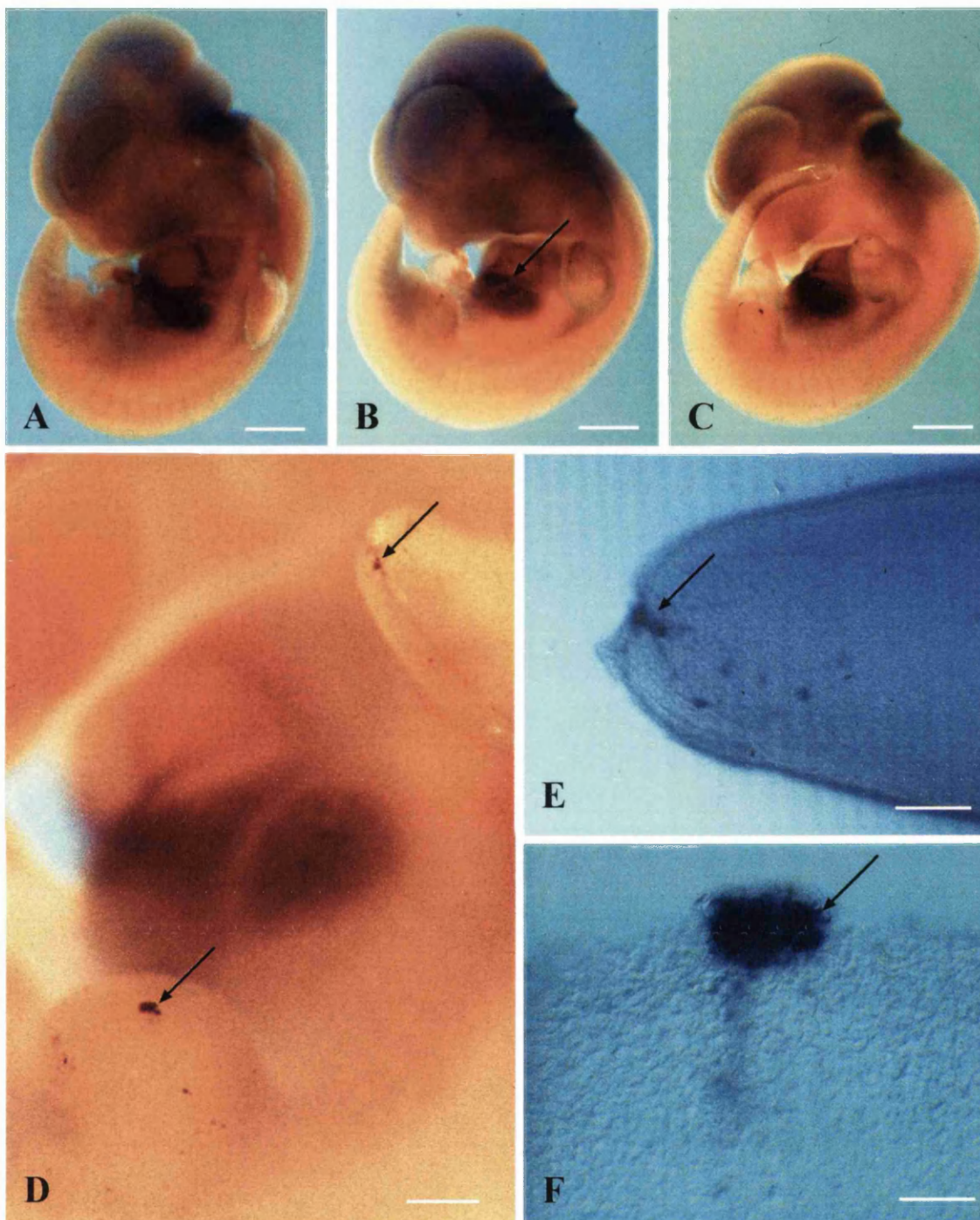


Figure 2.14 TGF-Beta-1 expression following wounding

- A-C. TGF-Beta-1 in situ hybridisation studies in E11.5 embryos wounded on the forelimb, flank and hindlimb prior to culturing for 0 min (A), 30 min (B) and 60 min (C). Arrow in B indicates normal expression of TGF-Beta-1 in the heart.
- D. Detail from 60 min wound (C), with arrows indicating TGF-Beta-1 expression at sites of forelimb and hindlimb wounds.
- E. Vibratome section of TGF-Beta-1 in situ stained wholemount viewed using Nomarsky optics. The section is transverse through the forelimb and shows expression (blue) in the wounded limb marginal view (arrow).
- F. High magnification detail of TGF-Beta-1 expression in a transverse section of the hindlimb amputation stump. Arrow indicates expression localised to the central blood vessel.

Scale bars: (A-C) 500 μ m; (D) 100 μ m; (E, F) 50 μ m.

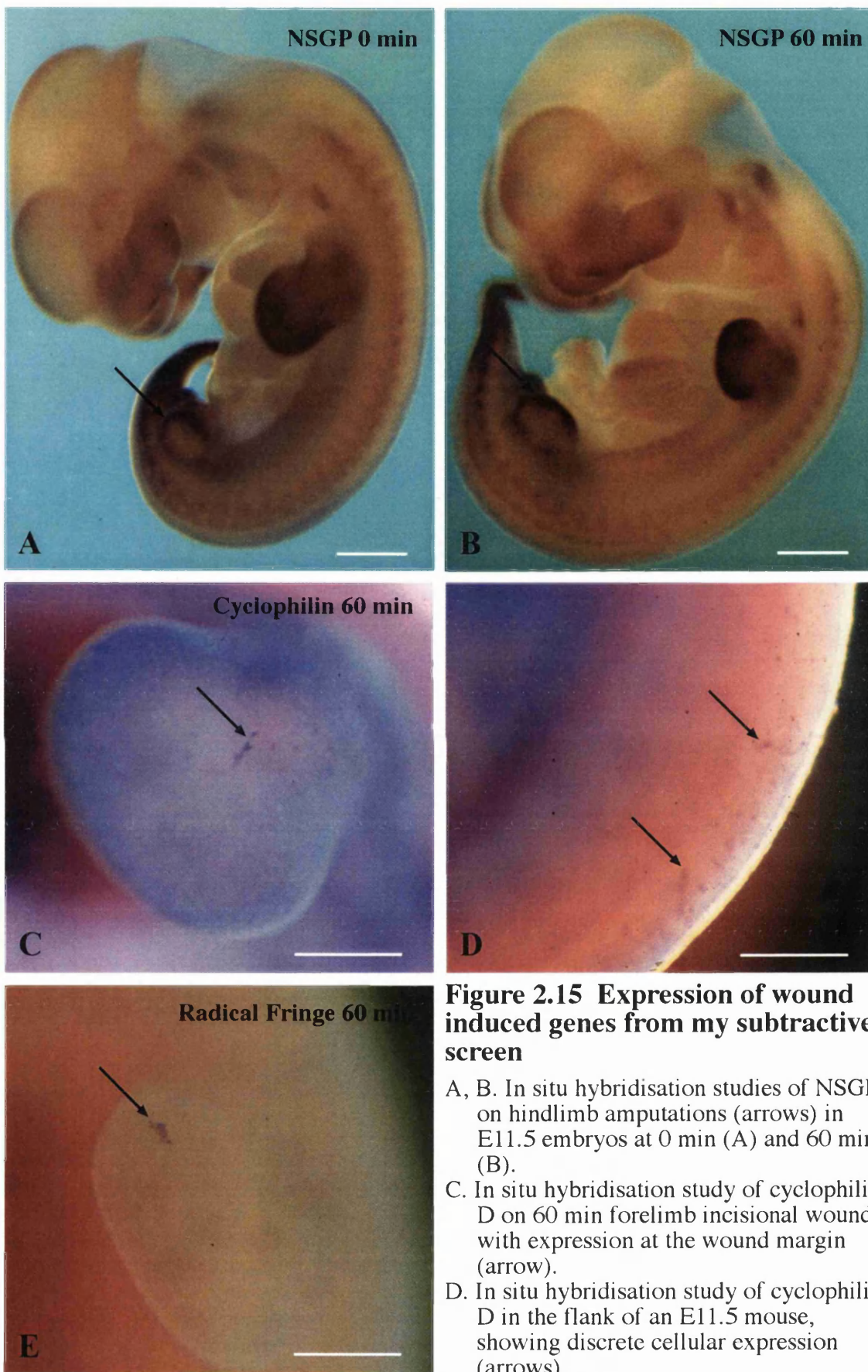


Figure 2.15 Expression of wound induced genes from my subtractive screen

A, B. In situ hybridisation studies of NSGP on hindlimb amputations (arrows) in E11.5 embryos at 0 min (A) and 60 min (B).

C. In situ hybridisation study of cyclophilin D on 60 min forelimb incisional wound with expression at the wound margin (arrow).

D. In situ hybridisation study of cyclophilin D in the flank of an E11.5 mouse, showing discrete cellular expression (arrows).

E. In situ hybridisation study for radical fringe in a 60 min E11.5 forelimb incisional wound, showing expression in the distal epithelial margin of the wound (arrow).

Scale bars: (A, B) 500µm; (C-E) 100µm.

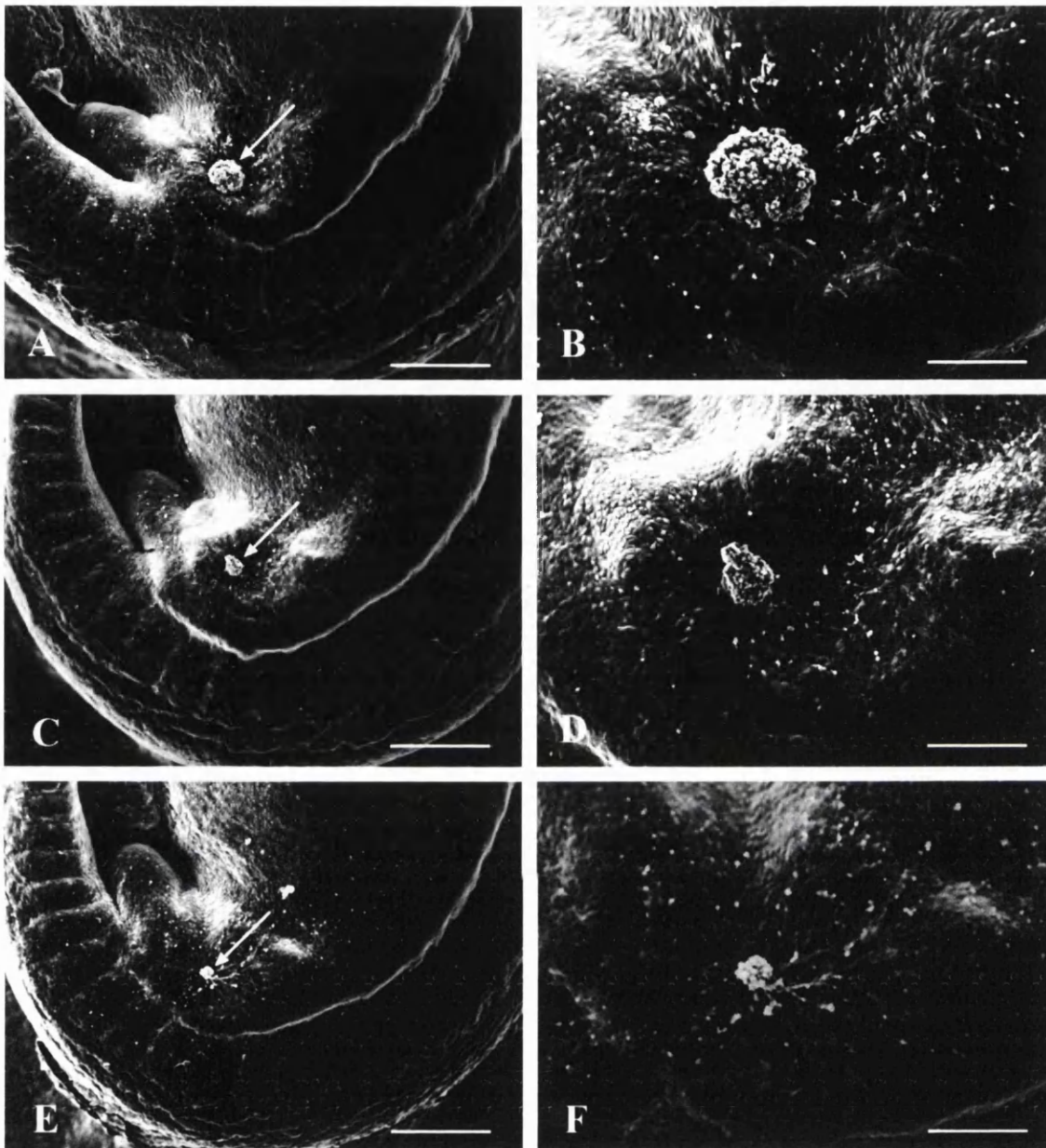


Figure 2.16 Wound closure in the presence of the collagenase blocker UK-221316

Scanning electron micrographs of E11.5 mouse embryos cultured for 24 hours following hindlimb amputation. Left hand panel shows low power views of closed wounds with characteristic wound pimple (arrows) and right hand panel shows higher magnification detail.

A, B. Control embryo cultured in standard embryo culture medium (see Materials and Methods).

C, D. Embryo cultured in standard embryo culture medium plus 5uM collagenase blocker.

E, F. Embryo cultured in standard embryo culture medium plus 50uM collagenase blocker.

Scale bars: (A, C, E) 250um; (B, D, F) 100um.

Discussion

In this chapter I report that three immediate early genes, *c-fos*, *krox-20* and *krox-24*, are upregulated rapidly in response to wounding in the E11.5 mouse. All of these genes are transcription factors which can control the expression of downstream effector genes, one such gene being *TGF β 1*, which is also described. In addition to detailed *in situ* studies describing the expression patterns of the above genes, I present results from my subtractive hybridisation screen for genes upregulated 1 hour after wounding which, although not as conclusive as those for the above genes, raise interesting points for discussion. In addition to my molecular data, I also report a morphological change in epithelial cells at the wound margin which may reflect underlying molecular events.

- ***In situ hybridisation for c-fos yields predicted results***

It has previously been reported that c-Fos protein can be detected at the wound site within 30 minutes in the E12.5 rat (Martin and Nobes, 1992) and in the E11.5 mouse (J. McCluskey DPhil. Thesis Univ. Oxford, 1995), and shows maximal expression around 1 hour after wounding. In agreement with this I observe *c-fos* message within 15 minutes of wounding, peaking at around 30 minutes and decreasing again to almost zero by 60 minutes (Figure 2.10). This is consistent with what one might expect from an immediate early gene such as *c-fos*, and similar patterns of expression have been described in tissue culture wounds (Wichelhaus et al., 1987).

- ***krox-24 upregulation is consistent with published studies***

Krox-24 was initially described as an early growth response gene that displayed *fos*-like induction kinetics in fibroblasts, epithelial cells, and lymphocytes following mitogenic

stimulation (Sukhatme et al., 1988). It is known to be expressed in response to wounding in several tissue culture models (Khachigian et al., 1996; 1997; Dieckgraefe and Weems, 1999). In the most recent study, a gut epithelial cell line, IEC-6, was used to test whether the extracellular signal-regulated kinase (ERK) operated as a mediator of wound-signal transduction and a possible regulator of epithelial restitution (Dieckgraefe and Weems, 1999). They found significant increases in *c-fos* and *krox-24* mRNA levels following wounding of monolayers, downstream of ERK activation and peaking by 20 minutes. This ERK activation and upregulation of *krox-24* and *c-fos* was inhibited in a dose-dependent fashion by the Parke Davis ERK kinase (MEK) inhibitor PD-98059. In addition, expression of a dominant negative *krox-24* construct also led to significantly reduced *in vitro* monolayer restitution.

These findings are especially exciting since they not only confirm my *in situ* timecourse of expression for *c-fos* and *krox-24*, but the dominant negative *krox-24* study suggests that we may well see a wound healing phenotype in the *krox-24* knockout mouse when it becomes available.

- *Similar expression patterns for c-fos, krox-20 and krox-24 can be explained by looking at their upstream regulatory sequences*

There is a striking similarity between the pattern of expression of the immediate early genes *c-fos*, *krox-20* and *krox-24* following wounding. All show detectable message levels within 15 minutes, although *krox-24* seems to be expressed at higher levels than the other two.

The *krox-24* (*Egr-1*) gene encodes a zinc finger transcription factor which binds to target DNA sequence at GC rich motifs, to regulate downstream gene expression. It is closely related to *Krox-20*, which binds the same target sequence, and it was first identified as a serum response gene with a similar pattern of regulation as the proto-oncogene *c-fos* (Janssen-Timmen et al., 1989). Both genes have a series of 5' serum response elements

(SREs), which bind serum response factor (SRF) in an identical fashion to the *c-fos* SRE.

Krox-20 was first reported as a transcription factor which showed remarkable rhombomere specific expression in the developing hindbrain (Wilkinson et al., 1989). The *krox-20* upstream SRE can functionally substitute for the *c-fos* SRE and binds the same nuclear protein (Chavrier et al., 1989). It is also expressed by Schwann cells, but not until later in development than *krox-24* (Topilko et al., 1997).

- *The krox-24/lacZ mouse offers hope for future wound healing studies*

In addition to being transiently expressed during cell cycle re-entry (Lemaire et al., 1990), *krox-24* has been studied in some detail in the developing nervous system, where it is expressed by Schwann cell precursors (Topilko et al., 1997). In the adult mouse, Krox-24 is strongly induced in Schwann cells following nerve lesion, and is thought to play a key role in their adopting a proliferative rather than myelinating phenotype (Topilko et al., 1997). The Charnay lab have generated a *krox-24/lacZ* reporter mouse to allow functional studies of Krox-24 protein (Topilko et al., 1998). Injecting a targeting vector containing *krox-24* sequence either side of an in frame insertion of the *E.coli lacZ* gene, into mouse embryonic stem cells, they generated a transgenic line where heterozygotes develop normally but express the *lacZ* reporter gene product, β -Galactosidase, at sites of *krox-24* expression. Homozygous *krox-24/lacZ* siblings make no functional *krox-24* protein. In their published study, mice lacking functional Krox-24 are born according to Mendelian ratios, averaging one quarter of the newborns, but are infertile, showing multiple pituitary and ovarian defects (Topilko et al., 1998).

