Macroscopic forces generated by cell-matrix interactions

I. Cells generate forces after becoming attached to a matrix.

II. How do cells attach to a matrix?

III. Cell-matrix interactions control the spontaneous closure of wounds in organs.

IV. What happens when wound closure occurs by induced regeneration?
Outline

A. Introduction: Synthesis of organs, in vitro or in vivo?

B. Irreversible organ injury.

C. Antagonistic relation between contraction and regeneration.

D. Synchronous and isomorphous replacement.

E. Two theories. 1. Immuno competence theory. 2. Contraction blockade + synchronized isomorphous replacement.
A. Introduction: Synthesis of organs, in vitro or in vivo?
Skin: In vitro or in vivo synthesis?

IN VITRO SYNTHESIS

IN VIVO SYNTHESIS

From Yannas, 2001
Peripheral nerves: In vitro or in vivo synthesis?

Diagram removed for copyright reasons.
See Figure 7.1 in [Yannas 2001]: Yannas, I. V. *Tissue and Organ Regeneration in Adults*. New York: Springer, 2001.
In vitro or in vivo?

Two published protocols, A and B, for synthesis of skin

A. First step is *in vitro*: Keratinocytes + Fibroblasts + Collagen gel → Implant

   Second step is *in vivo*: Implant → Skin

B. Directly *in vivo*: Keratinocytes + Dermis regeneration template → Skin

Direct *in vivo* synthesis is simpler:

- Investigator focuses on one reactor only.
- Uses the endogenous cytokine field* and endogenous FB. No need to add growth factors, including angiogenesis factors.

*Cytokine field: The unknown time- and space-dependent concentrations of growth factors and other cytokines in injured site.
B. Irreversible organ injury.
Why study the healing process?

1. In vitro or in vivo method → implant

2. Implant → injured anatomical site undergoing healing

3. Implant + healing → organ synthesis
Two adult healing modes

**Spontaneous healing in adults**
injury $\rightarrow$ contraction + scar formation

**Healing by regeneration in adults**
injury $\rightarrow$ implant an active cell-seeded scaffold $\rightarrow$ MECHANISM? $\rightarrow$ organ synthesis
Reversible injury in an amphibian

Spontaneous regeneration of amputated limb in the newt occurs independently of severity of injury

Goss, 1992
Irreversible injury in adult mammal

Burn victim suffering from severe contraction and scar formation

Tomasek et al., 2000
C. Antagonistic relation between contraction and regeneration.

• Methodology: defect closure rule.
• Four sets of data showing changes in importance of healing modes (C, S, R) with:
  I. Development.
  II. Severity of organ injury.
  III. Scaffold-induced regeneration in adults.
  IV. Impairment of healing.
Quantitative description of healing processes: The defect closure rule. 

- The initial state is the freshly injured wound. Wound area is $A_0$.
- The final state is the closed wound. $A_0$ eventually has closed up by three processes: contraction, scar formation, regeneration. No other processes involved in wound closure.
- Closure of wound by contributions from contraction ($%C$), scar formation ($%S$) or regeneration ($%R$).

Defect closure rule:

$$C + S + R = 100$$
Measurement of C, S and R in full-thickness skin wounds after wound has closed. Use only “final state” data!

Graph removed for copyright reasons.
See Figure 4.3 in [Yannas 2001].
Burn patient has closed severe skin wounds in neck partly by contraction and partly by scar.

Final state of healing of full-thickness skin wound in the human.
Final state of healing of full-thickness skin wound in the guinea pig.
### Representative data illustrating the defect closure rule

<table>
<thead>
<tr>
<th>Spontaneously healing defect</th>
<th>Configuration of final state</th>
</tr>
</thead>
<tbody>
<tr>
<td>general case</td>
<td>[C, S, R]</td>
</tr>
<tr>
<td>Ideal fetal healing</td>
<td>[0, 0, 100]</td>
</tr>
<tr>
<td>Dermis-free skin/ adult rodents</td>
<td>[96, 4, 0]</td>
</tr>
<tr>
<td>Dermis-free skin/ adult human</td>
<td>[37, 63, 0]</td>
</tr>
<tr>
<td>Peripheral nerve/ adult rat</td>
<td>[96, 4, 0]</td>
</tr>
<tr>
<td>Conjunctiva/ adult rabbit</td>
<td>[45, 55, 0]</td>
</tr>
</tbody>
</table>

Data reviewed in Yannas, 2001
Fact I: Change in healing modes (C, S, R) with development

- During the fetal-to-adult transition in mammals, contraction gradually replaces regeneration as the major mode of wound closure (Lorenz et al., 1992; Mast et al., 1992; Stocum, 1995; McCallion and Ferguson, 1996; Martin, 1997).

