A massive wooden tower in Lausanne, Switzerland (La Tour de Sauvabelin).
Is it bio-mimetic, or just good mechanics? It comprises an integrated spiral staircase and walls made in wood. Spiral structures occur in nature, for example in snails and collagen molecules, but they are just a geometric form with particular mechanical strengths. They are uncommon, at least at the gross scale, in human anatomy. Wood is clearly bio-mimetic in composition but, strictly speaking, only where we are mimicking advanced plants such as trees. Just because something looks natural, it does not automatically make it bio-mimetic.

2 Checking Out the Tissue Groupings and the Small Print

or: Avoiding the low aim that still misses

2.1 Checking the small print: what did we agree to engineer?

I have never worked for Airbus or Boeing. But you can be sure that before their huge design and engineering teams so much as reach for a pen to sketch a new aeroplane, they have a *very* clear analysis of what they are being asked to make. This is most definitely not the case (yet) in tissue engineering, although – for reasons even more pressing than those of the aerospace industry – that *should* be our aim. After all, if Airbus Industries need to switch from a metal alloy fuel line to plastic in the final prototype, they just fabricate the replacement, insert and verify that the new one performs as their model predicts. The additional costs should be modest. No one would reasonably expect them

Extreme Tissue Engineering: Concepts and Strategies for Tissue Fabrication, First Edition. Robert A. Brown.

to smash up their previous prototype planes and start again!

However, this is not the case (again at present) for an engineered tissue therapy, real or imagined. Our ability to predict, analyse and model the tissue systems we aspire to fabricate is minimal in comparison to non-biological, manufactured devices. The consequences of getting things wrong can be just as disastrous for both types of engineering, though perhaps more spectacular and immediate for passenger planes.

The regulatory authorities responsible for civil aircraft would ask for data and validation of predictions on the performance of the new fuel pipe in the existing device. This is most obvious when we remember that the odd Boeing or Airbus has crashed in the past. Occasionally this has been due to control software, engine parts or structural surface failure. Yet identical aircraft types remain in service; they have been checked out, replacement parts fitted and are back, better than before. No one normally expects all examples of that plane to disappear overnight to the breaker's yard.

Not so with tissue engineered implant devices. It is likely that regulators responsible for the quality/safety of human implants would send the producer right back to the beginning of the development, in the process incinerating all examples of the failed design. The simple explanation, of course, is that we do not yet understand enough about tissues, and exactly what they need to do, to make them as predictable as parts for modern aircraft. However, it cannot be accurate to describe our example structure here of the Airbus airliner as a 'simple' device. These 100-tonne machines routinely bullet around at 200 metres/sec on the edge of space, where outside temperatures are good for freezing your blood. Still, the risk to passenger life and limb is judged to be negligible.

This can be illustrated by a closer look at the aeroplane engines next time you fly. Many Airbus types, for example, are made to take one of perhaps two or three completely different engine types (i.e. produced by the main jet engine manufacturers, Rolls Royce, General Electric or Pratt and Whitney: see Figure 2.1). Yet still each aircraft version performs predictably and safely within tolerances which would make biologists weep with envy.

In contrast, when we change only single minor process steps or components in biological tissues

Figure 2.1 Airbus 320 can be fitted with either Rolls-Royce or General Electric jet engines. Spot the different cowlings.

and implantable materials, we really do lose all confidence in its subsequent performance. Until we 'try' the experiment, it is hard to predict whether performance will change totally, not at all or somewhere in between. Commonly, the extent or even the direction of change cannot be predicted – sometimes not even as basically as saying it is likely to be better or worse!

We can perhaps glimpse the extent of this difference from the history of knock-out mice and their informative surprises. In preparing a knock-out mouse, the biologist takes out just *one* gene (so eliminating one protein component from the entire mouse). But far from sitting back, confident that this modest, focused removal will elicit a single change in function, one shift in behaviour or a unique block in a signalling pathway, the biologist investigates the entire animal. Every tissue, every habit and every metabolic pathway is catalogued to look for 'the phenotype' – that *pattern* of changes which characterizes the deletion (but could not be predicted). Sometimes these changes can be so great or so numerous that the animals cannot breed or develop beyond embryonic stages. But sometimes, the complexity of cross-support systems or the process of duplication and protein redundancy means that no 'phenotype' is immediately detectable. On occasions, where repair/remodelling mechanisms are affected, the knock-out mice must actually be wounded before any effect is apparent.