Unfortunately I was unable to obtain any knockout embryos in five litters generated from heterozygous *krox-24/lacZ/+* matings. This tends to contradict the published observations of Mendelian inheritance, but the colony I was working has only recently

been established at UCL and we hope it will begin to provide knockout embryos in the near future. There is also a transgenic *krox-20/lacZ* line available for study, knockouts of which survive embryogenesis, but die soon after birth (Schneider-Maunoury, 1993). There are therefore an obvious set of functional studies to be performed looking at wound closure in single and double knockout embryos, as soon as the colonies are yielding the expected ratios of transgenic mice. My preliminary studies looking at healing of hindlimb amputations in these mice showed no difference between the ability of *krox-24/lacZ* heterozygous embryos and wild type littermates to heal wounds (data not shown). This is unsurprising since heterozygotes are phenotypically identical to wild type littermates as adults (Piotr Topilko, UCL, personal communication), with a single copy of the *krox-24* gene being sufficient for normal development.

- *TGFβ1 expression in my wound model is surprising in the light of previous immunocytochemical data*

Earlier in this chapter I described expression of *TGFβ1* message in the central blood vessel of a 1 hour E11.5 hindlimb amputation stump. *TGFβ1* mRNA levels at the wound site in an E11.5 mouse have been described previously (Martin et al., 1993), and it appears from their 3 hour wound wholemount *in situs* that they too see expression in the invading blood vessel, though this is not highlighted in the paper. Interestingly, this study presents conclusive immunohistochemical evidence that, in contrast to this mRNA expression domain, TGFβ1 protein is expressed at high levels throughout the wound mesenchyme and epithelium at the wound edge, from 1 hour post-wounding and persists until around 18 hours post-wounding, by which time the wound is almost healed. The only area in the wound bed not to stain positive for TGFβ1 protein is the exposed tip of the transected major blood vessel, the precise domain of TGFβ1 mRNA expression. This phenomenon of complementary domains of TGFβ message and protein localisation

has been reported previously in the mouse embryo, specifically in the lung (Pelton et al., 1991a), but also in many other tissues (Pelton et al., 1991b). In many tissues the two are co-expressed but in the developing lung, protein expression of all three TGF β isoforms is confined to the bronchiolar epithelium, while TGF β 1 mRNA transcripts are found in smooth muscle cells and connective tissue fibroblasts lying subjacent to the epithelium. These results suggest that TGF β s have a complex pattern of transcription, translation, and secretion during normal development in the mouse embryo, and support my observations of spatial segregation of TGF β 1 mRNA and protein in embryonic wound repair. It would be interesting to see if these results are recapitulated in the forelimb slash wound, where TGF β 1 transcription is restricted to the limb marginal blood vessel.

- *Assembly of microvilli by wound edge cells could reflect gene activation*

The small GTPase molecular switch Rho regulates formation of many diverse actin based structures, including stress fibres (Ridley and Hall, 1992), and microvilli-like structures at apical membranes (Shaw et al., 1998). It is also known to be an essential component of the signalling pathway controlling the formation of the actin cable in the leading edge epithelial cells at the margin of a chick embryo slash wound (Brock et al., 1996). At the margin of an E11.5 mouse embryo slash wound, high magnification SEM reveals large numbers of microvilli on the apical surfaces of wound edge epithelial cells. These are the same cells that are actively reorganising their cytoskeleton, and share this “hairy” appearance with epithelial cells distant from the wound margin that are undergoing cytokinesis, another cellular process requiring activation of the small GTPase Rho (Kishi et al., 1993; Gauthier-Rouviere et al., 1998).

- *Why does my screen fail to identify known genes?*

Only 200 clones from my subtractive library were sequenced, so this may well not have been a saturation screen, despite one gene, *type IV collagenase*, coming up more than 4 times. This fact coupled with my looking only at messages upregulated 1 hour post-wounding helps explain why I did not pull out any of the genes known to be upregulated in E11.5 mouse embryo wounds. Messenger RNA levels of all of the immediate early genes mentioned earlier; *c-fos*, *krox-20* and *krox-24*, have decreased to basal levels by 1 hour after wounding, so would not be predicted to show up in my screen, and TGFβ1 mRNA is only detectable in injured major blood vessels by *in situ* hybridisation at 1 hour post-wounding. When compared to a whole limb, this relatively minor upregulation may mean that TGFβ1 mRNA does not form a large percentage of the transcriptional response in limb tissue 1 hour after wounding.

With the benefit of hindsight it is possible to identify several ways in which my subtractive hybridisation screen could be improved. Fundamental to the success of a screen such as mine is the amount and quality of mRNA that one starts with. Since I was wounding amputated E11.5 limbs of around 500µm diameter I was only able to isolate the 2µg mRNA sufficient for starting the Clontech PCRselect™ kit protocol. This is in marked contrast to the amount of starting material used in the screen for Brachyury inducible genes published from Jim Smith's lab (Tada et al., 1998), where they were able to isolate mRNA from 1500 *Xenopus* animal caps for both control and dexamethasone treated tissues, generating more than 10µg mRNA. More recently, technology has become available to produce high quality cDNA from nanograms of starting mRNA using a protocol specifically designed for preparing cDNAs for use in the PCR-Select kit. Background levels of cDNAs in the subtracted, wound-specific library that are common to both the wound and control samples is reported to depend somewhat on the quality of

RNA purification as well as the performance of the subtraction (CLONTECH PCR-Select™ protocol, 1998).

A further improvement would be to expand the screen and analyse more clones using filter screening prior to *in situ* hybridisation analysis. It is possible that some of the results of my *in situ* hybridisation studies may be false negatives, due to the particular region of sequence I cloned being unsuitable for the generation of a reliable riboprobe. This is supported by the fact that my *in situs* for *radical fringe* fail to repeat the published non-wound expression pattern during mouse embryogenesis (Johnston et al., 1997). By probing identical filters, copied from colonies obtained after the cloning of suppression-PCR fragments, with radioactively-labelled cDNAs reverse transcribed from either wounded or unwounded tissue mRNAs, one could identify differentially expressed genes from a much larger sample group of clones than by sequencing (Hedrick et al., 1984). Promising clones could then be further screened by Northern blot analysis, before being sequenced and used to generate riboprobes for *in situ* hybridisation studies.

It would also be nice to repeat this type of screen to look at earlier timepoints following wounding, especially as I have identified at least three immediate early genes; *c-fos*, *krox-20* and *krox-24*, which would serve as positive controls for whether the screen was working.

It is possible that some false positives will be generated by my screen from failure to get complete hybridisation of all messages common to wounded and control tissues during the subtractive hybridisation step. Additionally, some genes identified from my screen may not be expressed in my wholemount *in situ* mouse embryo wounds. The wounding model used for my subtractive screen was designed to produce a large number of wound edges in a limited tissue sample, to try to maximise the percentage of tissue close to a wound site. Wound edges were created by multiple needle slash wounds to an amputated limb, which was subsequently cultured in isolation from the embryo. This may not be an ideal model, since multiple slash wounds will not result in the gaping that is seen upon

slash wounding of an intact embryo (Brock et al., 1996), as there is less tension in the epithelium of a multiply-wounded amputated limb. Finally, there is no intact blood supply in the amputated limb during the 1 hour post-wounding culture period, so the wound site is precluded from sensing any blood borne cues involved in the healing response.

I was in the fortunate position of being able to sequence many clones prior to any kind of screening, so that I could pick clones to follow up after I knew which gene they encoded. Obviously this approach would not allow me to follow up previously unsequenced cDNAs, but was a good initial step for identifying known genes which had not previously been studied in an embryonic wound model.

- *Several studies give support to genes identified by my screen being WIGs*

Despite the failure of my *in situs* to demonstrate expression of several of the genes identified by my screen at the wound site, there are published studies which suggest that each of them could play a role in embryonic wound healing. In the following paragraphs I shall discuss each of these genes in turn.

Non-Selenium Glutathione Peroxidase

Non-Selenium Glutathione Peroxidase (NSGP) was perhaps the most exciting gene that I identified, having already been described as a gene upregulated in both activated keratinocyte (Frank et al., 1997) and adult rat wound (Munz et al., 1997) models. NSGP is thought to be important in protecting cells at the wound site from damage caused by reactive oxygen species, such as hydrogen peroxide and oxyradicals. In the adult it is known that free radicals abound at the wound site, being released by macrophages and

other inflammatory cell types in a protective oxidative burst (Halliwell and Gutteridge, 1990).

My *in situ* data show only modest upregulation of this gene in a 1 hour amputation wound, but show a relatively high background level of expression. This is to be expected, since reactive oxygen species are generated in all cell types by a variety of metabolic processes and NSGP is therefore needed ubiquitously.

Cyclophilin D

In situ hybridisations for *cyclophilin D* mRNA in 1 hour mouse embryo wounds show the clearest wound site upregulation of any of those from genes identified in my screen. In contrast to NSGP, Cyclophilin has not been previously implicated in wound healing, although it has been described as an immune regulatory molecule produced by mouse embryo 3T3 cells following treatment with FGF (Davis et al., 1991). This study claimed to be an *in vitro* wound healing model, with FGF treatment simulating the local release of growth factors that occurs at the wound site. More interestingly, Richards and colleagues (1992) showed in cultured lymphocytes that transient inhibition of two phosphatases, PP1 and PP2A, affected the induction of several downstream genes including *krox-24* and *cyclophilin*. These phosphatases were inhibited by treatment with okadaic acid which resulted in an activation of the MAP kinase pathway, downstream of which *krox-24* was strongly but transiently upregulated, and *cyclophilin* was upregulated over a more prolonged timecourse, from a reasonably high basal level of expression to almost 2-fold higher by 3 hours post-treatment.

Type IV Collagenase

There were four hits for *type IV collagenase* (MMP-9) in my subtracted library, which would suggest that it is therefore relatively strongly expressed following wounding. This

is certainly consistent with published studies where *type IV collagenase* is seen upregulated in both *in vitro* (Salo et al., 1991) and *in vivo* (Salo et al., 1994) models. However, my *in situs* were unsuccessful and when I used the small-molecule drug UK221316 to block collagenase activity, there was no impairment of closure of an E11.5 mouse hindlimb amputation wound. This lack of effect could be because the primary role of type IV Collagenase at the adult wound site is in tissue remodelling, where it effects the lysis of the basement membrane to allow keratinocytes freedom to begin re-epithelialisation. This degradation is not required for re-epithelialisation of an E11.5 mouse embryo wound, since the basal lamina remains attached to the leading edge epithelium as it sweeps over the exposed wound bed mesenchyme (J. McCluskey DPhil. Thesis Univ. Oxford, 1995). From my limited results it appears that wounds which have healed in the presence of collagenase blocker show less of a wound pimple. One explanation of this would be that collagenase released from the migrating epithelial edge acts to free more cellular debris from the exposed wound bed mesenchyme, and the advancing epidermis sweeps this towards the centre of the wound, where it is extruded as a wound pimple. In the absence of collagenase activity the epithelium sweeps over most of this debris, which remains loosely bound in the mesenchyme, so forms a smaller wound pimple and may even advance more rapidly with less to push before its leading edge.

Phospholipase A2 Activating Protein

Although matrix metalloproteinases (MMPs) may not be needed in the healing of an embryonic wound, expression of type IV collagenase is interesting when considering one of the other genes upregulated. Phospholipase-A₂ activating protein (PLAP) is also identified in my screen and has been shown previously to regulate induction of MMPs (Shankavaram et al., 1998) in a tyrosine phosphorylation dependent manner. In Shankavaram's experiments, this induction was observed in human monocytes treated with lipopolysaccharide, a bacterially derived toxin. Their studies showed that tyrosine

phosphorylation of PLA₂ is one of the initial steps needed for the lipopolysaccharide induced MMP production in human monocytes.

PLAP is also implicated in the regulation of inflammation in the gut in diseases such as Crohn's and ulcerative colitis, where it regulates the production of arachidonic acid (Peterson et al., 1996). Several other important adult wound healing molecules have been identified as key mediators of inflammation in the gut including Activin (Hubner et al., 1997), Connective Tissue Growth Factor (Dammeier et al., 1998) and KGF (Brauchle et al., 1996).

Frizzled

frizzled (*fz*) was initially identified as a tissue polarity gene in *Drosophila*, where it forms part of the *wingless* signalling pathway. *Frizzled* encodes a serpentine receptor-like transmembrane protein required for reception and transmission of a polarity signal, downstream of which is the small GTPase switch RhoA (Strutt et al., 1997). *fz* is not normally expressed in the E11.5 limb and *in situs* fail to show convincing expression at the wound site after 1 hour of culture. The fact that it is highlighted in my screen hints at a role for the *Wnt* signalling pathway during wound closure, and this would be consistent with my screen also pulling out an APC-binding protein as being upregulated (see *Appendix II*). The adenomatous polyposis coli gene, *APC*, is downstream of *fz* in the *Wnt/wingless* pathway and these genes appear to function in determining cytoskeletal polarity in early *C. elegans* embryos (Rocheleau et al., 1997).

Radical fringe

The *fringe* gene was initially identified in *Drosophila*, where it is expressed at sites of boundary formation, particularly in the developing wing. *Radical fringe* has been reported as being expressed during neural development in the mouse (Johnston et al., 1997). My *in situs* did not show conclusive wound upregulation but there is a very recently published study which describes isolation of a cDNA encoding *radical fringe* from a regeneration-specific library in the regenerating newt limb (Cadinouche et al., 1999). In this study, *in situs* found *radical fringe* highly expressed during the regenerative phases of active cell division following limb amputation.

Supervillin

Supervillin is an actin-binding membrane protein, originally cloned from bovine neutrophils (Pestonjamas et al., 1997). It shows striking similarity to the actin binding proteins villin and gelsolin, particularly in regions known to bind F-actin, and is thought to be involved in actin filament assembly at adherens junctions. We know that assembly of a contractile cable comprised of actin filaments is fundamental to closure of an E11.5 mouse wound (McCluskey et al., 1995), so a protein involved in filament assembly would be a likely molecule to be expressed at the wound site.

In conclusion, it appears that the “informed guesswork” approach of searching for genes upregulated following wounding in my embryo model was far more fruitful than my subtractive screening. It is clear, however, that if we are to identify novel or unpredicted genes involved in wound healing that this predictive approach would not work. I have identified several ways in which my screening procedure could be improved for future screens and discussed the alternative strategies currently available. There are some key experiments still to be performed on the *krox-24* and *krox-20* transgenic mouse embryos

as soon as homozygous knockouts can be generated, and hopefully these will identify these genes as key players in embryonic wound repair.

CHAPTER THREE

A study of the molecular basis of embryonic wound healing in the fruitfly *Drosophila melanogaster*

Introduction

- *A historical perspective on insect wound healing studies*

Insect wound healing studies have been largely limited to three species; *Rhodnius prolixus*, a South American hemipteran insect responsible for transmitting Chaga's disease, the cockroach *Periplanetus*, and the fruit fly, *Drosophila melanogaster*. The first studies to address insect wound repair were published by Wigglesworth in 1937, and reported healing in *Rhodnius*, where wounds to the abdomen of an adult were seen to be sealed by a plug of haemocytes, the insect equivalent of macrophages. These wounds, either a 0.5mm incision or a 0.5mm² excision healed within 2 days. Later studies using a cockroach leg wound model showed similar clotting, with the epidermal cells using the hemocyte plug as a migratory support, in the absence of basal lamina, during wound reepithelialisation (Bohn, 1974 and 1976). These studies also showed hemocytes to have a growth promoting effect on cockroach epidermis *in vitro*.

More recently, insect wound healing studies have focussed on pattern regeneration, using *Drosophila* as a model system. The choice of the fruitfly is based on the huge volume of

research that has made it the most genetically tractable model organism in modern developmental biology.

- ***Drosophila wing imaginal discs are useful tools for a wound healing study***

The *Drosophila* wing imaginal disc consists of a folded sheet of columnar epithelial cells and an overlying sheet of squamous epithelium, the peripodial membrane, which together forms a continuous flattened epithelial bag enveloping the disc lumen. It is the columnar epithelium cells which proliferate to give rise to the adult wing structures and the thoracic musculature. Most of the studies on wing discs have been on discs isolated from third instar larvae, when the discs are about 400µm long, but still relatively flattened. As the larva matures towards pupation at the end of stage three, the discs begin to change shape, with the presumptive wing developing as an evagination from the 2-dimensional disc; at this stage, it becomes more tricky to wound reproducibly. Once isolated from the larva, discs can be cultured for short periods of a few hours in insect cell culture medium but, for longer term studies, they are cultured *in vivo*, in the abdominal cavity of an adult female virgin fly (Reinhardt et al., 1977 and 1981; Karpen and Schubiger, 1981; Dale and Bownes, 1985).

- ***Excisional imaginal disc wounds regenerate perfectly***

In 1976, French and colleagues described a polar coordinate model (after Wolpert, 1969) for pattern formation in epimorphic fields, whereby each cell's fate within a 2-dimensional domain is determined by its position on a hypothetical grid. One of the examples they chose to illustrate this model was the *Drosophila* wing imaginal disc. They proposed that it was the confrontation of cells not normally directly apposed to each other that drives pattern regeneration following an excisional disc wound.

Several further studies have addressed the capacity of wing imaginal discs to regenerate pattern, typically following an excisional wound in which approximately a quarter of the disc is excised (Karpen and Schubiger, 1981; Dale and Bownes, 1985; Bryant and Fraser, 1988). Structures which are missing following excisional wounding could potentially be replaced by one of two mechanisms; epimorphosis, where cellular proliferation and transdetermination follow blastema formation, or morphallaxis, where respecification occurs in the absence of growth. Several studies suggest that disc wounds regenerate structures via epimorphosis (Reinhardt and Bryant, 1981; Dale and Bownes, 1985; Bryant and Fraser, 1988).

- *Disc wounds can heal in two ways*

Initial wound closure in imaginal discs seems to happen in one of two ways; heterotypically, where the free edges of columnar and squamous epithelium on the same side of the wound fuse together first, or homotypically, where both epithelia fuse with their homologous counterparts on the opposite side of the wound when the wound edges meet (Reinhardt and Bryant, 1981). Where wounds initially heal heterotypically, there is a subsequent homotypic healing step during culture, which results in a fully healed wound. The healing process is outlined in the diagram below:

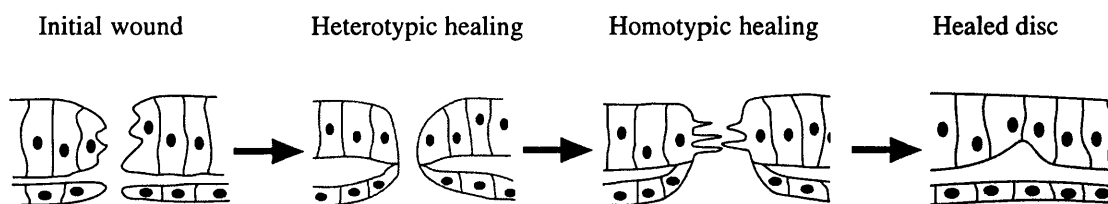


Figure 3.1 Wound healing in wing imaginal discs

It seems somewhat unpredictable which process will occur in any one situation, but the mechanism of healing seems to determine whether tissues regenerate or duplicate during the re-establishment of tissue pattern (Dale and Bownes, 1985). In this study, fragments

that healed homotypically regenerated missing parts, while fragments that first healed heterotypically duplicated parts. Where wounds heal heterotypically at first there is a subsequent homotypic healing step, with filopodial extensions from each of the epithelial types making contact across the wound gap to permit restoration of continuous epithelial layers (Reinhardt and Bryant, 1981). Whichever mechanism is used, disc wounds heal completely within 48 hours when cultured *in vivo*. No studies have addressed what happens when only one of the epithelial layers is wounded and this is one of the questions addressed in this chapter.