- During amphibian development, contraction becomes dominant and scar appears as regeneration recedes (Stocum, 1995; Tsonis, 1996; Yannas et al., 1996).
Tadpole development $\rightarrow$ Frog

Developmental changes in configuration of final state $[C, S, R]$:

Development $\longrightarrow$

$[41, 0, 59] \rightarrow [62, 0, 38] \rightarrow [66, 0, 34] \rightarrow [90, 10, 0]$

tadpole $\rightarrow$ frog

Yannas et al., 1996
Graph removed for copyright reasons. Percent wound closure across developmental stages. See Figure 8.3 in [Yannas 2001].
Fact II: Changes in healing modes (C, S, R) with severity of organ injury

- As injury progresses in severity in an organ from epithelial tissue to basement membrane to stroma, contraction replaces regeneration as the major mode of wound closure.
The tissue triad in skin and nerves

epithelial tissue: 100% cellular, no ECM
basement membrane: 100% ECM, no cells
stroma: cells, ECM, blood vessels

Figures by MIT OCW. After Figure 2.7 in [Yannas 2001].
The epidermis is a regenerative tissue. After excision, it regenerates spontaneously. Reversible injury. No contraction. No scar.

Figure by MIT OCW. After Figure 2.1 in [Yannas 2001].
The dermis is a nonregenerative tissue in the adult. After excision, it does not regenerate spontaneously. Irreversible injury. Closes with contraction and scar formation.

Figure by MIT OCW. After Figure 2.2 in [Yannas 2001].
Peripheral nerve: reversible injury

The myelin sheath is a regenerative tissue. Following nerve crushing with myelin disruption, the myelin regenerates spontaneously. Reversible injury. No contraction. No scar.

Figure by MIT OCW. After Figure 2.3 in [Yannas 2001].
Peripheral nerve: irreversible injury

The endoneurial stroma is a nonregenerative tissue. Following transection, it forms neural scar (neuroma). Irreversible injury. Closes with contraction and scar formation.

Figure by MIT OCW. After Figure 2.4 in [Yannas 2001].
### Summary:

**Increased severity of injury**

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SKIN</strong></td>
<td></td>
</tr>
<tr>
<td>epidermis</td>
<td>dermis (stroma)</td>
</tr>
<tr>
<td>BM</td>
<td></td>
</tr>
<tr>
<td><strong>NERVE</strong></td>
<td></td>
</tr>
<tr>
<td>myelin</td>
<td>endoneurial stroma</td>
</tr>
<tr>
<td>BM</td>
<td></td>
</tr>
<tr>
<td>BM</td>
<td></td>
</tr>
</tbody>
</table>
Fact III: Scaffold-induced regeneration in adults

a. Regeneration is induced when a scaffold blocks contraction. Three organs: Skin, conjunctiva, peripheral nerve.

b. Scar is abolished when contraction is blocked by a scaffold, even modestly.

Comment: At least in rodents, scar formation appears to be a process secondary to contraction.
<table>
<thead>
<tr>
<th>Organ/species</th>
<th>Treatment used</th>
<th>Spontaneous healing</th>
<th>Treated with template</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin/guinea pig</td>
<td>scaffold DRT</td>
<td>[91, 9, 0]</td>
<td>[89, 0, 11]</td>
</tr>
<tr>
<td>Skin/guinea pig</td>
<td>scaffold DRT+KC</td>
<td>[92, 8, 0]</td>
<td>[28, 0, 72]</td>
</tr>
<tr>
<td>Conjunctiva/rabbit</td>
<td>scaffold DRT</td>
<td>[45, 55, 0]</td>
<td>[13, 0, 87]</td>
</tr>
<tr>
<td>Nerve/rat</td>
<td>silicone tube+scaffold NRT</td>
<td>[95, 5, 0]</td>
<td>[53, 0, 47]</td>
</tr>
<tr>
<td>Nerve/rat</td>
<td>collagen tube+scaffold NRT</td>
<td>[95, 5, 0]</td>
<td>[0, 0, 100]</td>
</tr>
</tbody>
</table>

Data reviewed in Yannas, 2001
Standard injured skin model
(excise full-thickness skin, including entire dermis, then graft with scaffold)

The initial state of skin wound healing studies

Yannas, 2001
Kinetics of closure of skin defect area using three protocols

KC = keratinocytes
DRT = dermis regeneration template (active scaffold)

Graph removed for copyright reasons. See Figure 10.2 in [Yannas 2001].
Myofibroblast detected with antibody to $\alpha$-SM actin

Diagram removed for copyright reasons.