In fact, the difference with aircraft systems is not at all surprising, however complex and interlinked they may be. The aircraft systems were built up, from the bottom, by engineers. They have a full understanding of how each component part works, both alone and in combination with its co-parts. After all, they *made* the components. This is not true of the knock-out biologist, who is working by making alterations, top-down, on an already highly complex system, not knowing but guessing at the workings of the whole mouse.

This difference is reflected in how we modify and regulate the bio-fabrication of engineered tissues. When we 'grow' a tissue implant that produces a good result (e.g. satisfactory to both patient and surgeon), that is *it* – cast in stone! Changing pretty well any component (e.g. the sequence, timing, sometimes even reagent suppliers), fills us (and the government regulators) with a profound insecurity – so much so that we are sent right back to the start of our designing, testing and proving-what-to-expect process. It is as if our aircraft manufacturers did not know how to make devices fly in general, just how this type works, and even then only as long as it is an Airbus A320, serial number A320-000417-D, with Rolls-Royce engines, tuned for Shell kerosene. Getting the design of an engineered clinical tissue even slightly wrong can be, and often has been, disastrous because of this 'return-to-go' principle.

There may, then, be an opportunity for us eventually to fabricate tissues as if we were engineers. However, if this is our claim, then the non-engineering tribes will need to adjust to the reality of working like engineers. This means understanding what quantifiable functions we want to produce *and* how they can be measured once they are assembled. Ideally this should apply to our basic components as well as the finished article, so that we can change or improve components without 'surprises'. The problem is that this is not really a typical approach for biological scientists, and this how the gulf between aspiration and reality has been excavated in some areas. In brief, biological methods alone are rarely ideal for making structures that are expected to perform as if they were engineered.

Hence, it is critical to accept the implications of adopting the 'engineering' word. It will be interpreted (e.g. by engineers) in the manner that aircraft manufacturers ***engineer*** large planes. This involves understanding the operation of the wings, fuselage and engines to a high level of mathematical accuracy. Such mechanistic understanding allows them to compute (using their predictive models) that they want to fly 200 passengers for 3,500 km at 550 km/h, with tolerances for extremes of wind speed. The model predicts the ideal patterns of wing shape, dimensions, engine power, fuel consumption and maintenance intervals. The engineers make the plane and then identify *exactly* where there is the slightest deviation in performance from their prediction. If a parameter goes outside its performance range, alternative structures or

materials can be substituted and key adjustments made to other factors, which will be altered as a consequence.

For perfectly good reasons, this is not the understanding of the biological community:

- Bio-systems are extremely complex and integrated (so the mechanistic understanding is still simplistic) and mostly not quantitative or even fully reproducible.
- The properties of construction materials which are available to modern tissue engineers are largely uncertain under most biological conditions.

In fact, the biomaterials part of the tissue engineering partnership might feel more comfortable with the analogy shown in Figure 2.2, of early aircraft designs by A.V. Roe and Anthony Fokker. These early aviators knew roughly the tricks that should get a heavier-than-air-machine into the air – but only just. They sadly knew rather less about the tricks needed to get down in one piece, with the result that they did not always take flight and remain airborne for the required periods or in the intended direction. They made informed, and sometimes inspired, guesses, but all too often these were based more on emotional feelings than a knowledge of the material strengths, forces and durability of their creations. For a considerable period of the evolution of early aircraft, the plan focused on investigating the crashes! For example, with the luxury of modern retrospection, we can look at Figure 2.2 and question the wisdom of the rear, strapped-down fuel tank in (b) and the close-set pram wheels in (a). Neither of these was even likely to catch on.