- ***Fluorescent cellular tracking helps explain the steps of regeneration***

An elegant study in 1988 tracked cell movements during regeneration using intracellular injection of large fluorescent dextrans to label individual cells (Bryant and Fraser, 1988). This study utilised a standard model of culturing 3/4 fragments of wing disc *in vivo*, but also labelled discrete cells along the wound edge to allow tracking of cell movements and lineage. The wounded discs regenerated their excised fragments by cell proliferation, over a period of several days, and periodic fluorescent photography allowed a timecourse of events to be established.

During the first day of culture, the two wound edges were brought together by wound healing, such that the fluorescently labelled cells appeared as a single line. These marked cells then gradually moved apart over the next three days, as new cells were added between them by proliferation. This proliferative response, as detected by bromodeoxyuridine labelling of DNA synthesis, began at around 18 hours post-wounding, and was followed by the establishment of gap-junctional cell communications across the healed wound. Cell division was apparent only in the immediate vicinity of the healed wound, and up to several cell diameters back from the wound edge. It was proposed that excisional wounding, and the resulting repair mechanism, brings together cells from distant positions in the disc, and this apposition of cells with differing positional values is what triggers the local upregulation of cell proliferation. In this case,

one could foresee incisional wounding not triggering cellular proliferation, since the cut edges have neighbouring positional identities.

- *Regeneration genes can be identified by insertion screening*

The great strength of working with *Drosophila* is the scope for developing a screen to search for genes involved in mediating any particular process. Studies from the Russell lab have used enhancer-sensitive P-element screens to search for genes that are upregulated at sites of disc regeneration (Brook et al., 1993; Addison et al., 1995). In these types of study a construct comprising the β -galactosidase gene, downstream of a minimal promoter, is randomly inserted into the genome via its flanking P-elements, DNA sequences homologous to bacterial transposons. Where the construct insertion point is close enough to an endogenous enhancer sequence, the minimal promoter expression will be amplified sufficiently to enable reporter gene detection. This system allows mapping of gene expression in the heterozygote disc, potentially coupled with knockout phenotypic analysis in the homozygous disc.

These studies identified several genes upregulated at the wound edge following cell death induced by a temperature-sensitive cell-lethal mutation. After screening around 800 insertions, they found 23 lines where spatial and temporal correlations were found between lac-Z expression and regeneration blastema formation (Brook et al., 1993). These lines were categorized into 3 classes: 1) A small number of lines where upregulation appeared to occur solely in discs undergoing regeneration and not at any other stage, possibly representing genes active exclusively in regeneration; 2) A larger class expressed normally during oogenesis or in the embryo, but not normally in the imaginal discs; this class of genes must represent functions recruited from earlier stages of the developmental program; and 3) Finally, a class of genes whose expression normally includes spatial domains within the disc; this class included several insertions with expression associated with morphogenesis, including one at the decapentaplegic (*dpp*)

locus, and one at the *crumbs* (*crb*) locus, a TGF β family homologue, and an EGF-repeat gene, respectively.

- *Many morphogenetically important genes have distinctive expression domains in the wing imaginal disc*

A key feature of *Drosophila* wing imaginal disc development is the repeated use of some of the same patterning genes that were responsible for patterning the embryo, much earlier in development. Some of these genes, which display distinctive patterns of expression, are illustrated below (Fig. 3.2).

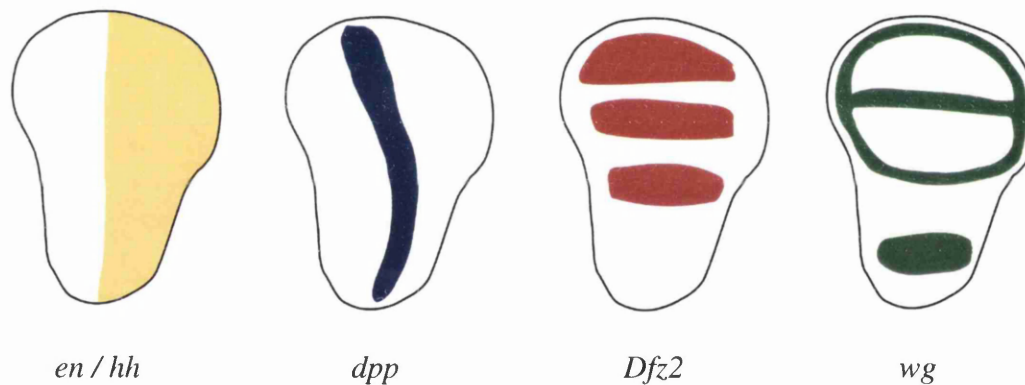


Figure 3.2 Gene expression domains in the developing wing imaginal disc

One of the earliest genes expressed is the segment polarity gene *engrailed* (*en*), which is expressed throughout the posterior compartment of the wing disc. Co-localised with *engrailed* expression is another segment polarity gene *hedgehog* (*hh*), and this secreted protein induces cells at the boundary of the anterior and posterior compartments of the disc to express a broad stripe of Decapentaplegic (Dpp) (reviewed in Wolpert, 1998). Later, during antero-posterior patterning, the secreted signalling protein Wingless (Wg), and its receptor Dfz2 are also expressed in distinct, complementary domains (Zhang and Carthew, 1998).

- *Wounding provides a means for fate-mapping the Drosophila embryo*

Marking or removing defined cell populations in the early embryo are two classic means for determining prospective cell fate, and have been used in various developmental model organisms. Several groups have used wounding as a means of removing specific groups of cells early in development to allow fate-mapping of the *Drosophila* embryo. The technique was first used in insects early this century by Hegner (1910) and in *Drosophila* in particular by Geigy (1931), who irradiated germ cells at the blastoderm stage. Soon after, cells were eliminated from the *Drosophila* embryo by pricking (Howland and Child, 1935; Bownes and Sang, 1974a), microcauterizing (Bownes and Sang, 1974b) or locally UV laser irradiating (Lohs-Schardin et al., 1979a and b). The fate maps determined from these studies showed reasonable correlation with each other, but there were problems of reproducibility, high mortality rates and high frequency of abnormal larvae. These problems highlight the fragility of the early *Drosophila* embryo, but since some embryos clearly do survive these traumas, it is evident that embryos do possess some wound healing capacity.

In 1980, Underwood and colleagues determined an embryonic *Drosophila* fate map by withdrawing cells at the early blastoderm stage (3 hours post-laying) with a micropipette and looking for defects at an early segmentation stage, about 6 hours later. Though not actually addressing the issue of wound healing in the embryo, their paper provides proof that the early embryo can heal a wound of at least 15µm, since such a wound results from their experimental procedure. Scanning Electron Microscopy pictures of 9 hour old embryos show that these micropipette stab wounds heal within 6 hours, resulting in missing segmental structures, where progenitor cells were removed, but leaving a continuous healed epithelium.

- *There are several ways of visualising the actin pursestring during dorsal closure*

To test whether *Drosophila* embryo wound repair is analogous to repair in vertebrate embryos, and to morphogenetic movements in the fly, requires investigation of actin structures assembled in wound edge cells. The contractile actin pursestring driving dorsal closure can be visualised in a number of ways, either in fixed or living embryos. Fluorescently tagged phalloidin can be used in exactly the same way as in the vertebrate embryo to bind to the actin filaments that form the pursestring. Alternatively, the myosin motors that provide the driving force for contraction can be labelled immunocytochemically using an antibody to Zipper, *Drosophila* non-muscle myosin (Young and Kiehart, 1993). This results in a similar, if somewhat weaker, staining pattern to that given by revealing the actin with phalloidin.

More recently, the advent of green fluorescent protein (GFP) as a tag for examining protein localisation within cells has provided a means for following the dynamics of the actin pursestring *in vivo*. Expression of a fusion protein where GFP has been fused to the actin binding domain of moesin, a membrane-cytoskeleton linker protein, provides a strong *in vivo* marker for cell shape and pattern during embryonic morphogenesis (Edwards et al., 1997). This GFP-Moesin construct can be expressed ubiquitously in the embryo or larva, under the control of a heat shock promoter, with no adverse phenotype.

- *Morphogenetic movements in the fly appear similar to wound closure*

In Chapter One I described the morphogenetic movements of gastrulation and dorsal closure in some detail. The following diagram illustrates the cellular movements involved in dorsal closure in a simplified cartoon form:

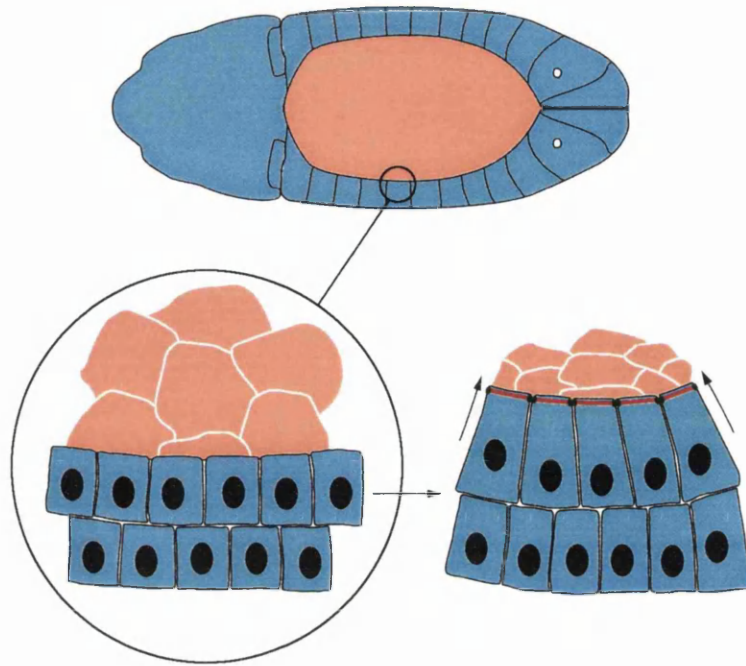


Figure 3.3 Cartoon of dorsal closure in the *Drosophila* embryo

About ten hours into embryogenesis, the dorsal surface of the *Drosophila* embryo is covered by a squamous extraembryonic membrane, the amnioserosa (pink). Over the next two hours, the lateral epithelium (blue) extends dorsally to enclose the entire dorsal surface in the process termed dorsal closure. The zoom-in figures highlight the two components that provide the motor to accomplish this. First, the leading edge epithelial cells can be seen to assemble an intracellular actin cable (red) just beneath their apical surface. Adherens junctions (green) allow coupling of these intracellular cables, resulting in one continuous contractile pursestring running the entire circumference of the dorsalmost row of epithelial cells. The amnioserosa is also an active player during this process - these squamous epithelial cells undergo shape changes, constricting their apices to become columnar, prior to dropping into the embryo where they apoptose and are engulfed by haemocytes (Rugendorff et al., 1994). The culmination of embryogenesis comes when these two processes, epithelial advance and amnioserosal contraction, combine to close the dorsal hole, enveloping the embryo in one contiguous epithelium.

The hypothesis I will explore in this chapter is that the tissue movements of wound healing are more than superficially similar to dorsal closure; they actually utilise the same structural and motor proteins, and are regulated by the same signalling cascades. To do this I will first provide a basic characterisation of wound healing in both embryonic and disc models, and then move on to look at gene expression in both dorsal closure and wound repair.

Materials and Methods

- ***Collecting, wounding and culturing developmentally synchronous *Drosophila* embryos***

Adult *Drosophila* were transferred from a stock bottle to an egg-laying cage, with a base comprising a 60mm petri dish filled with apple juice agar (ICRF, London, U.K.) with a smear of live yeast. Egg lays were collected over 1-2 hour periods at room temperature. Unstressed flies lay soon after mating, so time of laying can be taken as roughly equivalent to time of fertilisation. Stressed females may retain their eggs for several hours, so not all eggs collected are necessarily at the same developmental stage. For this reason the first egg lay of the day was always discarded, as it yielded the most developmentally asynchronous embryos.

A soft-bristled paintbrush was used to collect eggs from the surface of the agar, and the eggs washed off into an embryo sieve using distilled water. Embryos were dechorionated by washing in Sodium hypochlorite bleach diluted with 2 parts water (3% final concentration) for 2 minutes at room temperature. The embryos were then washed thoroughly in distilled water to remove any residual bleach and transferred to one half of a fresh apple juice agar plate using a paintbrush. A line was cut down the centre of the plate using a razor blade, and the embryos aligned alongside, so that they were all oriented the same way allowing subsequent wounds to be in the same region of each embryo. During this step one can check that the chorion has been completely removed by looking for the presence of dorsal filaments. These are paired "antlers" present at the anterior of each egg, adjacent to the micropyle. They allow air to reach the embryo, and come away along with the chorion if the eggs have been bleached effectively.

Embryos were then transferred to cover slips coated with Sellotape glue. This glue was prepared by adding heptane to crumpled Sellotape in a tube and rolling for 30 minutes, allowing the heptane to dissolve off the adhesive. The cover slip was laid gently on top of the line of embryos, then lifted off and stuck onto a glass slide for ease of handling, using a drop of water.

Embryos were then desiccated over anhydrous silica gel for approximately 6 minutes to prevent excessive leakage following wounding. Once desiccated they were overlaid with a thin layer of 10S Voltalef oil. This prevented further desiccation, whilst still allowing gas exchange.

At this stage, embryos were ready for wounding. A number of wounding strategies were tried, varying from a delicate 3 μ m glass needle pinprick to a 100 μ m slash wound, but a glass micropipette stab giving a wound of around 20 μ m was adopted. After wounding, the cover slip was removed from the slide and the embryos incubated at room temperature, in a humid chamber.

After incubation the embryos were prepared for fixation by rinsing the oil off using heptane, and the embryos washed from the cover slip with fixative. Optimum fixatives varied depending on the fate of the specimen.

For phalloidin staining, embryos were fixed for 25 minutes in heptane saturated with formaldehyde (obtained by shaking equal volumes of heptane : 37% formaldehyde, allowing to settle, and using the upper heptane layer), at room temperature.

For Scanning Electron Microscopy, embryos were fixed for 60 minutes in heptane saturated with glutaraldehyde (obtained by shaking equal volumes of heptane : 25% glutaraldehyde, allowing to settle, and using the upper heptane layer), at room temperature.

After appropriate fixation, embryos were transferred to a glass slide using a yellow Gilson pipette tip, and the heptane allowed to evaporate. The embryos were then carefully transferred onto a new glass slide covered with a layer of double-sided sticky tape. Under a dissecting microscope, a few drops of PBT were pipetted over the embryos, so they could be teased out of their vitelline membranes using a tungsten needle.

- ***Preparing embryos for Scanning Electron Microscopy***

Preparation of samples for SEM was similar to that already described in the Materials and Methods of the previous chapter for mouse embryos, with the exception of the initial fixation step. Having been fixed in heptane saturated with 25% glutaraldehyde, embryos were post-fixed in Osmium. This was done by washing twice for 10 minutes, in 0.1M sodium cacodylate buffer, before leaving in 0.1M sodium cacodylate buffer containing 1% osmium tetroxide for 30 minutes at 4°C. After two more 10 minute washes in 0.2M sodium cacodylate, embryos were dehydrated through graded alcohols, 5 minute washes in each. Rather than critical point drying, Hexamethyldisilazane (HMDS, Sigma, Poole, U.K.) was used to air dry the embryos ready for SEM. Embryos were rinsed once in HMDS, and then washed for 10 minutes prior to pipetting onto a glass slide where they were left to dry at room temperature. After at least 1 hour the embryos were transferred to carbon discs (Agar scientific) on SEM stubs using a paintbrush, then sputtercoated with 20nm gold in the usual way, prior to scanning in a Jeol 5410 LV scanning electron microscope.

- ***Preparing embryos for Transmission Electron Microscopy***

Fixation of samples for TEM was identical to that already described for SEM up until the end of the Osmium post-fix step. Embryos were then washed, in 1.5ml Eppendorfs, four times in distilled water, before staining for 30 minutes in 2% uranyl acetate, at room

temperature. Following three washes in distilled water, embryos were dehydrated through graded alcohols, and washed 3 times for 3 minutes in dry alcohol. After washing twice for 1 minute in xylene, embryos were washed for 30 minutes in a 1:3 mix of propylene oxide : xylene. Most of this mixture was then removed, and replaced with Araldite resin, leaving the tube open for several hours to allow the last of the xylene to evaporate. The bases of the embedding moulds were covered with Araldite resin and incubated at 80°C for 30 minutes, until the Araldite was the consistency of soft caramel. Specimens were then aligned in individual moulds, covered with more Araldite, and incubated for 24 hours, at 60°C.

Embryos were cut out of their the Araldite block with a hacksaw and mounted on stubs with superglue (Loctite). Thick, 10µm, sections were cut on a microtome (Reichert, OmU3), dried onto Tespa-coated slides, and stained with toluidene blue for 2 minutes, at 65°C. When mid-way through the embryo, ultra-thin sections (60-70nm) were cut on an ultra-microtome (Reichert ultra-cut E) and floated off into distilled water, prior to mounting on copper grids. Sections were stained with lead citrate (BDH) and viewed on a Jeol Transmission Electron Microscope-1010.

- *Preparing embryos for phalloidin staining*

Preparation of embryos for phalloidin staining was again similar to that already described in the Materials and Methods of the previous chapter, with the exception of the initial fixation step. Having been fixed and devitellinised, embryos were washed three times for 15 minutes in PBT, and then left at room temperature for 60 minutes in PBT containing 250ng/ml FITC-Phalloidin. Embryos were subsequently washed twice for 5 minutes and then three times for 30 minutes in PBT, at room temperature. Embryos were mounted in Citifluor (UKC, Kent, U.K.) under a coverslip and staining for filamentous actin imaged using a Leica Confocal laser scanning microscope.