Tomasek et al., 2000
Contraction blocked by scaffold (bottom)

Ungrafted. Contracting vigorously.

Red-brown: stained with antibody to α-SM actin. 10 d

Grafted with DRT. No contraction.

Troxel, MIT Thesis, 1994
Partly regenerated skin

KC + DRT

KC + inactive scaffold

Full-thickness skin wound (guinea pig) grafted with keratinocytes (KC) and either dermis regeneration template (DRT) or inactive scaffold

Orgill, MIT Thesis, 1983
KINETICS OF SKIN SYNTHESIS

E, neoepidermis
D, neodermis

Photos removed for copyright reasons.

Scaffold has partly degraded by 14 d

Butler et al., 1998
Normal skin

rete ridges with capillary loops and vascular plexus underneath

Diagram removed for copyright reasons. See Figure 5.2 (top left) in [Yannas 2001].

Burkitt et al., 1992
Regenerated skin (swine).
Immunostaining with Factor VIII for capillary loops.
No angiogenesis factors were added by investigators.

Staining for capillaries forming in loops as part of the rete ridge structure
This is our regenerated tissue, closely approximating the normal
All this tissue was ECM seeded with 50,000 keratinocytes per cm², porcine model (next three slides are histology following grafting of a 4x4 cm square wound cut down to the fat layer
Day 35:
Vascular remodeling is evident on stains for endothelium that reveal a well organized arcade of hairpin-loop capillaries interdigitating with nascent epidermal rete ridges in a normal pattern

Compton et al., 2000
Severely burned patient lost all skin on right side of face. 
Grafted with DRT scaffold and later covered with autoepidermal graft.
Injured conjunctiva model
(excise full-thickness conjunctiva including entire stroma, then graft with scaffold)

After Hsu et al., 2000
DRT graft blocked contraction of conjunctival wound

Figure by MIT OCW.

After Hsu et al., 2000
Ungrafted. Contracting vigorously.

Grafted with DRT. Blocked contraction.

Red-brown: stained with antibody to $\alpha$-SM actin. 14 d

Arrows: newly formed blood vessels

Photo removed for copyright reasons.

Hsu et al., 2000
Hsu et al., 2000

Fully excised conjunctiva (rabbit) grafted with DRT or ungrafted (14 d)
Regeneration of conjunctival stroma
(use microscope polarizing stage to study orientation of collagen fibers)

ungrafted
(conjunctival scar)

grafted with DRT scaffold

normal conjunctiva

Summary: alignment as indicated by birefringence when viewed under polarized light
These images are histological sections viewed under polarized light. Upper left, ungrafted wound.

Hsu et al., 2000
Standard injured peripheral nerve model
(transect nerve, then insert stumps inside scaffold tube)

Cooper & Schiller, 1975

Diagram removed for copyright reasons.

transected nerve stumps inside scaffold tube

The initial state of nerve wound healing studies
Inactive tube. Thin regenerated nerve.

Red-brown: stained with antibody to $\alpha$-SM actin.

No tube. Neuroma.
Inactive scaffold tube

60 weeks

15-20 myofibroblast layers

Poor quality of nerve regeneration.

Red-brown: stained with antibody to α-SM actin. 60 weeks

Nerve regeneration template (NRT) tube

60 weeks

1 myofibroblast layer

Superior nerve quality.

Chamberlain et al., 2000
Rat sciatic nerve transected. Stumps inserted in NRT tube or inactive tube.

Harley et al., 2003
Fact IV. Impaired healing of skin wounds

Dermis-free wounds in:
- genetically diabetic mouse
- genetically obese mouse
- infected wounds
- mechanically splinted
- treated with steroids

all impaired-healing wounds showed strong delay in contraction but did not show regeneration

Summary of Facts I-IV.