It is interesting to reflect on just how much of this analogy (including the wording) rings true of recent tissue engineering. Indeed, it is possible to extend the analogy one more step, to include the recent biological drive to use stem/progenitor cells to tackle our limitations in engineering tissues. This might be seen as an abandonment of 'wing design' altogether, in exchange for a completely different form of flight without wings (i.e. we cannot understand the heavier-than-air engineering mechanism, so we dodge it: see Figure 2.3).

(a)

(b)

Figure 2.2 Early (pre-1918) aircraft designs by Anthony Fokker (top panel) and A.V. Roe (lower panel), showing contemporary mono- and biplane formats, with traction (pulling forward (a)) and pusher (b) propellers, respectively.

It might be worth a passing thought at this stage, that we could re-examine the need for such regulatory rigidity if we ever hit on a way to fabricate biological structures from the bottom up, as we do with aeroplanes. When we can fabricate tissues from well-defined components that work together predictably through well-understood processes which can be mathematically modelled, *then* we can tune our systems and products. Perhaps a good target here is the pharmaceutical industry. Once it is established that a chemical compound has a series of desirable clinical effects, then that compound can be formulated in many different

Figure 2.3 Airship R34: flight without wings, demanding alternative, lighter-than-air technologies. © CSG CIC Glasgow Museums Collection.

ways and combinations. This often attracts only modest regulation, provided the chemical purity, concentration and sterility can be assured. We know what pitfalls to look for and what really matters for success and safety. Tissues are like that, except with spatial-mechanical complexities **and cells**!

The reader may wonder if we are shuffling off topic here, but it is an important analogy as it gives the biological part of our community some valuable context (and modesty). It should also help inform engineering partners about the void which we must bridge in concepts and expectations. Tissue engineering is well known for the tradition of progressively 'talking-up' its vision, often to attract valuable industrial and public support. But this positive, upbeat impression can sometimes lead new recruits to miss the enormity of our task ahead. This is *not* good for strategic thinking. When you perceive that you are 'nearly there', the plans you formulate are very different from those you make when you have a long journey before you. The aim here is to take a fresh look at the 'small print' of the tissue engineering contract we are signing so that we know exactly where we are.

2.2 Identifying special tissue needs, problems and opportunities

Each tissue type carries with it special requirements which represent its 'problems and opportunities'. Building up a rational and detailed profile of these is the key starting point for *engineering* that tissue. In practice, this involves a bottom-up or minimalist approach. It would be counter-productive – especially at the outset – to aim to engineer, say, a **left carotid artery**. This is both too variable (patient-specific) in its detailed anatomy and too specialized in its application to be a useful design starting point. Perhaps a better description of the target would be 'a visco-elastic vascular tube carrying clottable liquid under pulsatile pressure, with minimal turbulence' (Figure 2.4). It is certainly a sufficiently high hurdle. The important phrase to remember here is '**Key Functional Properties**' (KFP for short).

Notice that by denying ourselves the shorthand of using the anatomical name and instead identifying the KFP, we have been forced to list the *real* properties that we *really* need. This is a great start, particularly as it is likely to demand extensive discussions with the end user of your construct – perhaps a surgeon – rather than quick look at a textbook. It is also likely that these (KFP) properties will be useful across many vessels other than the left carotid artery. This can become a 'platform construct'. In other words, it may be possible to adapt it for use at all sorts of anatomical sites, simply by changing its shape.

Indeed, we can go further and start to put numbers to the KFPs, as allowable ranges for each property. In many cases, this will allow us to define

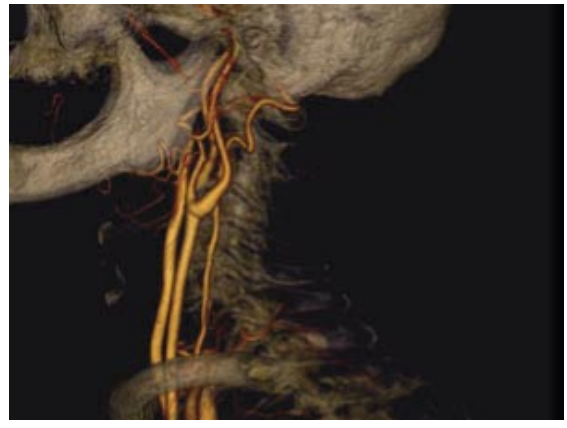

Figure 2.4 Left carotid artery, or 'a visco-elastic vascular tube carrying clottable liquid under pulsatile pressure, with minimal turbulence' to describe it using its KFP. © www.rime.pt.