- ***Dissecting, wounding and culturing wing imaginal discs***

Initially, wing imaginal discs were dissected from third instar “wandering” larvae using forceps. Larvae were held by their mouthparts and rear, and pulled apart whilst in disc medium (SF-900 serum-free insect cell culture medium, Gibco BRL). Discs were isolated by progressive removal of non-disc body parts. This method was only successful in around 50% of larvae, so I adapted the procedure such that discs were not actually dissected away from the body of the maggot for culturing. By cutting the larva in two roughly one third down its total length and subsequently inverting the head end, wing discs could be identified and extraneous material removed. Leaving the discs attached to the body of the larva meant they suffered less trauma and were easier to keep track of during subsequent wounding and culturing steps. They could also be left like this during fixation and downstream protocols, being dissected to isolation only just prior to photography or SEM.

Discs were wounded using an electrolytically sharpened tungsten needle to produce an incisional wound of up to 100µm in length, across the presumptive thoracic region of the disc. Discs were then cultured in disc medium at room temperature for up to 3 hours. They were subsequently fixed on ice either in half-strength Karnovsky’s fixative (Karnovsky, 1965) for 30 minutes (for SEM), or in 4% paraformaldehyde in PBS for 30 minutes (for phalloidin staining and *in situ* hybridisation).

- ***Preparing discs for Scanning Electron Microscopy***

Following fixation in half-strength Karnovsky’s fixative for 30 minutes at 4°C, wing discs were processed in exactly the same manner as *Drosophila* embryos, being post-fixed in Osmium, dehydrated in alcohols and dried using HMDS. Discs were then mounted on adhesive Carbon pads on SEM stubs, coated with Gold and viewed in the same SEM.

- ***Preparing discs for phalloidin staining***

Preparation of discs for phalloidin staining was identical to that used for embryos, once the initial fixation was complete. Discs were fixed in 4% paraformaldehyde for 20 minutes at 4°C, then washed three times for 15 minutes in PBT, and then left at room temperature for 60 minutes in PBT containing 250ng/ml FITC-Phalloidin. After washing twice for 5 minutes and three times for 30 minutes in PBT at room temperature, discs were mounted in Citifluor under a coverslip, and filamentous actin staining imaged using confocal laser scanning microscopy.

- ***In situ hybridisation on Drosophila embryos***

Unwounded embryos were dechorionated and fixed by shaking in a 1.5ml eppendorf for 20 minutes at room temperature, in a 1:1 mix of 4% paraformaldehyde in PBS:heptane. The aqueous phase was removed and replaced with methanol to devitellinise the embryos. Devitellinised embryos, which sink to the bottom of the tube, were rinsed twice in methanol and stored at -20°C. Wounded embryos were fixed by shaking in a 1.5ml eppendorf for 20 minutes at room temperature, in heptane saturated with 37% formaldehyde, then devitellinised into PBT as described above. They were then rinsed, once in 50% methanol in PBT and given two 10 minute washes in methanol, and stored at -20°C.

Stored embryos were rinsed in methanol at room temperature and rehydrated via a 1:1 wash to PBT. All the following steps were at room temperature unless stated. They were post-fixed in 4% paraformaldehyde in PBS for 20 minutes on a gently shaking platform. After 4 washes in PBT, embryos were treated with 50µg/ml Proteinase K for 2 minutes, and then rinsed twice in 2mg/ml Glycine in PBT. Embryos were then rinsed in PBT and post-fixed by gentle shaking for 20 minutes in 4% paraformaldehyde in PBS. Following 4 washes in PBT, embryos were rinsed once in a 1:1 mix of PBT : Fly hybridisation

solution (see *Appendix IV*), rinsed twice with hybridisation solution and finally pre-hybridised at 48°C or 70°C (for DNA and RNA probes respectively), for around 2 hours.

During the pre-hybridisation step, 1µl anti-DIG antibody (Boehringer Mannheim) was pre-absorbed in 1ml PBT, against PBT rinsed fixed embryos on a shaking platform, overnight at 4°C. Prior to use, the antibody was diluted in a further 1ml PBT, to give a final dilution of 1:2000.

The hybridisation step varied depending on whether a DNA or RNA DIG-labelled probe was being used. DNA probes were denatured by boiling for 20 minutes in 40µl hybridisation solution (1µg/ml final concentration), then added to pre-hybridised embryos from which as much hybridisation solution as possible had been removed, and incubated overnight at 48°C. RNA probes were diluted to 1µg/ml final concentration in hybridisation solution, added straight to samples at 70°C without denaturing and incubated overnight.

The next day, embryos were washed for 5, then 15 minutes in hybridisation solution at the appropriate hybridisation temperature, then given 5 minute washes in 30/50/70% dilutions of hyb B solution (see *Appendix IV*) in PBT, on a shaker. Embryos were then rinsed twice in PBT and given a further three 10 minute washes, prior to a 2 hour incubation with diluted anti-DIG antibody. After this antibody step, embryos were again rinsed twice in PBT, and given a further three 10 minute washes, then rinsed three times in staining solution (see *Appendix IV*), before staining with NBT/BCIP as with mouse *in situ*s. The staining reaction was stopped by washing with PBT. Embryos were then taken to 100% ethanol and returned to PBT by 5 minute washes through graded alcohols, before mounting for photography in 70% glycerol in PBT (Lehmann and Tautz, 1994).

- ***In situ hybridisation on Drosophila wing imaginal discs***

The *in situ* protocol for imaginal discs was identical to the above embryo protocol with the exception of fixation and pre-absorbing of the anti-DIG antibody. After fixing in 4% paraformaldehyde, as described earlier, discs were washed twice in cold PBT for 5 minutes, then dehydrated through graded dehydrated by washing in increasing concentrations of methanol : PBT (1:3, 1:1, 3:1, absolute methanol) and stored in methanol, at -20°C. Antibody was pre-absorbed overnight against fixed larval remains from which the discs were originally dissected.

- ***GFP induction in hsGFP-Moesin flies***

Heat shock induction of hsGFP-Moesin larvae was performed as described in the paper in which they were first reported (Edwards et al., 1997). Third instar larvae were collected on apple juice agar plates and placed in a humid chamber in a 37°C incubator for 1 hour. They were then taken out to room temperature for 1 hour recovery, returned to 37°C for a further hour, and finally left at room temperature for 2 hours to allow accumulation of GFP-Moesin protein, so that cytoskeletal fluorescence could be observed when discs were dissected.

Results

- *Scanning Electron Microscopy studies show a timecourse of dorsal closure in the Drosophila embryo*

The progress of dorsal closure from its start at stage 13 (10.5 hours post-laying) to completion, at stage 15 (12 hours post-laying) (Campos-Ortega and Hartenstein, 1985), can be clearly observed by scanning electron microscopy (Fig. 3.4A-F). In a stage 13 embryo, approximately 10.5 hours after fertilisation, the amnioserosa can be seen as a large expanse of squamous epithelium covering about 30% of the dorsal surface of the embryo (Fig. 3.4A). Over the next hour, the lateral epithelium sweeps dorsally to cover this membrane (Fig. 3.4C) and by stage 15, two hours after dorsal closure began, the process is complete. The epithelial leading edges meet at the dorsal midline and zip together, forming a contiguous epithelium (Fig. 3.4E).

Transmission electron microscopy on transverse sections through the leading edge epithelium, reveals finger-like protrusions from the advancing leading edge cells (Fig. 3.4G, H) as the epithelium sweeps forward over the amnioserosa. These projections have been described previously as microspikes, formed downstream of activation of the small GTPase molecular switch DRac (Harden et al., 1995). The front row cells are smooth and elongated, but show no obvious signs of lamellipodial adhesions to the amnioserosa (Fig. 3.4H).

- *An actin cable is formed at the leading edge of the advancing lateral epithelium and persists until completion of dorsal closure*

Confocal laser scanning microscopy studies of dorsal closure stage embryos, stained with FITC-phalloidin to visualise filamentous actin, allows the key cytoskeletal changes which

occur during closure, to be followed. By optically sectioning a stage 13 embryo one can observe a thick actin cable around the perimeter of the large expanse of exposed amnioserosa (Fig. 3.5A). Looking at higher magnification, this cable has clearly assembled at the leading edge cells of the front row of epithelial cells (Fig. 3.5B-E). This actin cable can still be seen in leading cells as they meet their opposite numbers, at the dorsal midline (Fig. 3.5G) in a stage 15 embryo, and it is disassembled as the epithelial cells interdigitate, to form a seamless join (Fig. 3.5H).

In *zipper* mutant embryos, which can assemble this cable, but lack the myosin motors to power its contraction, dorsal closure fails dramatically, resulting in a severe dorsal hole phenotype, clearly seen under the scanning electron microscope (Fig. 3.4I, J; Young et al., 1993).

- ***The dorsal edge of the lateral epithelium advances via dorsalwards cell elongation***

Cell shapes in the advancing epithelium can be clearly discerned by virtue of cortical actin fluorescence, when embryos are stained with FITC-phalloidin. This allows one to observe a definite dorsalward stretching of leading edge cells (Fig. 3.5C) and later, rows of epithelial cells further back (Figure 3.5F). This phenomenon, of a wave of epithelial cell elongation, has been reported as being controlled by short-range signalling via the TGF β homologue Dpp, which is released from leading edge cells following firing of the JNK signalling pathway (Riesgo-Escovar et al., 1997b).

- *Cells of the amnioserosa contract their apical surfaces to reduce the exposed dorsal surface*

Concomitant with this epithelial advance, contraction of cells of the amnioserosa is seen also. Early in dorsal closure, the amnioserosa cells appear as a squamous epithelium (Fig. 3.5D), but as closure proceeds tracking of cortical actin reveals that they undergo major contraction of their exposed apical surfaces (Fig. 3.5F). During this period of contraction, one can see distinctive rosettes of cells (Fig. 3.5C, D) where constrictions are occurring. The total number of amnioserosa cells visible from a dorsal view of the embryo does not decrease dramatically as closure progresses, suggesting that epithelial cells are not simply sweeping forward over a passive amnioserosa, but that it is very much a joint effort.

- *Dpp is transiently expressed in the leading edge epithelial cells during the early stages of dorsal closure*

As mentioned above, a key molecular signal underpinning epithelial cell stretching in dorsal closure is that of Dpp, which emanates from the specialised leading edge cells (Riesgo-Escovar et al., 1997). *In situ* hybridisation studies show that this signal is transient in nature, with *dpp* mRNA levels being high in the presumptive leading edge cells during germ band retraction, in a stage 11 embryo (Fig. 3.6A). These levels decrease gradually over the next 2 hours, so that in a stage 14 embryo, mid-way through dorsal closure, *dpp* mRNA is undetectable, by *in situ* hybridisation, in the advancing epithelium (Fig. 3.6B-D).

- *An actin cable is assembled around the margin of a Drosophila embryo wound*

Drosophila embryos made accessible for wounding, as described in the Materials and Methods section of this chapter, can be wounded using a glass micropipette, to give a wound of around 20µm diameter. Wounds such as these can be seen using scanning electron microscopy (Fig. 3.7A, B). If embryos are subsequently cultured for 30 minutes under oil, and stained with FITC-phalloidin, then confocal laser scanning microscopy reveals a large accumulation of filamentous actin at the wound site (Fig. 3.7C). Higher magnification views reveal that, in addition to the expected staining of cortical actin, there is also an accumulation of actin at the apical surfaces of epithelial cells around the margin of the wound (Fig. 3.7D, E), in exactly the same fashion as seen in a vertebrate embryo wound (Fig. 2.9E; Martin and Lewis, 1992). These high magnification views also allow one to see that the epithelial cells around the wound margin appear stretched, with their apices constricted in regions where the cable is clearly visible (Fig. 3.7E).

A problem that I encountered with this wounding model was that a large extrusion of cells often occurred from this wound site (Fig. 3.7F), and whilst this may not have prevented formation of an actin cable, this cellular mass seemed to prevent wound closure. This obstacle may explain why I failed to observe a clear timecourse of wound closure in the embryo. To investigate the mortality rate following wounding, I cultured groups of 25 embryos treated in 4 ways: 1) Embryos cultured without any interference; 2) Embryos dechorionated and overlaid with oil; 3) Embryos dechorionated, desiccated and overlaid with oil; 4) Embryos dechorionated, desiccated, overlaid with oil and wounded. I found that, whilst 80% of embryos in groups 1 and 2 successfully emerged as larvae, only 68% of group 3 survived, and no larvae emerged from group 4.

- *Wing imaginal discs can be wounded more reproducibly than embryos*

Drosophila wing imaginal discs, dissected from third instar larvae (Fig. 3.8A), are significantly easier to wound reproducibly, when compared to embryos. They can be cultured for periods of several hours, in serum-free insect cell culture medium, and wounded with a tungsten needle, without desiccation. Since the wing disc is exclusively epithelial tissue, one is not restricted to making extremely small wounds, and in this regard the model can almost be considered as a tissue culture model. Relatively large wounds, of over 50µm can be seen healing following culture (Fig. 3.8B, C), and these wounds are shown to assemble an actin cable around the wound margin also (Fig. 3.8G).

- *The epithelial margin of a wing disc wound assembles an actin cable within minutes of wounding*

Almost immediately following wounding, a cable of filamentous actin is assembled in the cells around the margin of an incisional disc wound. This cable is not present at the margin of a 0 hour wound (Fig. 3.8D), but is assembled in the same rapid fashion as in vertebrate embryo wound models (Martin and Lewis, 1992; McCluskey et al., 1995), being visible within 5 minutes of wounding (Fig. 3.8E), and persisting through until the completion of closure (Fig. 3.8F).

I had hoped to time-lapse wound closure in living discs, by wounding discs expressing heat-shock inducible GFP-moesin, which labels filamentous actin structures (Edwards et al., 1997). Unfortunately, despite labelling cortical actin, GFP-moesin failed to label the actin cable I see revealed by FITC-phalloidin staining (Fig. 3.8H).

- ***In situ hybridisation studies reveal that neither *dpp* or *Dfz2* are upregulated at the margin of a wing disc wound***

In situ hybridisation studies for expression of two genes, *dpp* and *Dfz2*, showed no upregulation of these genes at the wound site, when discs were cultured for up to 1 hour following wounding (Fig. 3.9A-F). Both of these genes are expressed in distinctive domains in the developing wing disc (Fig. 3.2), and the observation of these patterns served as positive controls for my *in situ* studies (Fig. 3.9A-D).

- ***In situ hybridisation studies reveal that *Dfz2* is upregulated at the wound edge in the larval epidermis***

As mentioned in the Materials and Methods section of this chapter, wing discs were cultured, fixed and processed whilst still attached to the larva, to ease handling and prevent loss. They remained attached to this tissue throughout the above *dpp* and *Dfz2 in situ* processing. This allowed the serendipitous observation that, whilst the wing discs failed to show wound-specific upregulation of either *dpp* or *Dfz2*, *Dfz2* showed distinct upregulation at the wounded margin of the larval epidermis, precisely where the larva had been bisected during disc dissection. This *Dfz2* expression was not discernible in a 0 hour wound (Fig. 3.9G), but was clearly visible in a disc preparation which had been cultured for 1 hour post-wounding (Fig. 3.9H).

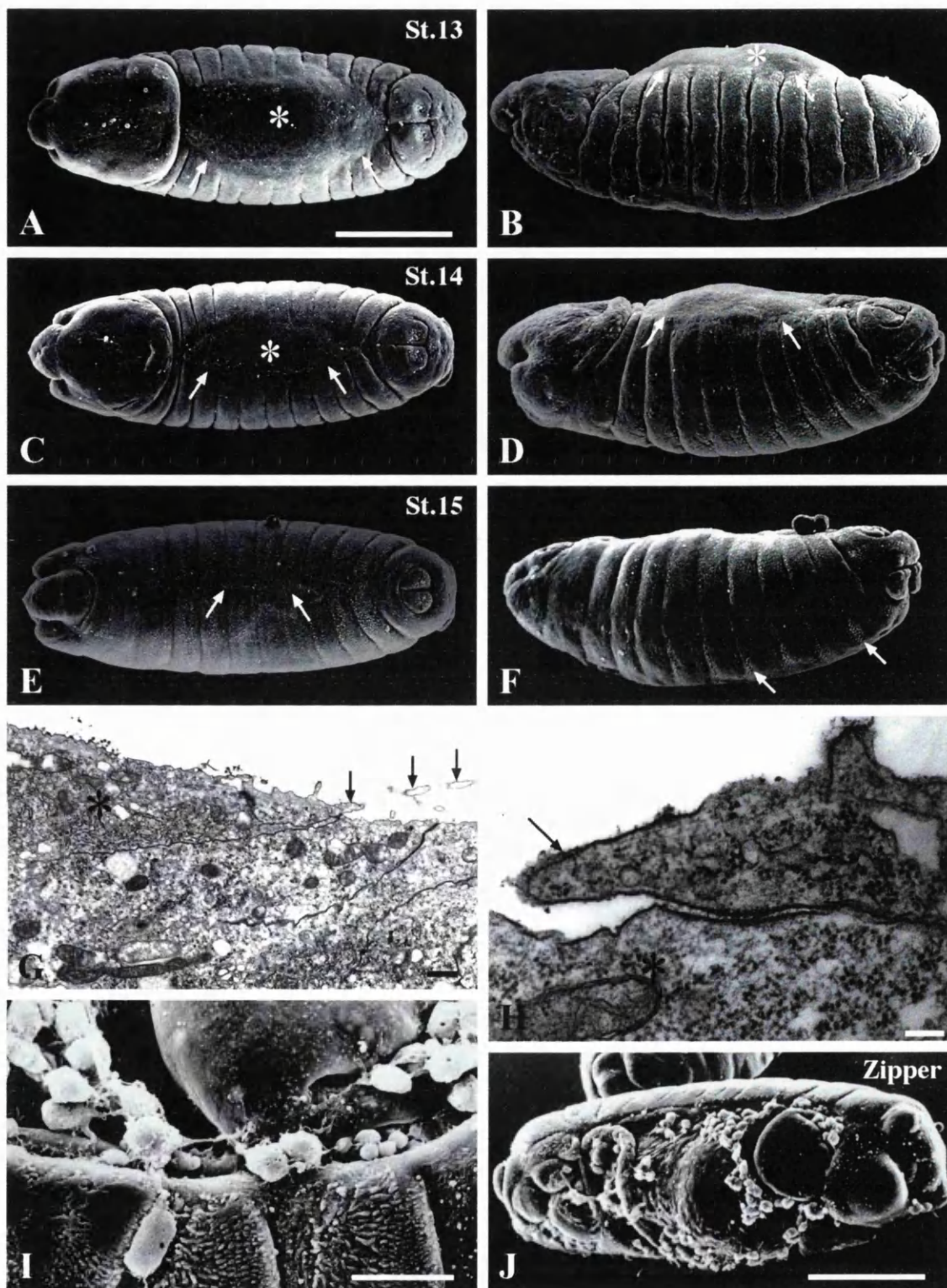


Figure 3.4 Electron microscopy study of dorsal closure in *Drosophila*

(A-F) Scanning electron microscopy timecourse of dorsal closure, viewed dorsally (A, C, E), and laterally (B, D, F), from start (stage 13) to finish (stage 15). The lateral epithelium (arrows in A-D) advances over the amnioserosa (asterisks in A-C), finally zipping up at the dorsal midline (arrows in E), to complete embryogenesis. Denticle belts can be seen developing on this late stage epithelium (arrows in F). (G) Transmission electron microscopy of a transverse section through a stage 14 embryo, showing a leading edge epithelial (asterisk) with a protruding microspike (arrows) advancing over the amnioserosa. (H) Zoom-in on the free leading edge of the lateral epithelium (arrow). (I) High magnification of a stage 15 zipper mutant epithelial front, with denticle-like projections. (J) Dorsal view of a stage 15 zipper mutant showing a severe dorsal hole phenotype. Scale bars: (A-F, J) 100 μ m; (G) 500nm; (H) 200nm; (I) 20 μ m.

