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Genetic approaches to disease and regeneration

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Cardiovascular disease is largely a consequence of coronary artery blockage through excessive proliferation of smooth muscle cells. It in turn leads to myocardial infarction and permanent and functionally devastating tissue damage to the heart wall. Our studies have revealed that elastin is a primary player in maintaining vascular smooth muscle cells in their dormant state and thus may be a useful therapeutic in vascular disease. By studying zebrafish, which unlike humans, can repair damage to heart muscle, we have begun to uncover some of the genes that seem necessary to undertake the de-differentiation steps that currently fail and prevent the formation of new proliferating cardiomyocytes at the site of damage in a mammalian heart.

Keywords: heart; regeneration; elastin; *msx*

1. ELASTIN AND VASCULAR DISEASE

(a) *Elastin prevents vascular disease*

Obstructive vascular diseases, such as atherosclerosis, cause stroke and myocardial infarction in millions of people. Extensive research has revealed key risk factors, including hypercholesterolaemia, hypertension and diabetes, but a central mechanism linking these diverse factors has not emerged. Our data suggest that elastin deficiency is the central molecular mechanism of many vascular diseases.

We discovered that elastin mutations cause obstructive vascular disease in humans (Curran *et al.* 1993; Ewart *et al.* 1993*a*,*b*, 1994; Li *et al.* 1997). We then demonstrated that mice lacking elastin die of arterial obliteration through uncontrolled proliferation of VSMC (Li *et al.* 1998*a*). Next, we discovered that elastin controls VSMC migration, proliferation and differentiation, maintaining a quiescent phenotype (Li *et al.* 1998*b*; Karnik *et al.* 2003). Elastin exerts its effects in a dose-dependent manner through a G_i-protein coupled signalling pathway (Karnik *et al.* 2003). Thus, elastin is both a structural protein and a signalling molecule that acts through receptors on VSMC, maintaining arterial integrity.

We propose that destruction of elastin by mechanical injury or inflammatory proteases plays a central role in the proliferative response of obstructive vascular disease. Therapy has not focused on this mechanism. We discovered that exogenous elastin inhibits VSMC proliferation and arterial obstruction in pigs; results similar to those achieved with drug-eluting stents (Karnik *et al.* 2003). Elastin, therefore, may prove valuable for preventing vascular disease.

One contribution of 13 to a Discussion Meeting Issue 'New directions in tissue repair and regeneration'.

2. REGENERATION GENETICS

(a) *Inadequate regeneration is a common mechanism of disease*

Through our studies of cardiovascular disease (see above), it became clear that recurrent injury followed by inadequate regeneration and fibrosis is a common pathogenic mechanism. This is the case for atherosclerosis, heart failure, arrhythmia and many other diseases. Humans regenerate liver and digit tips, but our regenerative capacity is limited compared with that of urodele amphibians, such as newts. These organisms recurrently regenerate retina, spinal cord, limbs and other organs with great facility and without scar. Morgan called this process epimorphic regeneration.

Although regeneration has fascinated man for millennia, study in the field has been slow to progress to a molecular level, possibly because traditional model organisms have been refractory to genetics. We collaborated with Stephen Johnson (Washington University) to develop regeneration genetics in zebrafish (Nechiporuk *et al.* 1999, 2003; Poss *et al.* 2002*a*). Zebrafish regenerate fins and are commonly used as genetic models for studying early vertebrate development, but genetic strategies for studying an adult phenotype have not, until recently, become available.

We used chemical mutagenesis to identify zebrafish fin regeneration mutants (Poss *et al.* 2002*a*; Nechiporuk *et al.* 2003). Assuming that most would be lethal in embryos, we screened for temperature-sensitive mutants. We used positional cloning to discover the first regeneration genes, *mps1* and *sly1* (Poss *et al.* 2002*a*; Nechiporuk *et al.* 2003) and a candidate gene approach to reveal *fgfr1* (Poss *et al.* 2000*a*) and *msx1* (Odelberg *et al.* 2000; Nechiporuk & Keating 2002) (table 1). Finally, we associated *fgf*, *lef1* and *hedgehog* with regeneration using expression analyses (Poss *et al.* 2000*a*,*b*, 2002*a*; Nechiporuk & Keating 2002; Nechiporuk *et al.* 2003). These studies revealed, to my knowledge, the first genetic insight into regeneration, and provided tools for uncovering the molecular mechanisms of this complex phenomenon.