those vessels, or families of tissues, for which our design will be appropriate. Equally important, it then becomes simple to exclude those for which it is unsuitable. Careful selection of KFPs at the outset will pay huge dividends later. Indeed, its iterations can make it one of the longest of stages.

We now have the skeleton of *good practice* for the planning stage:

1. Specify the key functional properties (KFPs), prioritized as 1st, 2nd, 3rd, etc., from the most to least important (i.e. critical to beneficial, or essential to desired).
2. Identify the range of values for which the performance of that tissue function would be:
   (i) ideal;
   (ii) acceptable;
   (iii) absolutely disastrous.
3. Specify (even in general) how the KFPs would be measured. Start by deciding which units (e.g. cells/ml; % live cells; MPa material stiffness; ml/min fluid flow rate) would be most useful to describe the function you need to measure.
4. List any caveats to this analysis, in particular at which stage(s) of the process these KFPs need to apply. The stages can go from initial assembly of the tissue components (e.g. cells, temporary scaffold, extra cellular matrix) to the end of bioreactor culture and on to post implantation. This can mean that for each caveat, a new set of slightly different KFP ranges need to be specified (e.g. range 1 = post-bioreactor culture stage; range 2 = one-month post-implantation stage).

These targets (KFPs), then, should ideally be the *first* section of a target application described or discussed by a tissue engineering partnership, as opposed to the anatomical site or surgical problem, which is more commonly the opening. Obviously, both the site and the problem play important parts in shaping decisions while the KFPs are being assembled, but their importance need not go far beyond assigning the priority order in the KFP list.

Once the tissue construct has its top rank KFPs, within their acceptable ranges, refinement of this generic construct can easily follow. To continue our analogy, we might hope that these specifications could become as easy to change as it is for Airbus to switch from Pratt and Whitney to Rolls-Royce engines to meet divergent airline needs. Throughout such iterations of design and testing, the aim is that KFP ranges come closer to their 'ideal' as they are better understood.

Clearly then, where the KFP model is used, constructs not only improve in function but also provide new knowledge of the factors which *control* that performance. This is a major, hidden opportunity as it represents a refining database which can grow into a model system for accurate prediction of future designs. It is a direct parallel of systems which now allow aircraft manufacturers to predict wing performance in a way that could never have happened if aeronautics had set out, say, simply, to mimic the wings of a crow, then of a vulture, then a swallow ... an albatross ... a dodo ...?

In some cases, the KFP progression may take the form of an evolution from an ultra-simple, almost embryonic tissue to a fully adult-functional, mature tissue. Alternatively, they can form a series of increasing complexity:

- 1st stage: fabrication of a model tissue (e.g. *animal sparing* for testing and research).
- 2nd (clinical) stage: as simple generic *spare part* surgical implants.
- 3rd stage: implants designed for a single *specific clinical* problem (such as the carotid artery in our initial example).

To summarize, describing our construct tissue targets as a rational series of KFPs, as opposed to naming the bio-anatomy, significantly shifts the early (and later) processing towards the 'engineering' benefits we want. It also lays a solid foundation of *function-based* design and generates a database of measureable, important factors to reduce the later incidence of 'surprises'. At least as important, it enhances the chance that our designs will have wider applications, based on the 'performance envelope' that we shall become able to produce for each key function. These should be applicable to similar sites or types of tissue or related defects. Quantifying how the engineered structures/components

perform, once they are made, makes it possible for future iterations to identify additional tissues or injury sites where they can be applied.