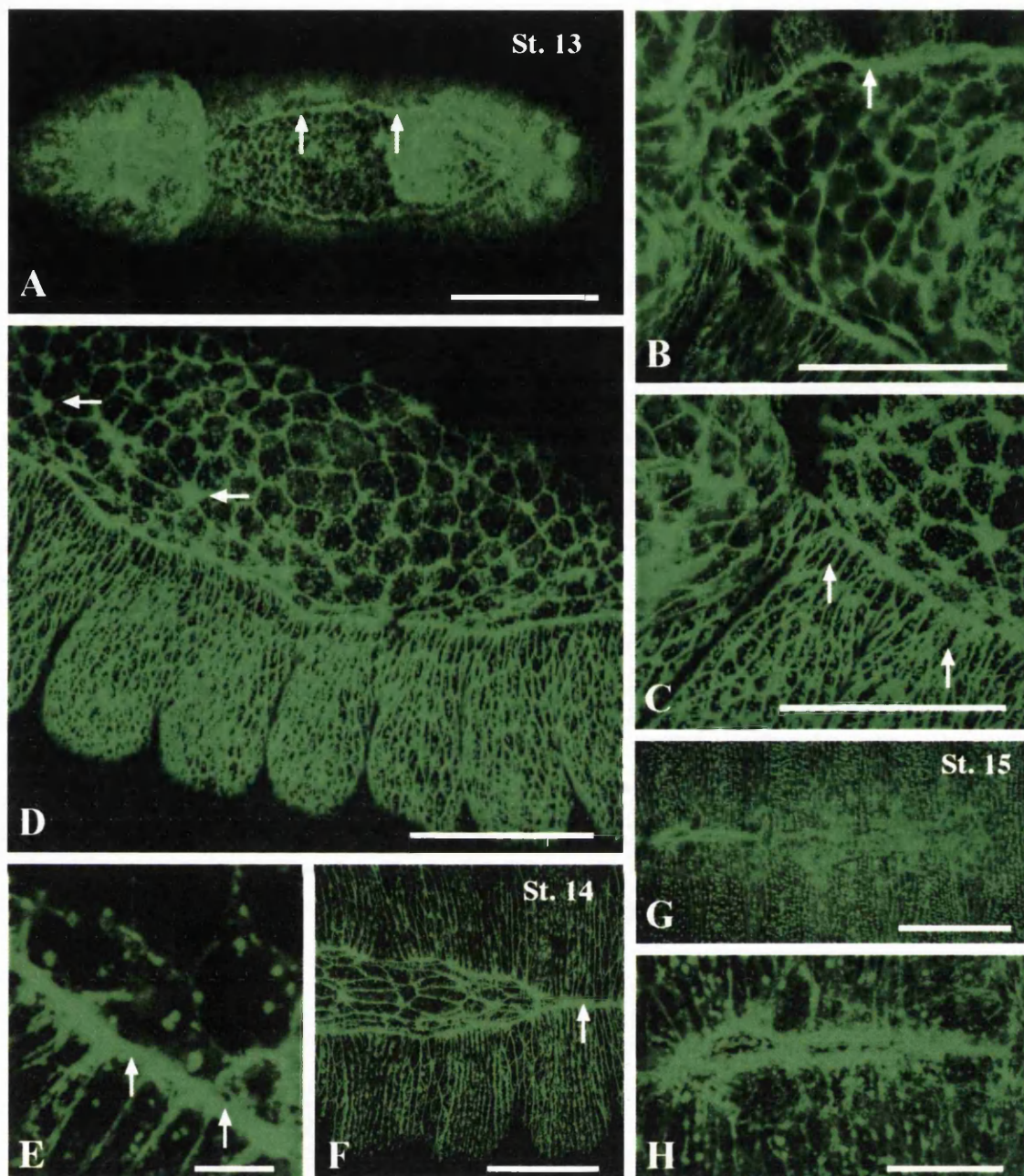


Figure 3.5 Confocal microscopy study of dorsal closure in the fruitfly

- A. Stage 13 embryo stained with FITC-phalloidin to show F-actin. Dorsal view allows visualisation of the actin cable (arrows) highlighted in higher magnification views in B, C, D and E. Front row epithelial cells can be seen elongating dorsally (arrows in C), and further magnification reveals a thick intracellular actin cable at the leading edge (arrows in E). The amnioserosa is also beginning to contract, which may explain the rosettes of constricting cells seen in D (arrows).
- F. Dorsal view of a stage 14 embryo. The cells of the amnioserosa have clearly changed their shape, so that they are significantly smaller along their dorso-ventral axes, when compared to an earlier stage (D). Epithelial cells further back from the leading edge have elongated dorsally, and the two epithelial fronts are zipping together at the dorsal midline (arrow).
- G. By stage 15 this zipping up is complete. The culmination of closure is seen in a zoom in (H).

Scale bars: (A) 100µm; (D, F, G) 50µm; (B, C, H) 30µm; (E) 15µm.

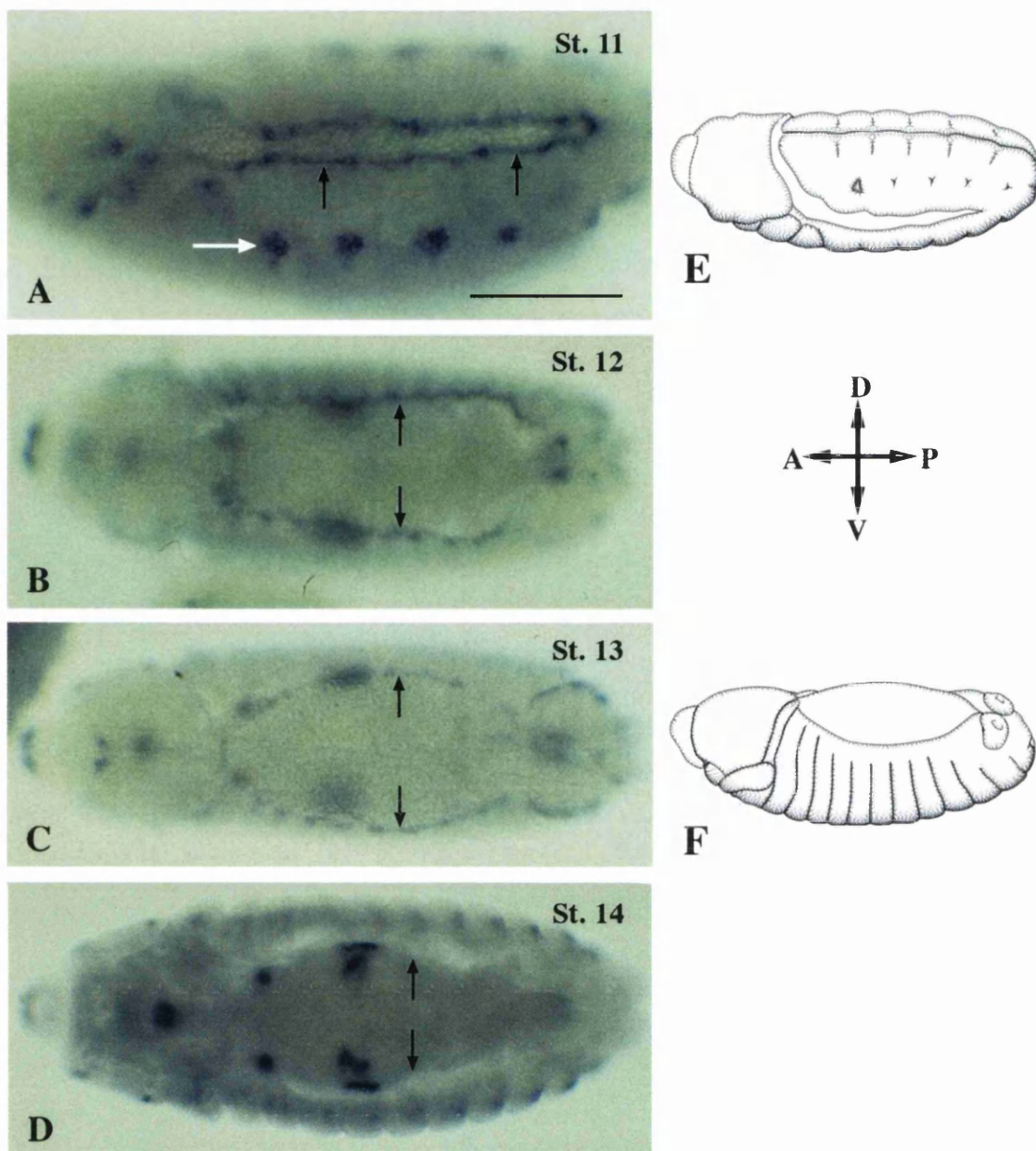


Figure 3.6 Decapentaplegic (dpp) expression during dorsal closure

- A. Lateral view of *dpp* expression in a stage 11 embryo, when the germband is fully extended. Expression is already strong in the presumptive leading edge cells (black arrows) and also in the tracheal pits (white arrow).
- B, C, D. Dorsal view of *dpp* expression during dorsal closure, with expression initially strong in the leading edge cells as the germband completes its retraction (arrows in B), then fades gradually during stage 13 (arrows in C) and has disappeared 1 hour after the start of closure (arrows in D).
- E, F. Cartoons (adapted from Flybase) illustrating germband extension (E) and early dorsal closure (F) stages of *Drosophila* embryogenesis.

Scale bars: (A-D) 100µm.

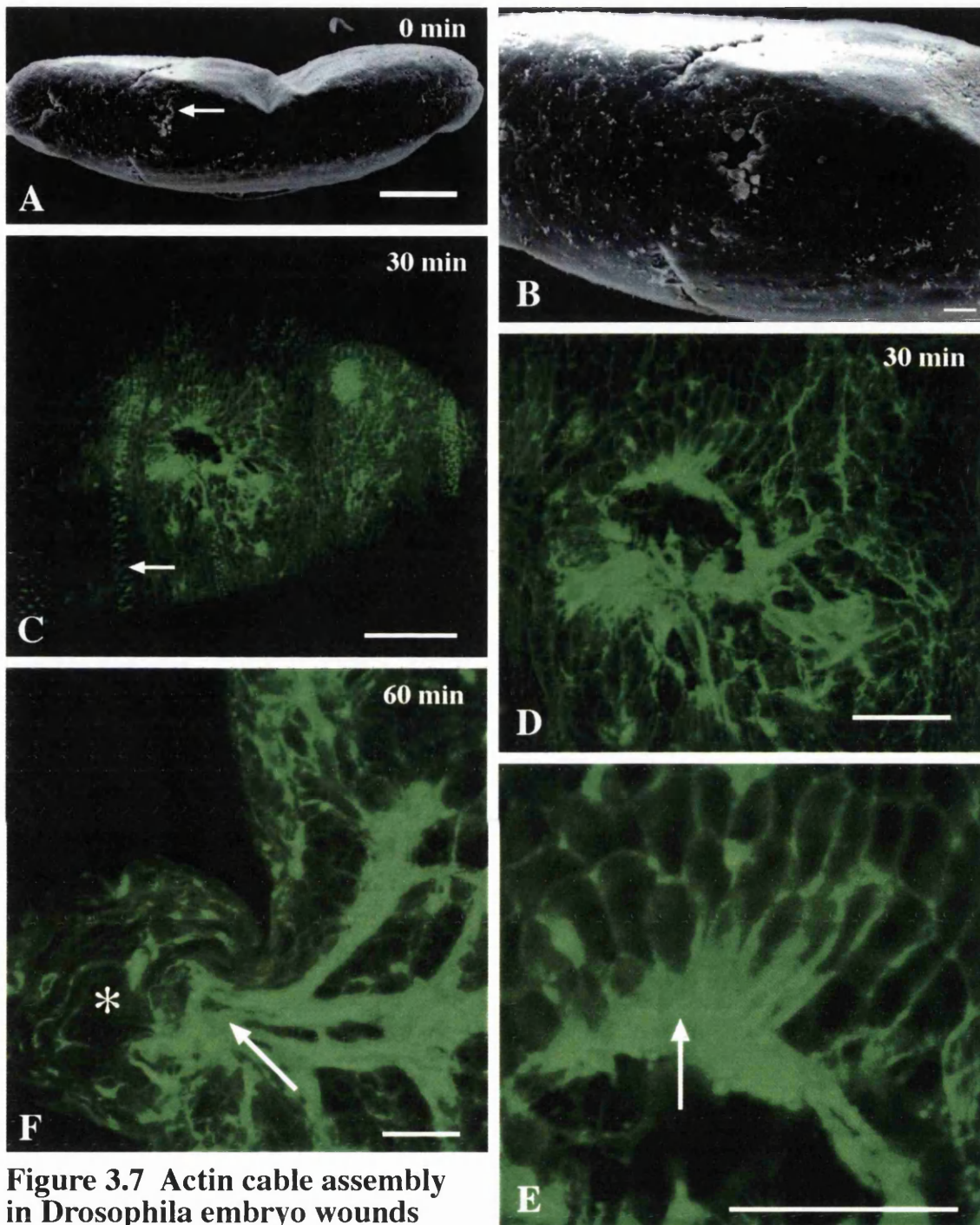
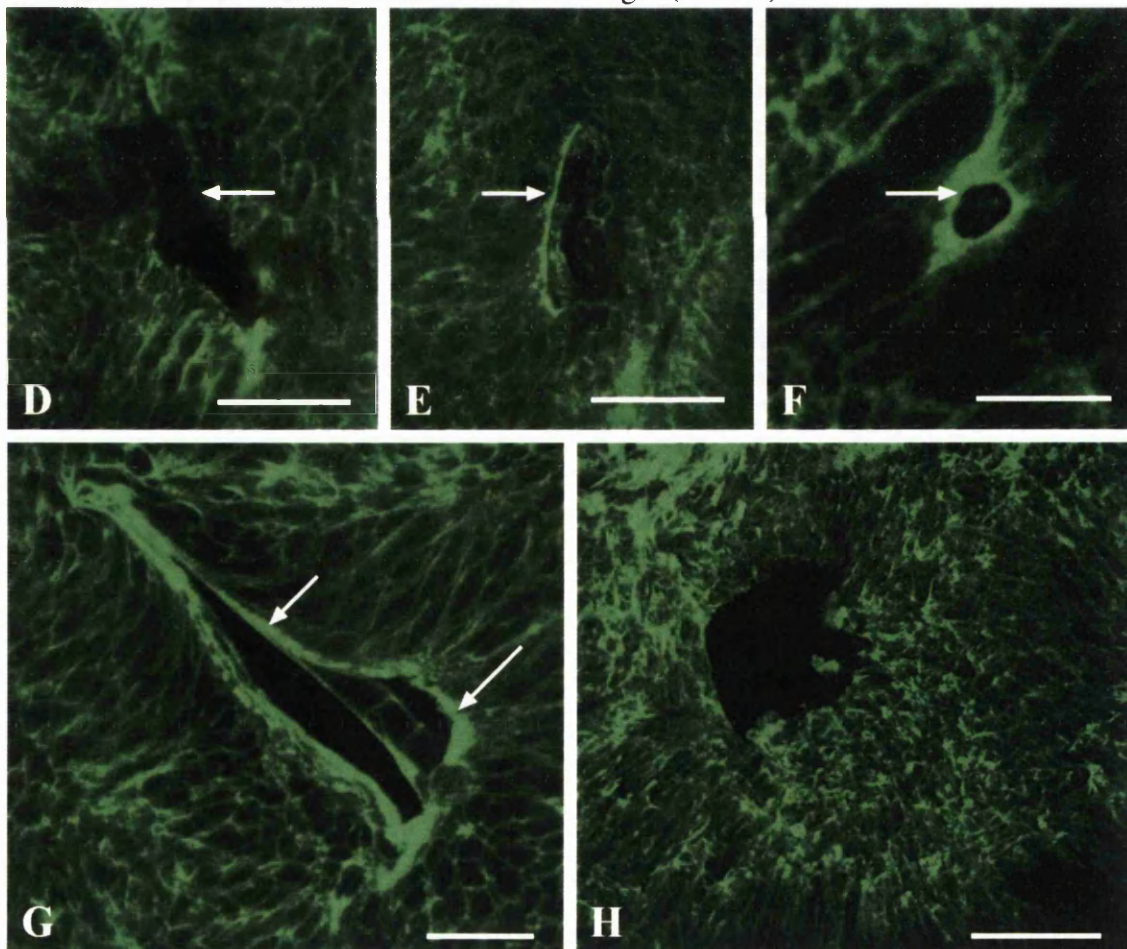
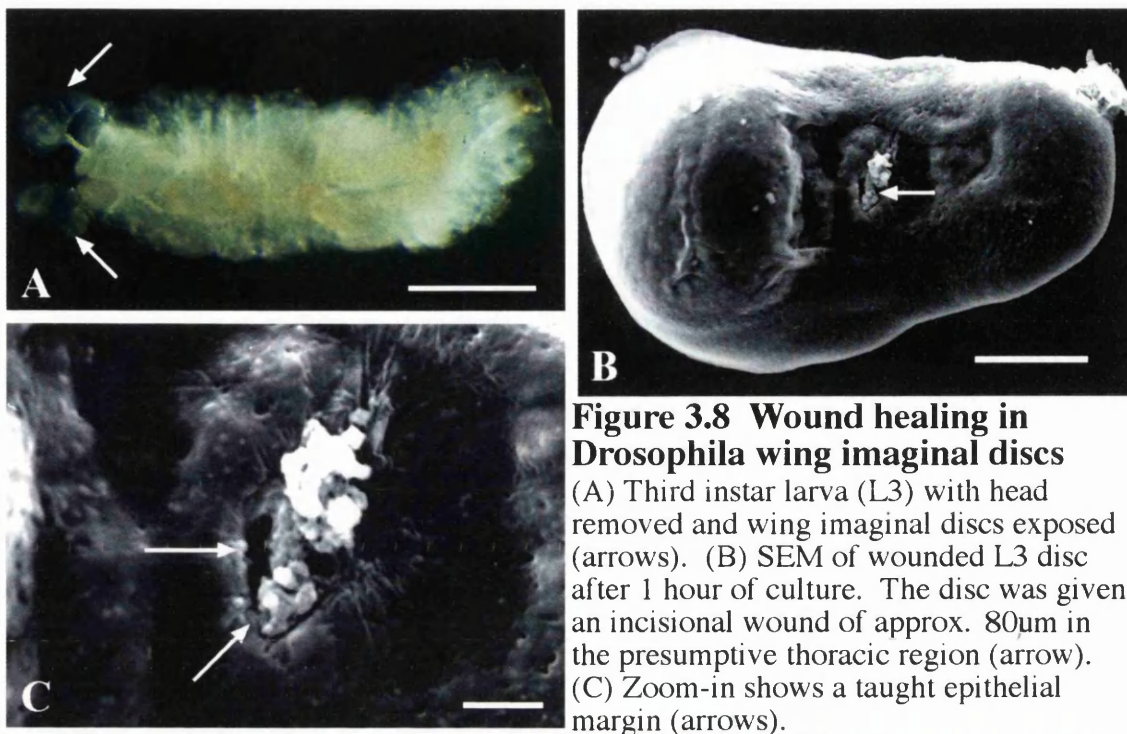


Figure 3.7 Actin cable assembly in *Drosophila* embryo wounds

- A. Ventral view SEM of a stage 11 embryo with a 0 hour stab wound on the ventral midline (arrow). Increased magnification shows that the wound is approximately 15µm in diameter (B).
- C. Lateral view of a stage 15 embryo stained with FITC-phalloidin to visualise filamentous actin at the wound site (see D, E) as well as the denticle belts (arrow).
- D. High magnification detail of wound site (in C), showing clear staining of cortical F-actin and actin accumulation around the wound margin. At higher magnification (E), the epithelial cells around the wound margin are seen stretching towards the wound, with an intracellular actin cable assembled at their apices.
- F. Optical section through a stage 16 embryo wound viewed 60 minutes post-wounding, showing extrusion of cells (asterisk) out through the wound site (arrow).