Table 1. Molecular and cellular mechanisms of regeneration.
(Asterisks indicate that the Keating laboratory was first, to my knowledge, to make this discovery.)

stage	gene	protein class	mechanism	references
regeneration cell formation*	*msx1**	transcriptional repressor	cell de-differentiation	Odelberg *et al.* (2000); Nechiporuk & Keating (2002)
	msx2	transcriptional repressor	?	Nechiporuk & Keating (2002); Nechiporuk et al. 2003)
blastema formation*	*fgfr1**	growth factor receptor	blastema cell proliferation and migration	Poss *et al.* (2000*a*)
regenerative outgrowth*	*mps1**	cell cycle checkpoint kinase	rapid blastema cell proliferation	Poss *et al.* (2002*a*)
	*sly1**	trafficking protein	rapid blastema cell proliferation	Nechiporuk *et al.* (2003)
pattern formation	*lef1**	wnt signalling molecule	cell differentiation and patterning	Poss *et al.* (2000*b*)
	*wnt3a**	wnt signalling molecule	?	Poss *et al.* (2000*b*)
	*wnt5**	wnt signalling molecule	?	Poss *et al.* (2000*b*)
	*beta-catenin**	wnt signalling molecule	?	Poss *et al.* (2000*b*)
	sonic hedgehog	signalling molecule	?	Poss *et al.* (2000*a*,*b*)

We next examined the function of proteins encoded by regeneration genes. Fgf is a signalling protein expressed in the wound epidermis shortly after injury (Poss *et al.* 2000*a*). An fgf receptor, fgfr1, is expressed in mesenchymal cells underlying the wound epidermis during blastema formation (Poss *et al.* 2000*a*). The blastema is a mass of cells immediately beneath the wound epidermis. Fgfr1 signalling is required for blastema formation. Msx proteins are homeodomain transcriptional repressors. These proteins are expressed in mesenchymal cells that coalesce to form pluripotent regeneration cells of the distal blastema (Nechiporuk & Keating 2002). Regeneration cells are like stem cells, providing daughter cells for regenerative outgrowth (Poss *et al.* 2002*a*; Nechiporuk & Keating 2002; Nechiporuk *et al.* 2003). Transgenic experiments suggest that msx induces cellular de-differentiation, creating regeneration cells (Odelberg *et al.* 2000; Nechiporuk & Keating 2002). *Mps1* encodes a mitotic checkpoint kinase critical for entry to the cell cycle in rapidly proliferating cells (Poss *et al.* 2002*a*). *Sly1* encodes an intracellular trafficking protein (Nechiporuk *et al.* 2003). Expression of both proteins is induced in, and restricted to, rapidly proliferating proximal blastema cells during regenerative outgrowth. Loss of *mps1* or *sly1* function eliminates regenerative outgrowth. Wnts and hedgehog proteins are signalling molecules expressed in the epithelium adjacent to the blastema (Poss *et al.* 2000*a*,*b*; Nechiporuk & Keating 2002). These proteins appear to be essential for patterning. Fgfr1 is required for their expression and function (Poss *et al.* 2000*a*). These data indicate that signalling molecules, transcriptional repressors and cell cycle proteins are essential for epimorphic regeneration.

(b) *Regeneration cells are central to epimorphic regeneration*

Together with previous developmental studies, our work provides a view of vertebrate regeneration at the molecular, cellular and organ levels (figure 1). The first step in fin regeneration is the formation of wound epithelium. We discovered that this is a non-proliferative event, involving the migration of existing epithelial cells to cover the wound (Nechiporuk & Keating 2002). The second step is the creation of the primary blastema. We found no evidence that these cells are derived from slow-cycling stem cells (Nechiporuk & Keating 2002). Instead, they are derived from existing mesenchymal cells beneath the wound epithelium, presumably though cellular de-differentiation. FGFr1 signalling is required for creation of blastema cells (Poss *et al.* 2000*a*). *Msx* genes are expressed in regeneration cells of the fin, and may induce de-differentiation (Odelberg *et al.* 2000; Poss *et al.* 2000*a*,*b*, 2002*a*; Nechiporuk & Keating 2002). These loosely distributed cells coalesce into the primary blastema, just beneath the wound epidermis (Nechiporuk & Keating 2002).