2.3 When is 'aiming high' just 'over the top'?

It is difficult to point to many forms of '**non-living** heavier-than-air' flight (excluding the short duration, seasonal (though passive) migrations of Oklahoma trailer homes in the tornado season). On this basis, it might be argued that human airliners are a form of bio-mimesis, though in this case of bird rather than human function. Oddly enough, this is an interesting thought that is relevant to contemporary tissue engineering, as it demonstrates how society's attitude has, over the last 100 years, already come to accommodate *de facto* 'bio-mimetic engineering'.

The point here is we are perfectly able to be pragmatic about 'how bio-mimetic' our flying devices need to be. We all can now visit colleagues or relatives a continent or two away, in Shanghai, Boston, Mumbai or Sydney. Yet we never blink an eye at the sight of the featherless Boeing that will fly us there. What could go more to the core of bird-flight-function than feathers? But ever since Icarus* and

* Who was, in fact, a mythological character who over did it with the Gods and came to a sticky flying-accident end for his trouble!

the earliest glider pioneers, it has been obvious that we **do not need** this particular bio-mimetic component (see Chapter 9); instead, we cross the oceans in a casing of sheet metal and rivets! So, by what good logical reason can we automatically assume which part of any given 'bio' we need to 'mimic' in our target tissue?

This is a fascinating question in contemporary tissue engineering and regenerative medicine (TERM). How close a 'copy' or mimic of the target tissue does our construct need to be? Equally, how can we assess when the 'imaginative and visionary' has drifted into 'wacky' dreams, especially where hyper-focused enthusiasts are the dreamers? The question has its roots in the tension (probably essential to TERM) between two contradictory needs. Let's call this the '***safe-hype***' tension. The first is for forward-looking analyses of potential applications which have imagination and vision. But the second requires that these same analyses are scientifically balanced, prudent and defensible.

Of course, pretty well anything *may* be possible if we work long enough at it, but sometime-never is not a permissible time frame. The *imaginative-vision* side of the strategic planning must have a more critical analysis of time scales than a Hollywood Sci-Fi movie. Hence, there is a need to balance this with the *prudent and defensible*. The question of how long it will take us to acquire the key understandings we are lacking is the pivot-point for assessing this 'reality' balance. Who can tell? Maybe a feathered fuselage will *eventually* improve our flight ... but in the meantime ...

Arguably more than many modern scientific fields, TERM has a reputation for hype (over-selling or exaggeration of its objectives). This matters, as the public are particularly interested in the prospects of having new parts of their body made painlessly available when vital bits are injured, decay or drop off. Indeed, they have every right to be interested, and realistically informed, as their taxes pay for the research. This interest is evident from the most casual glance at national newspapers and TV channels, with their seemingly endless series of upcoming miracle-systems for new hearts, eyes or skin. Clearly, TERM has more than its share of optimistic 'amazing-but-true' stories. But aside from feeding scientists' dreams and filling newspaper columns, this is one important half of valuable *safe-hype* tension – the half that drives us on.

Like all essential tensions, though, there is never a fixed stable balance point – no such thing as 'safe hype'. Scientists must inform and inspire their paymaster-sponsors but, at the same time, they must be balanced and cautious. This is particularly tricky where the subject matter detail is so complex and uncertain but the overall idea

seems so simple. One of the most important points where this tension can be effectively balanced is that between the optimism of building *biology-as-it-was* and the prudent-pragmatism that knows we can often get *function-without-perfect-recapitulation* (Text Box 2.1).

By looking for a timely appropriate balance to the *safe-hype* tension, we are moving to the possibility of putting stages or graded-milestones against our application targets from:

(i) modest-achievable (success soon); to
(ii) difficult-with-hard-grind (success in the mid-term); and
(iii) that-might-take-a-while (success for our descendants).