Scale bars: (A) 50µm; (C) 20µm; (B, D-F) 10µm.



Scale bars: (A) 500µm; (B) 50µm; (C, D, E, G) 10µm; (F) 5µm; (H) 20µm.

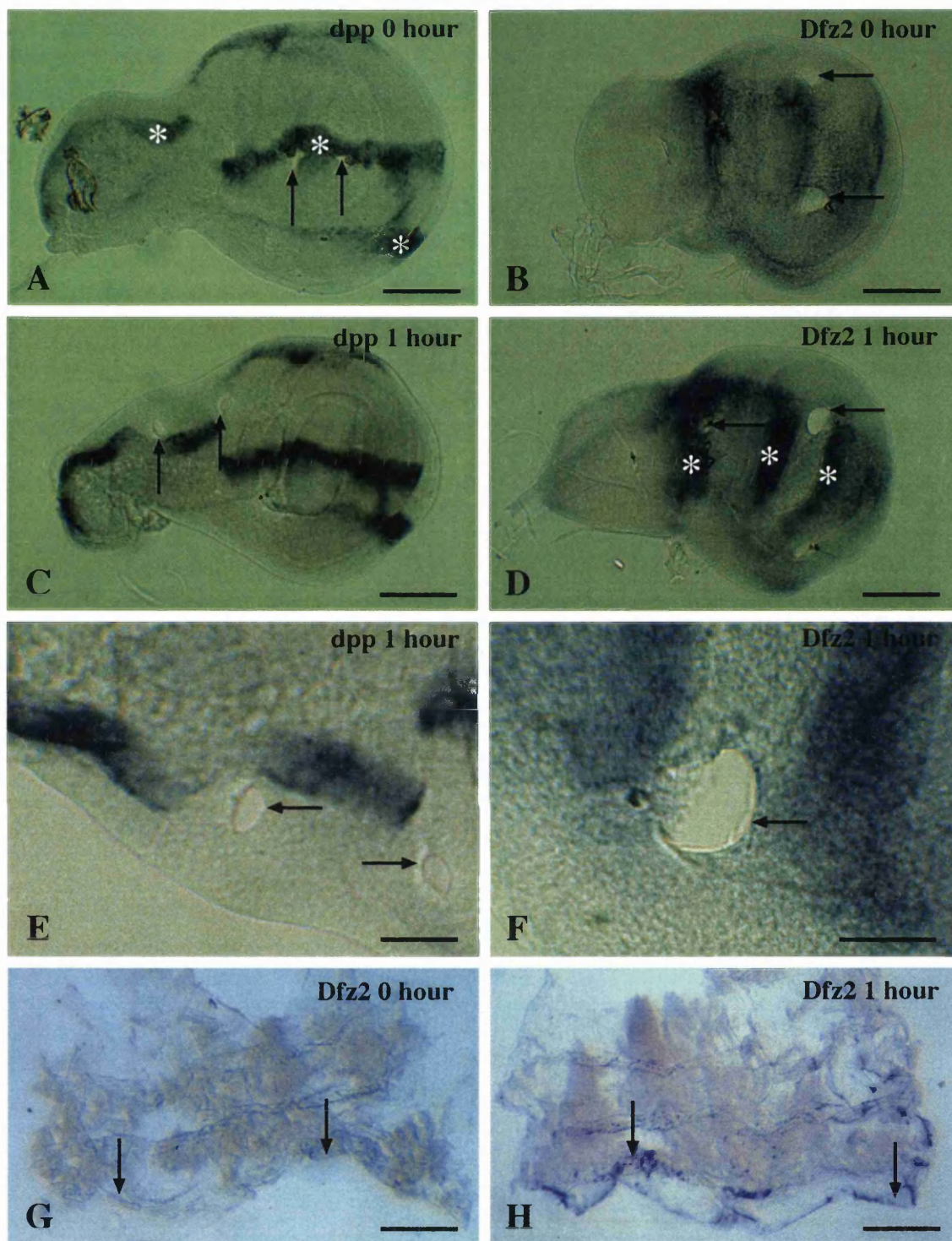


Figure 3.9 Dpp and Dfz2 expression during wound healing in wing imaginal discs and larval epidermis

A, C, E. Expression of *dpp* in wounded 3rd larval instar wing imaginal discs. In a 0 hour wound (arrows in A), there is no expression at the wound margin, except where the wound coincides with the normal expression domain of *dpp* (asterisks in A). After 1 hour in culture there is still no upregulation of *dpp* at the wound site (arrows in C), as can be seen by a higher magnification view (E).

B, D, F. Expression of *Dfz2* in similar wing disc wounds. Again, there is no increase in expression at the wound margin between a 0 hour (B) and 1 hour (D,F) wound. The normal expression domain of *Dfz2* is clearly visible (asterisks in D).

G, H. *Dfz2* is clearly upregulated following wounding of the larval epidermis, with no background expression in a 0 hour wound front (arrows in G) and clear expression after 1 hour in culture (arrows in H).

Scale bars: (A-D) 50µm; (E) 15µm; (F) 10µm; (G, H) 500µm.

Discussion

- *Actin cables in embryogenesis reveal similarities to vertebrate embryo wound healing*

The first suggestion that aspects of *Drosophila* morphogenesis might be analogous to embryo wound healing studies came with the report of a contractile actin purse-string in leading edge cells during dorsal closure (Young et al., 1993). This study provided evidence that embryos lacking the *zipper* gene product, non-muscle myosin II, failed to complete embryogenesis (Fig. 3.4J). The myosin motors, encoded by *zipper*, co-localise to an intracellular cable of filamentous actin around the epithelial margin of the amnioserosa of dorsal closure stage embryos, clearly visualised by FITC-phalloidin staining (Fig. 3.5A-E). This cable is just like that assembled around the epithelial margin of a vertebrate embryo wound (Fig. 2.9G), and is present in leading edge epithelial cells throughout both closure processes. It is assembled before epithelial advance can commence, and persists until shortly after the epithelial front zips together.

- *Epithelial spreading and contraction of the amnioserosa combine to facilitate dorsal closure*

Recent dorsal closure studies have focussed almost exclusively on the JNK signalling pathway, and its likely role in regulating the dorsalward elongation of cells in the lateral epithelium, just behind the leading edge (reviewed in Noselli, 1998). However, it is clear, from looking at cell shape changes during dorsal closure, that the amnioserosa is not simply a passive membrane over which the lateral epidermis sweeps. Both electron microscopy studies (Rugendorff et al., 1994), and my own phalloidin-stained preparations of embryos at different stages of dorsal closure (Fig. 3.5) suggest that active

contraction of the amnioserosa, to decrease the exposed dorsal surface, is probably responsible for much of the closure effort.

This combination of two tissue movements - leading edge purse-string based epithelial advance, and contraction of the exposed amnioserosa, bears a striking similarity to the two mechanisms we know are responsible for healing a mouse embryo hindlimb amputation wound - re-epithelialisation and mesenchymal contraction. In this sense, it is tempting to regard embryonic dorsal closure in the fly as being analogous to embryonic wound healing in the vertebrate.

- *Actin cable assembly in embryo and disc wounds reveals similarity to vertebrate embryo models*

Within minutes of wounding either a *Drosophila* embryo or wing imaginal disc, a concentration of actin filaments accumulate just beneath the membrane of epithelial cells adjacent to the wound. At low magnification, a clear cable of actin can be seen running within the wound margin cells (Fig. 3.7D; Fig. 3.8G). This cable is assembled with very similar kinetics to that which we see in vertebrate embryo wounds, and its appearance is indistinguishable from its vertebrate counterpart.

One difference between the *Drosophila* embryo and current vertebrate embryo wound healing models is the differentiated state of the repairing epithelium. In the fly embryo this epithelium is a single layer, whilst in the limb bud stage mouse embryo, the epithelium is bilayered, with a squamous periderm overlying a cuboidal basal layer. It would seem that the fly epidermis is analogous to the basal epidermal layer in the mouse embryo since, in the mouse, it is this basal layer that assembles an actin cable in its leading edge. The squamous periderm rides passively, piggy-back on the motile basal layer (McCluskey et al., 1995). In the fly embryo, the epithelial cells around the margin of a healing wound appear to elongate towards the wound, as contraction of the actin cable shrinks the wound circumference (Fig. 3.7E). This cell elongation is not so

apparent during closure of vertebrate embryo wounds, but is very characteristic of the cell shape changes that occur in leading edge cells during dorsal closure.

Due to the flat, simple epithelial structure of the wing imaginal disc, assembly of an actin cable following wounding is much easier to visualise, compared to the wounded embryo (Fig. 3.8D-G). As in a vertebrate embryo wound, assembly within disc wound edge cells is too rapid (within 5 minutes) for new actin transcription or translation to be playing a driving role (Fig. 3.8E). Rather, the actin required for assembly of the wound cable must derive either from the rapid polymerisation of the cell's pool of monomeric actin, or from redistribution of cortical filamentous actin.

- *Wounding embryos during dorsal closure reveals epithelial tensions in the embryo*

When the vertebrate embryo is wounded, the cut epidermis gapes open as though under tension, and the leading wound edge cells are initially stretched along the wound axis. These tensions within the epithelium may provide some of the primary wound signals that initiate actin cable assembly (Martin and Lewis, 1992; Brock et al., 1996). Recent studies in *Drosophila* embryos reveal that similar tensions may exist in embryonic epithelia, and might be important during dorsal closure. The Kiehart lab (1999) used embryos expressing GFP-Moesin (Edwards et al., 1997) to image the actin cytoskeleton, and followed changes of morphology in both the lateral epithelium and the amnioserosa, after wounding regions they predicted to be under tension. Using microneedles or UV laser irradiation, these workers made lesions in either the lateral epidermis or the amnioserosa. When they ablated a small region of the epithelial leading edge, just a few cells in diameter, there was a rapid recoil of wound margin leading edges, suggesting that the advancing epithelium is indeed under tension parallel to its direction of travel. Wounding of the amnioserosa also caused gaping, suggesting that this too was under tension. These data confirm that the amnioserosa is probably playing an active role in drawing the dorsal hole closed. Kiehart does not follow how these wounds heal, but his observation of

wounds initially gaping, confirms that cell stretching at the epithelial wound margin is a potential “kick-start” cue to leading edge cells, and may be the cue that triggers assembly of an actin cable.

- *Dpp is expressed during dorsal closure but is not upregulated in a healing disc wound*

My control *in situ* hybridisation studies for *dpp* expression during the last few stages of embryogenesis, and in third larval instar wing imaginal discs, correlate well with published expression patterns (Capdevila et al., 1994; Riesgo-Escovar et al., 1997a). Expression is strong in the leading edge epithelial cells during germband retraction, and in the early stages of dorsal closure, but this signal is transient, and has faded to background levels mid-way through closure (Fig. 3.6A-D). If signalling events during wound closure are identical to those of dorsal closure, one would expect transient expression of *dpp* in wound margin cells, but at none of the times examined did my *in situ* studies reveal even faint expression, at the wound site in discs (Fig. 3.9E), or in wounded larval epidermis (data not shown). There are several possible explanations for this, the simplest being that wounding does not lead to induction of any transcription factors upstream of Dpp. In this regard, it will be important to look at expression of *Dfos* and the AP-1 signal in wounded fly embryos and discs. Alternatively, it could be that Dpp is actively repressed in all zones in the disc bar the normal expression domains. Some of my wounds straddle the expressing and non-expressing domains (Figure 3.9A, C), giving the best opportunity to see an upregulation if this were to occur.

- *Dfz2 expression in the larval epidermis provides my first fly “wound” gene*

Dfz2, which encodes a functional Wingless receptor (Bhanot et al., 1996) is also expressed in a distinctive pattern in the wing disc (Zhang and Carthew, 1998). My

control *in situs* match those reported previously (Fig 3.9D) but again this gene appears not to be upregulated in cultured disc wounds. However, in the wounded larval epidermis, which is cut during disc dissection, *Dfz2* is markedly upregulated by 1 hour, in disc culture medium. Perhaps as for *dpp*, *Dfz2* expression is tightly regulated in the developing larval discs, but these restrictions are lifted in the larval epidermis. In fact there is evidence that DFz2 might be a regulator of epithelial movements in the embryo. It is generally considered as a segment polarity gene with functions in patterning, but it is expressed at low levels throughout early embryogenesis, with strongest expression in the strip of cells that will invaginate during ventral furrow formation (Bhanot et al., 1996). Expression of *Dfz2* during dorsal closure is extremely dynamic, but can be seen specifically in leading edge cells as dorsal closure proceeds (Alfonso Martinez-Arias, personal communication). What might the role of DFz2 be in all of these tissue movements? Perhaps it plays a role in maintenance or re-establishment of tissue positional value, and thus patterning, since this is its main function during development (Nusse and Varmus, 1992), or possibly it plays a novel role upstream of Rho small GTPases in a signalling cascade that leads to cytoskeletal reorganisations, as it does in the eye (Strutt et al., 1997). The Carthew lab recently reported that DFz2 function could be ablated in *Drosophila* using a double-stranded RNA interference approach (Kennerdell and Carthew, 1998). Whilst they were interested in looking at the requirement for DFz2 in Wingless signalling, their technique could also be used for perturbing DFz2 in wound healing studies, since no flies mutant for *Dfz2* have yet been identified.

- ***The future's bright, the future's green***

Key to our understanding the cell movements which underlie healing, will be the use of green fluorescent protein (GFP) to facilitate *in vivo* tracking of cell and tissue movements during wound repair in our fly models. My attempts to visualise the actin cable in a wounded disc, using GFP-Moesin, did not work. This could be because the purse-string only recruits moesin to the late stage cable, which might explain why GFP-Moesin labels the actin cable during dorsal closure, but not during wound repair. An attractive

alternative construct for looking at the dynamics of actin cable assembly at the wound margin is the newly reported GFP-actin construct (Verkhusha et al., 1999), which will allow direct tracking of actin in fly embryos. Other GFP constructs that tag nuclei (Davis et al., 1995) will be excellent for following the cell shufflings and movements that occur at the wound margin as the epithelium repairs.

In addition to timelapse studies, GFP will be an invaluable marker of cell populations either expressing, or not expressing, dominant negative forms of morphogenetically important genes. For example, using the yeast based FLP-FRT (reviewed in Theodosiou and Xu, 1998) and GAL4-UAS (reviewed in Phelps and Brand, 1998) systems available in *Drosophila*, it will be possible to generate mutant dominant negative N17-DRac expressing “white” clones of cells in a “green” otherwise wildtype disc. One could then wound discs, under a fluorescent dissecting scope, aiming specifically for “white” clones, and determine whether or not such a gene is required for wound closure. The steps for generating such a clone of cells in a wing disc are illustrated below (Phelps and Brand, 1998):

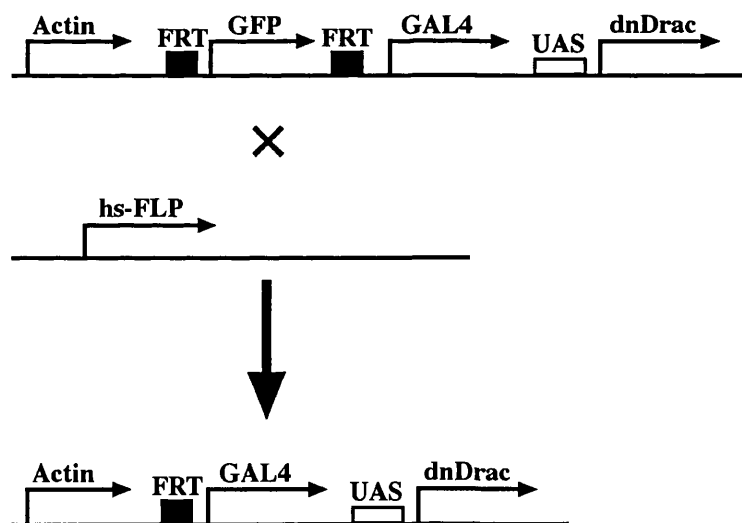


Figure 3.10 An ectopic gene expression strategy in *Drosophila*

GAL4 is a yeast transcription factor which binds to an upstream activating sequence (UAS), to drive transcription of a downstream target gene. FLP is a yeast site-specific recombinase which mediates recombination between target FRT sites. In the case

illustrated (Fig. 3.10) discs would initially express GFP under the control of the ubiquitously expressed actin promoter. However, the *GFP* gene insertion would prevent expression of downstream GAL4, and therefore of dominant negative Drac (*dnDrac*) also. Following heat shock, *FLP* expression would be induced in a small proportion of cells, mediating recombination between the two FRT sites, thus excising the *GFP* gene. GAL4 would then be expressed, and would in turn induce dnDRac expression. Subsequent cell divisions will lead to the generation of a clone of “white” cells which do not express GFP, but do express dnDRac. An incisional wound in one of these regions, followed by TRITC-phalloidin staining, would demonstrate whether DRac function is necessary for actin cable assembly.