The third step in regeneration is blastema maturation and regenerative outgrowth. We discovered that the blastema is organized into two compartments (Nechiporuk & Keating 2002; Poss *et al.* 2002*a*): (i) a distal blastema that contains non-proliferating, msx+/mps1− regeneration cells; and (ii) a proximal blastema that contains msx−/mps1+ daughter cells. Proximal blastema cells proliferate intensely, with a 50-fold step-up of proliferation across 10 cells, driving regenerative outgrowth (Nechiporuk & Keating 2002; Poss *et al.* 2002*a*).

The fourth stage of regeneration is differentiation and patterning. Epithelial cells adjacent to the proximal blastema express signalling molecules like *wnts* and hedgehog (Poss *et al.* 2000*a*,*b*; Nechiporuk & Keating 2002). These proteins appear to organize the formation and alignment of scleroblasts, forming new bone, nerves and blood vessels. Regeneration ceases when the original size and appearance of the fin have been recreated.

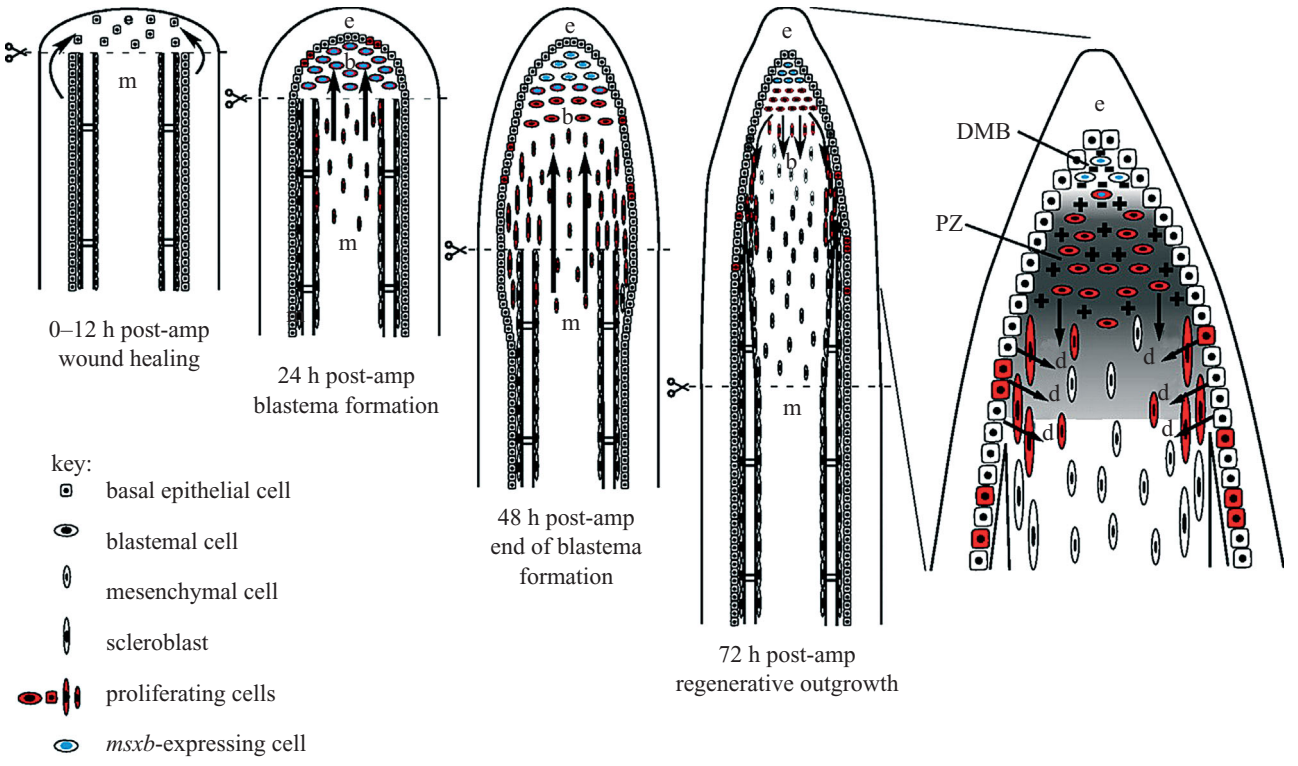

Figure 1. A cellular model of zebrafish fin regeneration. During stage 1 (0–12 h; wound healing), the wound epidermis is formed by migrating epithelial cells. In stage 2 (12–48 h; blastema formation), the basal epidermis is formed, mesenchymal tissue proximal to the amputation plane begins to disorganize and intraray mesenchymal cells proliferate and move upwards. A subset of early mesenchymal proliferating cells (red) express *msxb* (blue nuclei). The number of *msxb*-positive proliferating cells continues to increase throughout blastema formation. Just before the onset of regenerative outgrowth, blastemal cells segregate into *msxb*-positive non-proliferating distal-most blastema (DMB) and *msxb*-negative proliferating proliferation zone (PZ), with a gradient of proliferation between the two domains. During stage 3 (48 h to one week; regenerative outgrowth), the gradient of *msxb* expression and proliferation is maintained, controlling the direction of outgrowth. Cells in the PZ proliferate vigorously and move in the proximal direction to differentiate. A zone of negative proliferation in the DMB maintains the directionality of the outgrowth by inhibiting proliferation. d, differentiation signal; '+', positive proliferation signal; '−', negative proliferation signal; b, blastema; e, wound epidermis; m, mesenchyma; DMB, distal-most blastema; PZ, proliferation zone.

(c) *Zebrafish hearts regenerate*