Listening and reading about the 'in process' progress in regenerative medicine generally can leave the impression that most initiatives are in the (i) tending to (ii) category. This is the effect of the *positive vision*. Though experienced individuals may learn to recognize these levels, there is a guideline which can make it easier for the newcomer. This uses the principle that some long journeys bring smaller benefits en route *before* we reach the great destination. So we can judge the balance of time-risk against end-point benefit of any engineered construct, based on what smaller or earlier outputs will emerge en route. There are three such application targets (matching the milestones in the list above):

1. Model 3D tissues (research and screening lab tools).
2. Simple spare parts for general surgical reconstruction/repair.
3. Fully integrated (regenerated) tissue and organ replacements.

Text Box 2.1 Looking for the 'functional' compromise?

Crisp, defining lines of logic are hard to find in this area, as there is a tendency for all things to overlap. However, one way to plot research progress or the evolution of strategies is to identify where the requirement for improved function really is. This can vary in any given decade and at any one tissue or lesion site. For example, early prosthetic hips were valuable *replacements* where no alternatives were available. Later versions have concentrated on longer life and simpler surgical fitting. These functions are now all so good that research focuses on making them easier to replace once they wear out (which they must, eventually!) or avoiding them completely with engineered tissues.

Tissue *repair* was initially revolutionized by 'simple' technologies (at least they are now) to prevent massive infection or bleeding. These improve repair by allowing the patient to survive long enough to mount a repair response at all. Approaches to 'engineer' the natural repair process have evolved subsequently to include the full spectrum of approaches, from genetic engineering of repair cells to supporting temporary scaffolds and manipulation of local growth factor levels. Out from this spectrum has emerged the idea that we can use classical engineering and bio-engineering processing to improve the final *repair tissue function*. This leaves tissue engineering as one (particularly appropriate) approach amongst a number of others.

Finally, then, we reach the pinnacle of functional restoration embedded in the idea of tissue *regeneration*. This involves producing an exact replica of the failed tissue. Consequently, its function will, by definition, be perfectly matched to the target tissue. For example, an advance/future target for loss of a patch of eyelid would be to restore it with new eyelid skin, as opposed to forearm, buttock or a generic/average skin. These would be the last generation solutions, OK for tissue replacement or repair strategies – it would, of course, be a Utopian vision at present. At its extreme, this vision is rooted in the concept that it will one day be possible to recapitulate embryological growth and development in order to 'regenerate' *perfect function*, in the way that seems to occur in some amphibia (see Chapter 1).

This illustrates the full spectrum of aspirations, ranging from the pragmatic baseline of replacing *some* function (with all its implied compromises) to the Utopian end-stop of perfect regeneration, with a near-infinite variety of fine-functional matching. That would seem to be no compromise at all – except for the time compromise! So, we may be tacitly accepting the biggest compromise of all where we have no idea *when* these ideals will be achieved.