Genetic trickery such as that illustrated above is one of the most attractive aspects of developing a *Drosophila* wound healing model. However, problems of wound reproducibility and post-wounding mortality still remain an issue, particularly in embryos. There can be little selective pressure on an embryo to heal a wound, since each adult female lays thousands of eggs during her lifetime. Still, our question is not really “Do *Drosophila* embryos have specialised wound healing machinery?”, but rather “Can a *Drosophila* embryo, or disc, re-use signalling pathways and cytoskeletal rearrangements, used to drive the tissue movements of morphogenesis, to heal a wound?”. This question still remains to be fully answered, but the experiments I have reported in this chapter confirm that, at the cytoskeletal level, it appears that similar actin based contraction underpins both morphogenesis and wound healing. It is now possible to begin testing how far these analogies can be stretched, and I have outlined several ways in which one could investigate these problems, using *Drosophila* as a model.

CHAPTER FOUR

General Discussion

Embryogenesis bears witness to the morphogenetic movements of various tissues resulting in a beautifully sculpted final body plan. Wound healing involves similar cell movements, in response to an artificial, rather than a developmental stimulus. Embryonic wound healing appears strikingly similar, in many ways, to the tissue movements that have earlier played key roles in morphogenesis. Both morphogenesis and repair are dependent on co-ordinated cell shape changes and shufflings which, in turn, are driven by spatially regulated contractions of the actin cytoskeleton. In this thesis I describe my studies into the wound closure process in the mouse and *Drosophila*, drawing attention to the homologies that exist between repair processes and several morphogenetic movements, particularly concentrating on one movement during fly embryogenesis, dorsal closure, which shares close homologies with vertebrate wound repair.

I begin with my embryonic mouse studies, where I have used two very different strategies to uncover what genes are rapidly upregulated at the wound site, and might therefore be key components of the repair machinery. I move onto my *Drosophila* wound healing studies, where I have begun to test whether the signalling cascades, shown genetically to be critical for dorsal closure, are also active during the tissue movements of wound healing.

- *The mouse embryo shows a rapid upregulation of immediate early gene expression following wounding*

In *Chapter 2*, I described three immediate early genes, *c-fos*, *krox-20* and *krox-24*, whose expression is upregulated within minutes of wounding. All three of these genes have previously been shown to be rapidly induced following serum stimulation of fibroblasts, and in tissue culture wound healing models.

This early response is striking in its rapidity and strength of expression (maximal expression within 15-20 minutes). What could the function of such a dramatic transcriptional response to wounding be? Perhaps a straightforward answer is that these are simply serum response genes, which are upregulated in wound site cells following exposure to culture medium. However, embryos cultured in a chemically defined medium, in the absence of any exogenous growth factors, show an equally rapid and dramatic upregulation of c-Fos (Martin and Nobes, 1992).

Wound repair in the E11.5 mouse embryo is both rapid and perfect, with a hindlimb amputation wound healing flawlessly within 24 hours in culture. Whilst small tissue culture wounds are seen to heal in the presence of protein synthesis blockers such as cycloheximide (Nobes and Hall, 1999), it is unlikely that the same would be true for repair of a relatively large wound in the embryo. It is likely that downstream of this rapid wound healing response, is a co-ordinated transcriptional programme, resulting in expression of proteins essential for contraction of the wound bed mesenchyme, and maintenance of the wound margin epithelial advance. Genes such as *c-fos*, *krox-20* and *krox-24* are excellent candidates for “kick-starting” such a response, and their transient expression profiles (all turned off by 1 hour post-wounding) fit nicely into this kind of model. The upregulation of *krox-24* may help explain the lack of a wound healing phenotype in *c-fos* knockout mouse embryos, who heal wounds in a fashion indistinguishable from their wildtype littermates. The perfect healing seen in *c-fos* deficient embryos can be explained by genetic redundancy - a phenomenon whereby lack

of a functional protein is compensated for either by a related family member (any one of the seven of the Fos/Jun family members in the case of *c-fos* knockout mice), or by another unrelated protein, which serves a function equivalent to that of the targetted gene product.

So what are the targets downstream of these signals? The number one wound healing gene throughout the last decade, *TGFβ*, is a prime candidate (Clark, 1996), particularly since in addition to a pair of AP-1 sites (*c-Fos* target sequences) in its upstream regulatory domain, it also possesses two EGR domains (Krox-20/24 target sequence). I have shown that *TGFβ1* mRNA is expressed at the wound site within an hour of injury, and previous studies have described TGFβ1 protein transiently expressed within the wound mesenchyme and marginal epithelium (Martin et al., 1993). This TGFβ may well be the signal which tells the cells of the wound bed mesenchyme to contract, since it is well known that exogenous TGFβ will stimulate cultured fibroblasts to contract a collagen gel (Montesano and Orci, 1988). In adult wounds, many other growth factors are upregulated at the wound site, including Activin, Connective-tissue growth factor (CTGF) and Platelet-derived growth factor (PDGF) (reviewed in Martin, 1997), but the role of these genes has not been studied at the vertebrate embryonic wound site. They may not all be expressed in response to embryo wounding, since platelets and macrophages - major sources of some of these growth factors, are not present at this stage of embryogenesis (Rugh, 1990; Hopkinson-Woolley et al., 1994).

Some of the other downstream targets in the embryo have been highlighted in my subtractive screen for genes upregulated 1 hour after wounding. Of the genes I identified, there are obvious roles for some at the adult wound site, and perhaps these exist in the embryo also. Non-Selenium glutathione peroxidase (NSGP) has been identified as a protective factor at the adult wound site, guarding exposed wound site cells against free radical oxidative damage (Munz et al., 1997). In the cultured mouse embryo, the

environment at the wound site may also be hostile to the exposed cells, especially with the high concentration of oxygen (95%) in the roller culture set-up, so an anti-oxidant enzyme like NSGP may be extremely useful. Another gene, *type IV collagenase*, does not appear so essential, since wounded embryos cultured in the presence of a collagenase blocker appear to heal their wounds perfectly.

This study of mouse embryo “woundy” genes could now be extended to look at different timepoints following wounding, either using similar subtractive technology to that which I have used, or using chip-based microarraying, if the technology becomes more widely available. A chip-based approach would simplify the determination of a detailed timecourse of wound gene expression profiles, compared to using a subtractive protocol. The best way to test gene function in the mouse is to use a transgenic approach, so wounding mouse embryos lacking copies of “woundy” genes, where such transgenic lines exist, will allow identification of genes key to wound healing in the embryo.

- *What cues initiate the wound response?*

Lying upstream of the activation of these genes, there must be other primary cues which act to kick-start the wound healing response. What these are is still largely a mystery, but there are several good candidates. In an adult wound situation, the initiation cues are thought to be growth factor signals released by degranulating platelets, but platelets are not yet present in the circulation in a mid-gestational mouse embryo (Rugh, 1990). The best guess in the embryo is that mechanical cues rather than diffusible chemical signals might be what initiates the wound healing response. The unwounded embryonic epidermis is evidently under tension and it gapes open when cut, leading to mechanical stretching of cells along the axis of the wound margin. This stretching, coupled with membrane ripping (McNeil, 1993), generates a transient leakiness in the cells along the wound margin, as shown by their ability to take up large fluorescently labelled dextrans (Brock et al., 1996). This transient leakiness would then allow an extracellular signal, thought to be calcium ions (Errington and Martin, unpublished data), to flood into wound

edge cells. This signal is thought to travel back, to cells further from the wound margin, via gap junctions - direct channels of communication linking the cytoplasm of adjacent cells, and act directly to trigger upregulation of immediate early genes.

Concomitant with this transcriptional wound response is the even more rapid assembly of an actin cable at the wound margin, and it is quite possible that both events are triggered by the same stretch stimulus. Kolega (1986) showed that pulling on cultured epidermal cells provokes rapid re-alignment of actin along the principal axis of stress, and similar primary cues, mediated by Rho signalling, may activate assembly of the wound purse-string (Brock et al., 1996).

- ***Actin dependent movements are critical to *Drosophila* embryogenesis***

As I have mentioned earlier, embryonic wound healing is not the only tissue movement involving regulated contractions of the actin cytoskeleton. As the culmination of embryogenesis, the *Drosophila* embryo undergoes dorsal closure, whereby the lateral epithelial margins cover over a transient dorsal extraembryonic membrane - the amnioserosa - and zip together at the dorsal midline to form one contiguous epithelial sheet. This movement appears to be partially achieved by shrinkage of the amnioserosa (Rugendorff et al., 1994), in a way analogous to mesenchymal contraction of a wound, and partially by active forward sweeping of epithelium. Not much is known about how amnioserosa cell shape changes are regulated, but the epithelial spreading movement has been very intensively studied in recent years. This movement is preceded by formation of an intracellular actin purse-string which assembles at the apices of cells in the leading edge. Contraction of the cable, just as that of the wound purse-string, is facilitated by myosin motors, with adherens junctions at cell-cell contacts providing the links between cells to form a continuous cable around the leading edge of the lateral epithelium. Contraction of this cable is clearly necessary because in *zipper* mutant flies, which lack these myosin motors, dorsal closure fails (Young et al., 1993).

An earlier actin-dependent morphogenetic movement, bearing homologies with embryonic wound healing, ventral furrow formation, occurs just over 7 hours before dorsal closure begins. We know, from mutant analysis, that *Fog*, *Cta*, *RhoGEF2* and *DRhoA* are all parts of the signalling machinery that coordinate and activate assembly of the contractile actinomyosin machinery which drives this invagination (reviewed in Leptin, 1999). Mutation of *RhoGEF2* results in a much more severe phenotype than seen in *fog* or *cta* mutants, suggesting that there is more than one pathway regulating activation of the small GTPase *DRhoA*. *Rho* plays the pivotal role of coordinating the reorganisation of intracellular actin, to drive formation of this furrow, just as it regulates assembly of the wound induced actin cable (Brock et al., 1996; Barrett et al., 1997; Strutt et al., 1997; Hacker and Perrimon, 1998).

- ***Two genetically tractable movements during C. elegans development are partially driven by actinomyosin contraction***

Epiboly of the *C. elegans* hypodermis involves the spreading of an epithelial sheet which wraps around the embryo until the free edges meet at the ventral midline. This movement of ventral enclosure somewhat resembles an upside-down version of *Drosophila* dorsal closure. Normarsky timelapse studies reveal that the epithelium is first dragged by four leader cells that extend filopodia and crawl downwards pulling the hypodermis from its initial position on the dorsal side, down and over the equator of the embryo. Subsequently, the epithelium is drawn closed by an actinomyosin purse-string (Williams et al., 1997), which again resembles that of vertebrate embryo wound closure. Laser ablation of groups of cells in this purse-stringing margin causes loss of tension and failure of epiboly. Later ablation of pairs of cells along the midline seam after the hole has closed, results in rupture of the embryo at this site and reveals how critical the junctional fusion between leading edge cells is in order to create a strong contiguous epithelium. Worms that are mutant for genes encoding the homologues of α - and β -catenin, and of *cadherin*, fail in ventral enclosure, demonstrating the key role of cell:cell junctions in

purse-string assembly (Costa et al., 1998). Mutant analysis also suggests that ephrin signalling may be pivotal in this process since worms null for *vab-1*, an eph receptor tyrosine kinase, are also disrupted in this movement (George et al., 1998).

Shortly after completion of ventral enclosure, the worm begins to elongate by constriction of actin microfilament bands that are circumferentially aligned (Priess and Hirsch, 1986). This squeezing of the embryo transforms it from relatively spherical in shape into a long thin worm. Embryos mutant in *let-502*, which encodes a protein with high similarity to Rho binding effector kinases, leads to a failure in elongation, and a screen for suppressors of *let-502* reveals a role for *mel-11*, which encodes a homologue of the regulatory subunit of smooth muscle myosin phosphatase (Wissman et al., 1997). These genetic data again suggest a pivotal role for the small GTPase, Rho which probably mediates the signal directing actin alignment prior to worm elongation by modulation of smooth muscle myosin binding of actin.

- *Vertebrates show examples of actin-dependent morphogenesis also*

Of course the invertebrates do not have a monopoly on the use of actin dependent contractions to drive the tissue movements of morphogenesis. One example of the cytoskeleton playing a pivotal role in vertebrate embryogenesis is in gastrulation of the frog, *Xenopus*. At the start of gastrulation, endodermal bottle cells at the site of the blastopore constrict their apices, by contracting apically localised actin filaments, and cause the endoderm to buckle inwards, defining the site for involution to form the dorsal lip of the blastopore (reviewed in Wolpert, 1998). Later in vertebrate development, during neurulation, actin dependent cell shape changes in the ectodermal cells of the neural plate are important for causing the invagination that leads to the formation of the neural tube (Schoenwolf and Smith, 1990).

- *What are the similarities and differences between embryonic wound healing and natural morphogenetic tissue movements?*

From the examples described above it is clear that many parallels can be drawn between wound healing in embryos and natural morphogenetic movements in embryos across several Phyla. A clear link between all of the epithelial movements I have described so far, is that Rho small GTPases appear to act as crucial molecular switches mediating the signals that direct appropriate cells to change their shape. This rule is likely to hold true for more complex movements like vertebrate neurulation also. In 3T3 fibroblasts it has been shown that Rho, Rac and Cdc42, each trigger assembly of unique actin based structures: Rho activation leads to stress fibre assembly, whilst Cdc42 and Rac mediate formation of filopodia and lamellae respectively (Ridley and Hall, 1992 a and b; Nobes and Hall, 1995). At the simplest level, an actin purse-string resembles an oriented bundle of stress fibres and so it is not surprising that Rho mediates wound-induced actin cable assembly. However, dorsal closure in the fly apparently requires all three Rho family members as well as Ras, suggesting that dorsal closure is more complex and may involve more than just purse-string assembly. In fact, whilst the purse-string of dorsal closure in flies and that which closes an embryonic wound appear superficially very similar, there are clear differences in how they operate. Dorsal closure does not involve rearrangement of cell positions in the leading edge – cells get thinner and taller but none appear to leave the front row. By contrast, there is good evidence that the number of cells at a wound circumference gets less as the wound closes (Brock et al., 1996); presumably, contraction of the intracellular purse-string causes a cell to shrink its leading edge until that edge is infinitesimally small, and then the cell slips out of the front row into a row further back.

Morphogenetic movements like gastrulation in flies and epiboly in the worm are very rapid movements that are complete in minutes. There is no time for cell division to play a driving role and it seems that this is probably true for epithelial closure of a wound also. A late-onset surge of cell proliferation, around 6-12 hours post-wounding, is seen in cells

at the perimeter of large wounds in the embryonic mouse (McCluskey and Martin, unpublished data). This may be important in closure of large wounds since it supplies replacement cells for those that were lost at wounding, but clearly cell division cannot be playing a role in pushing the epidermis forward. At the time of gastrulation, all cell division is arrested and none occurs at the leading edge during dorsal closure (Foe et al., 1989). It may be that cell division is actively repressed at the onset of morphogenetic tissue movements and initially at the wound site, which would make good sense since cytokinesis and functional cell shape changes are likely to be somewhat mutually exclusive.

One clear analogy between vertebrate embryo repair and dorsal closure in flies is the use of TGF β /Dpp to emit paracrine signals to adjacent tissues. In the fly it is presumed that the responding tissue is adjacent epithelium and that the signal allows this epithelium to follow the leader cells, but it might be the case that Dpp also directs the amnioserosa to contract in an analogous fashion to the way it is thought TGF β triggers mesenchymal contraction during wound closure. AP-1 activation is upstream of TGF β /Dpp signalling in both wound healing and dorsal closure. Because of genetic redundancy it has not been possible to properly test whether AP-1 activity is vital for wound healing using transgenic mice, but *kayak* flies, mutant in *D-fos*, have a dorsal open phenotype (Zeitlinger et al., 1998), and it does seem likely that AP-1 may be one of the key transducers of any gene inductions associated with these tissue movements.

In light of the expression of *dpp* in a mouse embryo wound and during dorsal closure, it was disappointing when *dpp* did not scream on at the wound site in my imaginal disc wounds. This could mean that the analogies between wound healing and morphogenesis do not extend to Dpp signalling, but it is also clear that, with such tight expression of *dpp* in the disc, it could be that its upregulation is prevented by a mutually exclusive signal. However, when one looks back to my mouse embryo wound *in situs* for *dpp* expression, there is a huge difference between mRNA levels and protein levels (Martin et al., 1993).

Therefore an important next step will be to look for Dpp protein in disc wounds, to see whether localisation of the protein is as tightly regulated as that of the message.

The future directions for this fly wound healing project are going to be fascinating. Of the dorsal closure pathway genes, *d-fos* is a key potential “kick-start” signal to be studied, especially since *kayak* mutants lacking *d-fos* show a dorsal hole phenotype, indicating that there is no genetic redundancy in the pathway. It will also be interesting to see whether Puckered, the phosphatase which appears to act as the brake on dorsal closure (Martin-Blanco et al., 1998), acts as a control mechanism during wound healing too. In addition to these genes, it will be interesting to look for activation of the components of the JNK signalling cascade following wounding, and with mutants for each step available it should be possible to really determine how close the homologies between wound repair and morphogenesis actually come.

In addition to the dorsal closure story, there are gastrulation signals which are tempting to investigate. Of these, *folded gastrulation* and *RhoGEF2* are particularly exciting - *fog*, because it appears to be expressed wherever invaginations occur which require cells to undergo actinomyosin-based apical constrictions, like those seen during wound closure; and *RhoGEF2* because flies mutant for this vital Rho activation factor show total failure in these same constriction movements, where *fog* mutants show a weaker phenotype.

Following my exciting *in situ* findings that *Dfz2* is upregulated at the margin of a wound in the larval epidermis, it may well be worth expanding the fly wounding studies to look at the previously unstudied healing of the larval epidermis. It appears, from my preliminary data, that the epidermis may lack the tight regulation of gene expression that exists in the wing disc, and may therefore be a more fruitful area to study wound healing. It would still be possible to generate labelled mosaic patches of epidermis expressing mutant forms of target genes in the same way as I described for the wing disc, and third stage larvae would certainly survive wounding long enough to look at gene expression, and would most likely heal a small tungsten needle wound. With my mouse screen also

pulling out *frizzled*, maybe signalling via this receptor is playing an important role during healing. With *Dfz2* being expressed during gastrulation in the precise region that forms the ventral furrow (Bhanot et al., 1996), it forges another link between morphogenesis and tissue repair.