I. During amphibian larval (tadpole) development; also, during the fetal-to-adult transition in mammals:
   \[ C \uparrow \quad R \downarrow \]

II. As the severity of injury in an organ is increased from epithelia alone to epithelia with stroma:
   \[ C \uparrow \quad R \downarrow \]

III. Certain scaffolds block contraction and induce partial regeneration in adult mammals (rodents, swine, human).
    \[ C \downarrow \quad R \uparrow \]
    Also scar is abolished when contraction is blocked, even partly.
    \[ C \downarrow \quad S = 0 \]

IV. Impaired healing blocks contraction but does not induce regeneration.
    \[ C = 0 \quad R = 0 \]
Contraction blockade theory explains the facts symbols refer to \([C, S, R]\)

- Inhibition of contraction is necessary but does not suffice to induce organ regeneration in adults

\[\Delta R > 0 \text{ and } S \rightarrow 0 \text{ if } \Delta C < 0\]
How does an active scaffold block contraction? Identify structural determinants of scaffold activity.
Mechanism of contraction inhibition by DRT scaffold in skin wound

1. Fact: Reduction in number of myofibroblasts. Scaffold does not aggregate platelets. Collagen banding, but not triple helix, deliberately abolished in scaffold. Hypothesis: Early release of TGF-β is downregulated.

2. Fact: Disruption of myofibroblast organization. Myofibroblasts bind on scaffold extensively, chiefly via the $\alpha_1\beta_1$ integrin. Hypothesis: myofibroblast contractile axes disoriented, cells separated; cell population loses its cohesion and force loses its uniaxial character.
Critical structural features of biologically ECM analogs used as scaffolds

1. chemical composition (ligand identity)
2. pore structure (ligand density)
4. macromolecular structure (scaffold duration)

3. orientation of pore channels (ligand spatial coordinates)

Diagrams removed for copyright reasons.
The graphic shows many scaffolds but dermis regeneration template (DRT) is the active scaffold (template). Ligand density is optimal between 20 and 120 µm,

Figure by MIT OCW. After Yannas, 1989.

Yannas et al., 1989
Pore size vs bound cell density

- The volume density $\rho_c$ of cells is equal to the product of the specific surface of template, $\sigma$, and the surface density of bound cells, $\Phi_c$:
  \[ \rho_c = \sigma \Phi_c \]
- We will compare $\rho_c$ for two types of scaffold, differing only in average pore size.
- Observations of myofibroblast density inside a template with average pore diameter of about 10 $\mu$m have yielded typical values of the volume density, $\rho_c$, of order $10^7$ myofibroblasts per cm$^3$ porous template. For this template the specific surface $\sigma$ is calculated, using a standard model for a porous solid with pore volume fraction of about 0.95, to be approximately $8 \times 10^4$ mm$^2$/cm$^3$. The cell surface density is accordingly $\Phi_c = \rho_c / \sigma = 10^7 / 8 \times 10^4 = 125$ cells/mm$^2$. 
• With a template of identical composition but average pore diameter as large as 300 µm, \( \Phi_c \) is the same as above since the two solids have identical types of ligands and ligand density; however, the specific surface is now calculated to be about \( 3 \times 10^3 \text{ mm}^2/\text{cm}^3 \) template. In this case, the volume density of cells is only \( \rho_c = \Phi_c \sigma = 125 \times 3 \times 10^3 = 3.75 \times 10^5 \) per cm\(^3\) porous template. We conclude that the template with an average pore diameter of 10 µm binds a volume density of myofibroblasts which is about 27 times higher than does the template with a pore diameter of 300 µm. The density of ligands clearly drops as the pore diameter increases; eventually, the ligand density becomes insufficient to bind all or most of the myofibroblasts and contraction resumes, as observed.

• These considerations suggest a maximum pore diameter requirement for the template, simply to ensure a specific surface which is large enough to bind a number of myofibroblasts that is appropriately large to block contraction across the entire scale of the defect.
Microscopic to macroscopic contraction force

- Exerting each an individual contractile force vector, $f_i$, a number $N$ of in-plane myofibroblasts scale up the force to the level of the macroscopic force vector, $F_c$, where wound contraction occurs:

$$F_c = Nf_i$$

- Measurement of the macroscopic force to contract a skin wound (in the absence of a template) yielded a force of order 0.1 N. Calculation of $f_i$ with dermal fibroblasts bound \textit{in vitro} on the DRT surface led to a value of order 1 nN per cell. Use of Eq. together with these measured values of the force per cell and the macroscopic force leads to an estimated

$$N = 10^{-1} \text{N}/1\text{nN} = 10^8$$
cells participating in contraction of the template-free injured site. This calculation suggests, therefore, an apparent force scale-up factor of $10^8$ (cell $\rightarrow$ wounded organ).
Reduction of contraction force by template