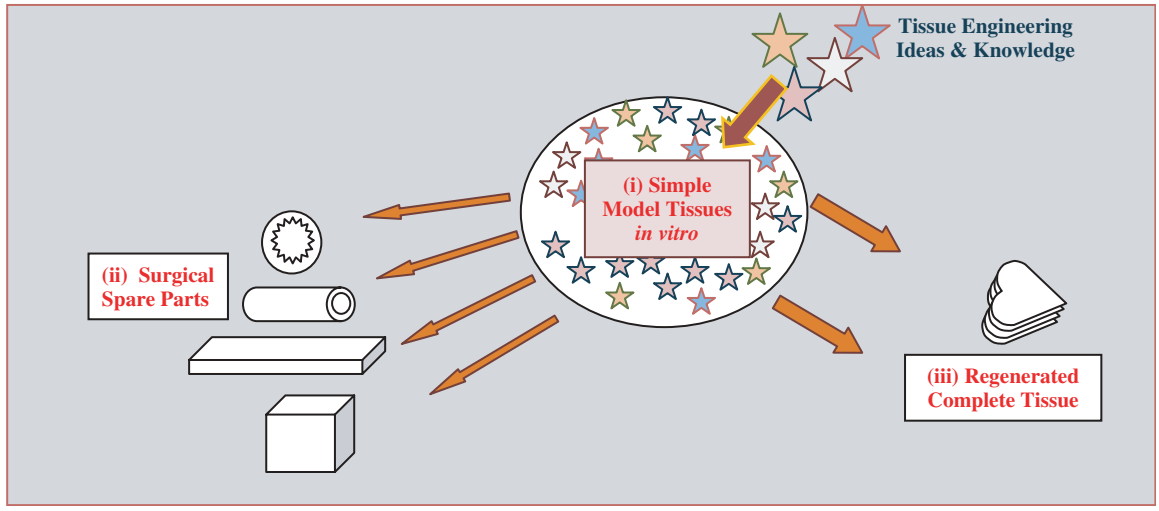

Figure 2.5 Scheme of the route map leading away from basic tissue engineering and the inter-relationship of: (i) tissue models (experimental & testing systems); (ii) spare parts (surgical repair kits); and (iii) complete tissue systems (regenerated implants).

To summarize this section, it is a characteristic of our subject that it must constantly balance between, on one hand, a vision of the promised benefits, and on the other, a sound assessment of what *is* possible. The safe-hype balance is shorthand for this tension, between the conservative drive to make tissues exactly as they are in nature and the vision of quick, low-cost tissues rolling off a production line. But there are questions that help us to balance this tension. At one end, we can ask how we *really* know that this molecule or that pattern of channels is essential for function. At the other end, we must ask ourselves how long it will realistically take to make the key process work.

2.4 Opportunities, risks and problems

The focus in this section will be on comparing the special needs and opportunities offered where we aim to produce model tissues. The corresponding analysis of engineering clinical implant tissues can be brief as it is largely the mirror image of, and the comparator for, model tissues.

2.4.1 Experimental model tissues (as distinct from spare-parts and fully regenerated tissues)

In its early days, tissue engineering was forged with a strong focus on clinical application targets (replacing specific high demand/high value tissues). As a result, the idea of preparing model (not-for-clinic) tissues was for many years overlooked, and it has only recently become a popular aim. However, the potential benefits and undoubted logic of using tissue models means that this is a growing field of activity. In effect, the very process of fabricating and assembling model tissues provides a natural spin-off of biological knowledge, as outlined in Chapter 1, Section 1.4. Consequently, while there are many sub-plots and alternative high- and low-risk clinical targets, the *en route* production of model tissues is a distinct and early target (Figure 2.5).

The special feature of **tissue models** is that they exist only *in vitro* (in our case probably 3D; culture), as simplified but strongly biomimetic forms of the tissue being modelled. We can cut rapidly to explain just what makes tissue models so attractive through two central points:

1. There is a major unmet need for 3D models for:
   (a) pharmaceutical and other screening
   (b) toxicity testing
   (c) clinical diagnosis/research.

   Simple but well-defined 3D *in vitro* model tissues have major potential uses in their own right, by providing alternatives to *in vivo* testing (i.e. they offer animal-sparing alternatives).
2. Much (though not all) of the work involved in generating 3D model tissues is essentially the same as that needed for clinical implant tissues, so they can represent a translational payback stage (i.e. a valuable output) on the way to conventional engineered tissue implants. In effect, it is essential to understand the performance, tolerances and limits of model tissues for testing, and these provide an excellent foundation for more complex clinical implant technology. Also, regulatory demands for testing systems have a far lighter (different?) touch than for clinical uses.

2.4.2 The pressing need for 3D model tissues

A well-documented and mature example is that of skin equivalent engineering. The huge and unmet demand for skin implants and grafts for patients is only too well known. But the demands on pharmaceutical, cosmetic and chemical industries for rapid, accurate, reproducible (and ideally humanized) test systems is equally pressing, though perhaps less well appreciated. In particular, under current European legislation on animal procedures, the need for alternative test models to replace or reduce animal use is becoming still more pressing, particularly in the cosmetics sector. Without new and well-characterized 3D tissue models, particularly of barrier functions, industrial progress will be hampered. What is more, where the new model tissues succeed, they can generate new industries of their own. It is a double win.