- *Rho small GTPase may be responsible for regulating assembly of the fly wound actin cables*

My wound healing studies in the fly have shown that, as with the vertebrate embryo, a contractile actin cable is assembled within minutes of healing. In contrast to the genetic complexity of the mouse embryo, working with *Drosophila* offers a fantastic chance to genetically dissect out mechanisms controlling wound repair. My disc wound healing model, in particular, is an excellent one in which to experimentally perturb the activity of the Rho family of small GTPases. There are several compounds available that allow one to tinker with the activity of the Rho family of small GTPases: C3-transferase specifically ADP-ribosylates Rho, effectively inactivating it; Toxin B glucosylates Rho, Rac and Cdc42, and renders them inactive; and CNF1 activates all three of them (reviewed in Hall, 1998). Downstream of Rho activation lies the Rho kinase, p160ROCK, and this too can now be blocked specifically using a small molecule drug (Uehata et al., 1998). Any of these compounds could be added to disc culture medium to try to block, or induce, small GTPase activity in disc wounds, or alternatively a scrape-loading approach could be adopted as with chick embryo wounding (Brock et al., 1996). With the Rho kinase blocking drug, it would be possible to inject compound into the yolk of an early embryo and, in addition to looking at effects on wound healing, see any subsequent morphogenetic defects.

- *Can morphogenesis teach us anything about stop signals?*

Compared to the signals initiating repair, the primary cues that stop tissue movements are more difficult to analyse. When two wound edges confront one another, forward movement halts because of a well described, but poorly understood process known as contact inhibition (Abercrombie, 1979). In the embryo, there is a slight overrun of the wound epidermis before the actin purse-string disassembles, presumably as a response to the patterns of stresses in the epithelium reverting to those of unwounded skin (Martin and Lewis, 1992; Brock et al., 1996). To understand the cues that halt wound closure, it may again be productive to extrapolate from morphogenetic events in fly and worm. It seems clear that the phosphatase Puckered is acting as some sort of brake during fly dorsal closure, and similar feedback mechanisms acting on kinase cascades must be operating during wound closure also. There is some evidence that eph receptor/ligand interactions play a role during ventral enclosure in the worm and clearly these cell:cell inhibitory signals might be the ideal means for announcing that contact has been made and that a particular movement should stop. Just as for the start signals, it seems certain that these stopping signals will be transduced through small GTPase regulation of the cytoskeleton, this time by switching off, rather than switching on, of the cell motility machinery.

In summary, my studies investigating the molecular events of wound repair in the mouse embryo have highlighted several new “woundy” genes, and future experiments on knockout mouse embryos will hopefully confirm the importance of at least two of these, Krox-20 and Krox-24, as potential kick start signals for embryonic wound repair. In my fly studies, I have shown that the tissue movements of morphogenesis and wound repair are remarkably similar. Dorsal closure, in particular, not only looks grossly like wound healing, but seems to be driven by the same cytoskeletal machinery also. I have demonstrated that *Drosophila* embryos and imaginal discs use exactly the same cytoskeletal machinery to close a wound as vertebrate embryos, assembling a contractile actin cable within minutes of wounding, suggesting that mechanisms of embryonic wound repair are remarkably well conserved. I have also shown that it is possible to investigate gene expression at the wound site in imaginal discs, and serendipitously struck upon another wound healing model in the larval epidermis. The characterisation of the *Drosophila* wound models described in this thesis will allow further genetic dissection of the molecular events governing wound repair, in a genetically tractable model. These future studies will allow us to determine whether the cellular and molecular tools facilitating tissue repair are the same ones that drive natural morphogenetic tissue movements in the embryo.

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APPENDIX I

Drosophila morphogenetic mutants

The following list summarises the main *Drosophila* mutants which show a defective dorsal closure phenotype:

Gene	Encodes	Reference
<i>anterior open protein</i>	Transcriptional repressor	Riesgo-Escovar and Hafen (1997)
<i>armadillo</i>	β -catenin	Cox et al. (1996)
<i>basket</i>	DJNK	Riesgo-Escovar et al. (1996)
<i>coracle</i>	Band 4.1 protein	Fehon et al. (1994)
<i>d-jun</i>	Transcription factor	Hou et al. (1997)
<i>d-pkn</i>	Protein kinase	Lu and Settleman (1999)
<i>drac / dc42 / drhoA / dras</i>	Small GTPases	reviewed in Harden et al. (1999)
<i>hemipterous</i>	DJNKK	Glise et al. (1995)
<i>kayak / d-fos</i>	Transcription factor	Zeitlinger et al. (1998)
<i>l-myospheroid</i>	β -integrin	Leptin et al. (1989)
<i>puckered</i>	DJNK phosphatase	Ring and Martinez-Arias (1993)
<i>schnurri</i>	Transcription factor	Arora et al. (1995)
<i>shotgun</i>	E-cadherin	Tepass et al. (1996)
<i>thick veins</i>	Dpp receptor	Affolter et al. (1994)
<i>zipper</i>	Non-muscle myosin II	Young et al. (1993)

APPENDIX II

Potential Wound Induced Genes (WIGs)

The following list summarises all of the potential WIGs obtained from my subtractive hybridisation screen. Cloned sequences which are not detailed below are stored on files at Pfizer Central Research Bioinformatics department, Sandwich, U.K.

calmodulin

chaperonin-containing TCP-1 epsilon subunit

type IV collagenase

cyclophilin D

fibroblast growth factor receptor K-sam

fibroglycan (syndecan-2)

frizzled

alpha-4 integrin

Non-selenium glutathione peroxidase

phospholipase A2 activating protein

radical fringe

supervillin

APPENDIX III

Table of transgenic mouse wound healing studies

Transgenic	Wound healing phenotype	Reference
<i>BMP-6</i> skin overexpressor	BMP-6 is upregulated after skin injury in keratinocytes at the wound edge and in the newly formed epithelium as well as in fibroblasts in the wound bed. In mice overexpressing BMP-6 in the epidermis, reepithelialization of skin wounds is significantly delayed, suggesting that strict spatial and temporal regulation of BMP-6 expression is necessary not only for formation but also for reestablishment of a fully differentiated epidermis.	Kaiser, S. et al. (1998)
<i>BPAG1</i> knockout	BPAG1 is a key protein of the hemidesmosomal junction, probably supplying the link between $\alpha6\beta4$ integrins and the keratin cytoskeleton. Wounded <i>-/-</i> mice show severely delayed re-epithelialisation, but wounds do eventually heal.	Guo, L. et al. (1995)
<i>c-fos</i> knockout	c-Fos is member of the AP-1 family of transcription factors and is upregulated rapidly in both the epithelium and mesenchyme at the wound site in vertebrate embryos (Martin & Nobes 1992). Despite this large upregulation, embryos lacking c-Fos heal wounds identically to their wild type littermates.	McCluskey, J. (DPhil. Thesis Univ. Oxford, 1995)

<i>FGF-2</i> knockout	<p>FGF-2 $-/-$ mice are viable, fertile and phenotypically indistinguishable from wild type littermates. However, the healing of excisional skin wounds is delayed in mice lacking FGF-2, suggesting a specific role in skin wound healing in mice, which, despite the apparent redundancy of FGF signaling, cannot be carried out by other FGF family members.</p>	Ortega, S. et al. (1998)
<i>Gelsolin</i> knockout	<p>Gelsolin is an important actin severing protein and is thus implicated in the regulation of actin based cell motility. In $-/-$ mice, leukocyte migration is impaired and primary $-/-$ fibroblasts heal a tissue culture wound more slowly than wild type fibroblasts. In vivo wounding studies show significantly impaired healing.</p>	Witke, W. et al. (1995)
<i>$\alpha 3 \beta 1$ integrin</i> knockout	<p>$\alpha 3 \beta 1$ integrin $-/-$ neonatal mice develop micro-blisters at the epidermal-dermal junction. These micro-blisters are associated with poor basement membrane organization. This study provides evidence for a role of $\alpha 3 \beta 1$ in regulating stress fiber formation and as a trans-dominant inhibitor of the functions of the other integrins in mouse keratinocytes. These results have potential implications for the regulation of keratinocyte adhesion and migration during wound healing.</p>	Hodivala-Dilke, K.M. et al. (1998)

Transgenic	Wound healing phenotype	Reference
<i>IP-10</i> skin overexpressor	IP-10 is a chemotactic cytokine which acts to recruit leukocytes and which can be detected at high levels in several chronic inflammatory conditions, including psoriasis. The transgenic mice reported overexpress IP-10 in keratinocytes and show an impaired wound healing response, when compared to wild types, with a more intense inflammatory phase and a prolonged and disorganized granulation phase with impaired blood vessel formation.	Luster, A.D. et al. (1998)
<i>Keratin 8</i> knockout	Keratin filaments are abundant in wild type embryonic epidermis but missing from the epidermis of -/- embryos. Nonetheless, re-epithelialisation is just as rapid and perfect as for wild type individuals.	Brock, J. et al. (1996)
<i>KGF</i> knockout	KGF (FGF-7) is significantly upregulated at the wound site but the knockout mice have no wound healing impairment. The reason may be genetic redundancy, because aFGF, which also signals via the KGF receptor, is upregulated at the wound site in both wild type and -/- mice. This study was published before the identification of KGF-2 (FGF-10), which is also upregulated at the wound site and signals via the KGF receptor.	Guo, L. et al. (1996)
Dominant negative KGF receptor	Mice with a dominant-negative KGF receptor targeted to basal epidermal cells have severely impaired healing. These results support the redundancy hypothesis explaining the lack of wound healing phenotype in KGF -/- mice.	Werner, S. et al. (1994)

Transgenic	Wound healing phenotype	Reference
<i>lef-1</i> skin overexpressor	Lymphoid-enhancer factor (<i>lef-1</i>) is a transcription factor involved in inductive epithelial-mesenchymal interactions. Ectopic <i>lef-1</i> expression induces Keratin 17 protein in the skin of adult transgenic mice. K17 is also induced, along with K6 and K16, early after acute injury to adult wild type skin. The pattern of K17 gene expression during development has direct implications for the morphogenesis of skin epithelia, and suggests a molecular relationship between development and wound repair.	McGowan, K.M. et al. (1998)
<i>MMP-1</i> skin overexpressor	Transgenic mice exhibited a 2-3 d delay in the time required to reach 50% closure of 6 mm wounds. Histologic analysis of the transgenic wound bed revealed the retarded migration of the epithelium across the open wound. The results are consistent with the hypothesis that control of MMP-1 expression is important for reepithelialization during wound healing and indicate that collagenase regulation is critical to the kinetics of normal repair.	Di Colandrea, T. et al. (1998)
<i>MMP-9/lacZ</i> reporter	Lac-Z is cloned downstream of a minimal region of MMP-9 promoter sequence that drives appropriate developmental and injury-induced reporter gene expression in transgenic mice. This study also shows that the expression and activity of three transcription factors (NF-kappaB, AP-2, and Sp1) that control the activity of the MMP-9 promoter are selectively induced in the epithelium migrating to heal a wound.	Mohan, R. et al. (1998)

Transgenic	Wound healing phenotype	Reference
<i>iNOS</i> knockout	Experiments were carried out to determine the requirement for iNOS in closing excisional wounds. Wound closure was delayed in iNOS $-/-$ mice compared with wild-type animals. An identical delay in wound closure was observed in wild-type mice given a continuous infusion of a partially selective iNOS inhibitor. Delayed wound healing in iNOS-deficient mice was completely reversed by a single application of an adenoviral vector containing human iNOS cDNA at the time of wounding. These results suggest a key role of iNOS in wound closure, and advocate a gene therapy strategy to improve wound healing in iNOS-deficient states such as diabetes or during steroid treatment.	Yamasaki, K. et al. (1998)
<i>Osteopontin</i> knockout	Knockouts are viable but, following incisional wounding, show a significantly decreased level of debridement, greater disorganization of matrix, and an alteration of collagen fibrillogenesis leading to small diameter collagen fibrils in the OPN mutant mice. These data indicate a role for OPN in tissue remodeling in vivo, and suggest physiological functions during matrix reorganization after injury.	Liaw, L. et al. (1998)
<i>Plasminogen</i> knockout	Without their main fibrinolytic enzyme, epidermal fronts remain blunt edged and fail to bore a pathway through the fibrin matrix. Even after 45 days some $-/-$ mice had failed to re-epithelialise a wound.	Romer, J. et al. (1996)

Transgenic	Wound healing phenotype	Reference
<i>P-Selectin</i> and <i>E-Selectin</i> knockouts	As independent knockout mice both E and P-Selectin nulls show no significant impairment in tissue repair capacity. However, double KOs show delayed neutrophil extravasation, reduced macrophage recruitment to wound sites and significantly impaired rates of re-epithelialisation.	Subramaniam, M. et al. (1997)
<i>Skn-1a</i> knockout	<i>Skn-1a</i> is a POU domain transcription factor expressed selectively in the epidermis. Wound re-epithelialisation is significantly faster in <i>Skn-1a</i> null mice and in situ reveal K14 and Spr-1 expressed at higher levels in the leading edge than in wounded wild type litter mates	Andersen, B. et al. (1997)
<i>Tenascin C</i> knockout	Tenascin C immunostaining is generally seen beneath the leading epidermal edge of a healing wound and is thought to play a role in epidermal migration (Mackie et al., 1988). Knockout mice have reduced fibronectin expression at the wound site but healing capacity is unaffected.	Forsberg, E. et al. (1996)
<i>TGFα</i>	Wound healing in double KGF and <i>TGFα</i> $-/-$ mice is unimpaired suggesting that <i>TGFα</i> is not essential for triggering re-epithelialisation. Genetic redundancy is the likely explanation with EGF the likely substitute growth factor for the missing <i>TGFα</i> .	Guo, L. et al (1996).
<i>TGFβ</i>	Knockout phenotype depends on background strain, with impairment in mesenchymal contraction in excisional embryonic wounds.	McCluskey, J. (personal comm.)

Transgenic	Wound healing phenotype	Reference
<i>uPA</i> receptor knockout	In a vascular wound model, u-PAR deficiency did not affect reendothelialization. In addition, <i>in vitro</i> studies showed no impairment of migration of wounded cultured -/- smooth muscle cells. These results suggest that binding of uPA to uPAR is not required to provide sufficient uPA-mediated plasmin proteolysis to allow cellular migration into a vascular wound.	Carmeliet, P. et al. (1998)

APPENDIX IV

Solutions

Mouse embryo explant saline

	300ml	500ml	1litre	2 litre
	(g)	(g)	(g)	(g)
NaCl	2.07	3.45	6.9	13.8
KCl	0.09	0.15	0.3	0.6
MgSO ₄ .7H ₂ O	0.03	0.05	0.1	0.2
MgCl ₂ .6H ₂ O	0.015	0.025	0.05	0.1
NaHPO ₄ .2H ₂ O	0.03	0.05	0.1	0.2
CaCl ₂ .2H ₂ O	0.078	0.13	0.26	0.53
Glucose	0.45	0.75	1.5	3.5
NaHCO ₃	0.15	0.25	0.5	1.0

Mouse embryo culture saline

	300ml	500ml	1litre	2 litre
	(g)	(g)	(g)	(g)
NaCl	2.07	3.45	6.9	13.8
KCl	0.09	0.15	0.3	0.6
MgSO ₄ .7H ₂ O	0.03	0.05	0.1	0.2
MgCl ₂ .6H ₂ O	0.015	0.025	0.05	0.1
NaHPO ₄ .2H ₂ O	0.03	0.05	0.1	0.2
CaCl ₂ .2H ₂ O	0.078	0.13	0.26	0.53
Glucose	0.45	0.75	1.5	3.5
NaHCO ₃	0.6	1.0	2.0	4.0

Half strength Karnovsky's fixative (for SEM/TEM)

1g Paraformaldehyde

25ml dH₂O

Stir on hotplate until almost dissolved, then add

50µl 1M KOH

Cool to 4°C, then add

18ml 0.2M Sodium cacodylate pH7.4

5ml 25% Glutaraldehyde

Hybridisation solution (for mouse *in situ* hybridisation)

50% Formamide

5x SSC

1% SDS

50µg/ml heparin

50µg/ml yeast tRNA

Solution 1 (for mouse *in situ* hybridisation)

50% Formamide

5x SSC

1% SDS

Solution 3 (for mouse *in situ* hybridisation)

50% Formamide

2x SSC

10x TBST (for mouse *in situ* hybridisation)

8g NaCl

0.2g KCl

25ml 1M Tris-HCl pH 7.5

10ml Tween-20

make up to 100ml with dH₂O

Dilute to 1x and add levamisole to 2mM on day of use.

Embryo powder (for mouse *in situ* hybridisation)

E11.5 mouse embryos were harvested and homogenized in a minimal volume of ice-cold PBS. One part of the resulting homogenate was mixed thoroughly with three parts ice-cold acetone and left on ice for 30 minutes. The resulting suspension was aliquotted into 1.5ml eppendorf tubes and these were centrifuged at 13000rpm for 10 minutes. Pellets were washed once with ice-cold acetone, spun again for 5 minutes at 13000rpm, then removed with forceps, into a watch glass, and allowed to air dry overnight at room temperature. The following day the pellets were ground to a powder using a pestle and mortar, and the powder stored in an eppendorf tube at -20°C.

NTMT (for mouse and *Drosophila* *in situ* hybridisation)

100mM NaCl

100mM Tris-HCl pH 9.5

50mM MgCl₂

0.1% Tween-20

DNA extraction buffer (for PCR-genotyping)

10mM Tris-HCl pH 7.9

1mM EDTA

0.5% SDS

X-Gal staining fixative

1% Formaldehyde
0.2% Glutaraldehyde
1x PBS
0.02% NP40

Can store at -20°C

X-Gal staining solution

1x PBS
5mM $\text{K}_3\text{Fe}(\text{CN})_6$
5mM $\text{K}_4\text{Fe}(\text{CN})_6$
2mM MgCl_2

Can store above at -20°C

then add 1mg/ml X-Gal when defrosted

Hybridisation solution (for *Drosophila in situ* hybridisation)

50% Formamide
5x SSC
1mg/ml heparin
0.1% Tween-20
1µg/ml Salmon sperm DNA (Sigma D9156)

Boil salmon sperm DNA for 10 minutes, then cool for 5 minutes in an ethanol/ice bath before adding to the hybridisation solution. Make up to final volume with dH₂O.

The final mix can be stored at -20°C.

Hyb B solution (for *Drosophila in situ* hybridisation)

50% Formamide
5x SSC

Make up to final volume with dH₂O.