We next examined the capacity of zebrafish to regenerate heart tissue. Human hearts do not regenerate. Instead, damaged myocardium is replaced by a scar. This is a significant medical problem, leading to an epidemic of heart failure, arrhythmia and death. Cardiomyocytes, the major structural cells of the heart, undergo hypertrophy to increase muscle mass after cardiac injury. Although recent findings suggest that cardiomyocytes within the diseased human heart may proliferate (Beltrami *et al.* 2001), most evidence to date indicates that cardiomyocyte proliferation is not a significant component of the mammalian response to cardiac injury (Pasumarthi & Field 2002).

We discovered that zebrafish fully regenerate hearts within two months of 20% ventricular resection (Poss *et al.* 2002*b*). Regeneration occurred through proliferation of cardiomyocytes localized at the epicardial edge of new myocardium. Hearts of zebrafish with mutations in *mps1* failed to regenerate and formed scars (Poss *et al.* 2002*b*). Thus, injury-induced cardiomyocyte proliferation can overcome scar formation, enabling cardiac regeneration. Our work provides, to my knowledge, the first demonstration of scarless regeneration of the heart and establishes a model system for genetically dissecting the mechanisms of cardiac regeneration.

(d) *Mammalian cells can de-differentiate to form pluripotent cells*

A key feature of epimorphic regeneration is cellular de-differentiation, creating regeneration cells (Nechiporuk & Keating 2002; Poss *et al.* 2002*a*). However, terminally differentiated mammalian cells, like cardiomyocytes, were previously thought to be incapable of undergoing a reversal of cell differentiation. These cells have permanently exited the cell cycle in response to the expression of cyclin-dependent kinase inhibitors, activation of members of the retinoblastoma family and downregulation of cyclins and cyclin-dependent kinases.

We discovered that terminally differentiated murine myotubes can de-differentiate (Odelberg *et al.* 2000). Ectopic expression of *msx1* in C2C12 myotubes reduced the nuclear muscle proteins MyoD, myogenin, MRF4 and p21 to undetectable levels in 20–60% of myotubes. Approximately 8% of myotubes cleave to produce smaller multinucleated myotubes or proliferating mononucleated cells. Finally, clonal populations of myotube-derived mononucleated cells can be induced to redifferentiate into cells expressing chondrogenic, adipogenic, myogenic and osteogenic makers. We achieved similar results using an extract derived from regenerating newt limbs (McGann *et al.* 2001). These results indicate that terminally

differentiated mammalian cells can de-differentiate when stimulated with the appropriate signals, and that *msx1* can contribute to the de-differentiation process. If de-differentiation and proliferation of cardiomyocytes can be achieved *in vivo*, it may be possible to enhance mammalian cardiac regeneration.

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GLOSSARY

VSMC: vascular smooth muscle cells