• The overall effect of the template inside the wound is, therefore, to reduce the factor $N$ and, separately, the factor $f_i$, in Eq. and thereby lead to a very significant drop in the macroscopic contraction force $F_c$ to near zero.
# Structural determinants of regeneration template activity

<table>
<thead>
<tr>
<th>Structural parameter of scaffold</th>
<th>Scaffold induces SKIN regeneration*</th>
<th>Scaffold induces NERVE regeneration**</th>
<th>Contribution to regenerative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I collagen/GAG, w/w</td>
<td>98/2</td>
<td>98/2</td>
<td>Ligand identity → Myofibroblasts (MFB) bound on scaffold</td>
</tr>
<tr>
<td>Average pore diameter, µm</td>
<td>20-120</td>
<td>5-10</td>
<td>Ligand density → Almost all MFB bound on scaffold</td>
</tr>
<tr>
<td>Pore channel orientation</td>
<td>random</td>
<td>axial</td>
<td>Spatial coordinates of ligands → Morphology of new organ</td>
</tr>
<tr>
<td>Average molecular weight between crosslinks****, $M_c$, kDa</td>
<td>5-15</td>
<td>40-60</td>
<td>Duration of scaffold topology → Synchronization with synthetic process</td>
</tr>
<tr>
<td>Degree of residual collagen fiber crystallinity (residual banding)***</td>
<td>ca. 5% of native collagen</td>
<td>ca. 5% of native collagen</td>
<td>Inhibition of platelet-aggregation → Reduce number of myofibroblasts</td>
</tr>
</tbody>
</table>
D. Synchronous and isomorphous replacement.
Synthesis of stroma

Isomorphous replacement
Replacement of template topography by a similar one.

Synchronous tissue synthesis
Synthesis of dermis (regeneration) was therefore observed when the time constants for template degradation and new tissue synthesis were approximately equal
\[ \frac{t_d}{t_h} \approx 1 \]

Summary of stroma synthesis. Synthesis occurs at a rate that matches the rate of template degradation and in a spatial configuration that replicates the departing template.
Dermis regeneration template (DRT)

Photo removed for copyright reasons.

Yannas, 2004
Nerve regeneration template (NRT)

Photo removed for copyright reasons.

100 \(\mu\text{m}\)

Yannas, 2004
Similarity of configuration + synchronization of template degradation rate.

Fact #1: A scaffold with regenerative activity (templates) in skin, conjunctiva and peripheral nerves has a three-dimensional configuration (topology) that is very similar to that of the stroma in the regenerating organs (Dagalakis et al., 1980; Chang et al., 1990; Hsu et al., 2000).

Hypothesis: Organ regeneration proceeds on the surface of a matrix that is a replica of the native stroma of the organ.

Fact #2: Regeneration templates lose their activity unless their degradation rate is roughly equal to the timescale for synthesis of tissue during healing (Yannas et al., 1979: Yannas and Burke, 1980).

Hypothesis: The template is required to remain intact long enough to initiate synthesis of new stroma but not long enough to block sterically the synthesis of new tissues.
Isomorphous tissue replacement rule

Similarity of configuration + synchronization of degradation rate of template → Rule of isomorphous tissue replacement (Yannas, 1997):

A scaffold cannot induce organ synthesis unless it is a configurational replica of the desired stroma and unless it degrades at a rate equal to the rate of stroma synthesis at the injured anatomical site.
Explain facts of regeneration using unified theory:

Contraction blockade +
  + Synchronized and Isomorphous replacement →
  → Regeneration
E. Two theories of regeneration:

1. Immunocompetence theory.

2. Contraction blocking and synchronized isomorphic replacement.
Immunocompetence theory: Loss of regenerative potential with development of immune system (amphibian data).

Diagram removed for copyright reasons.

Harty et al., 2003
Alternative theories of induced organ regeneration in adults

1. Increase in immune competence during development controls the gradual loss of regenerative potential that accompanies metamorphosis in amphibians and the fetal-adult healing transition in adults (Heber-Katz, 1999; Harty et al., 2003).

2. Regeneration is induced in adults by a scaffold that blocks contraction and provides a topology similar to the stroma being regenerated, remaining intact only for the duration of organ synthesis (Yannas, 2001).
Two theories of transition in healing response

1. Fetal $\rightarrow$ immune competence development $\rightarrow$ Adult

2. Adult $\rightarrow$ template $\rightarrow$ Fetal