The argument against using of animal testing models has important lessons for tissue engineering, once they are understood. First, there is the obvious moral aspect of testing on live animals, especially in the numbers currently used. In order to guarantee that this, at least, involves the minimum of suffering, governments have instituted many demanding requirements and control processes. This makes animal testing both an expensive and a time-hungry option, dramatically increasing the cost of drug research. Finally, though, it is becoming clear just how *wrong*, as well as variable, results from animal models can really be when we extrapolate them to human physiology, drug responses and diseases.

This means that engineered model tissues present particular and special opportunities. As we might expect, they are relatively inexpensive, not least because of the minimal bureaucracy involved. Their relative simplicity tends to make them more rapid and reproducible to use and easier to interpret. Beyond this, though, they have the potential to be tailored to mimic exactly the site or function we require (i.e. customization). Finally, the potential in many cases to use human cells makes it feasible to fabricate human test tissues. In other words, the small print in this particular tissue engineering contract points to the potential for an absolutely enormous radiation of highly specialized applications. This depends, however, on us (the extreme tissue engineers) actually delivering on the clauses headed 'reproducible', 'customized' and 'humanized' – in that order!

Typical targets and testing applications include:

- drug access through the skin, gut and blood-brain barrier;
- agent toxicity in the liver, lung and kidneys;
- prediction of pathological/age-related changes in the joint surfaces (cartilage) and bone;
- drug responsiveness of tumours, blood vessel wall and skin.
- testing if, or how an individual patient will respond to a drug, i.e. which version of the drug is best (customized medicine)? (Text Box 2.2)

2.4.3 Tissue models can be useful spin-offs on the way to implants

Engineered model tissues for testing do not have the baggage of having to function *in vivo* after implantation. They do not, for example, need to

Text Box 2.2 Non-animal testing

Topicality of this field can be judged from the near disappearance of animal testing in the European cosmetics industry (as of 2008/9). Paris-based L'Oreal is the largest cosmetics company in the world (the total value of the European cosmetics industry sector was estimated to be €35 billion in 2007).

L'Oreal ceased to rely on traditional animal-based testing over a decade ago and emphasised this recently by acquiring the tissue model (epithelial engineering) company, Skin Ethic Laboratories. Along with other model epithelial cell structures, Skin Ethic manufactures a model skin, comparable to that of MatTek, around which much of the parent company bases its product development.

In yet another sector, the multinational company Unilever, better known for soaps and foods, has had over two decades of research experience in producing and applying skin equivalent models of various levels of complexity.

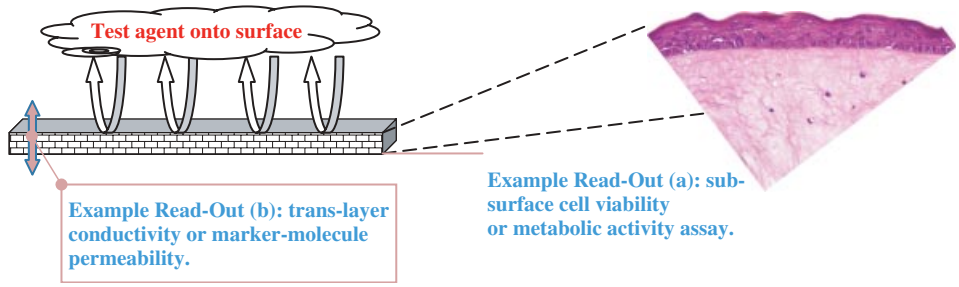

Figure 2.6 This summarises example testing strategies for one commercially available epidermal (MatTek (Inc.) EpiDerm skin-substitute: http://www.mattek.com). This consists of a multi-layer of epidermal skin cells (keratinocytes) grown in culture to form a sheet, using original technology of Reinwald and Green. The inset micrograph (right) shows the appearance of a dermal-epidermal skin 3D model with a similar epithelial layer, but grown on a compressed collagen-fibroblast layer. The spatial organisation provides the capacity for a 3D readout, in this case measuring the rates at which agents pass through the epithelial barrier to cells and matrix of the lower dermal layers. Agents can be drugs, toxins, chemicals, cosmetic components, UV or other radiation, applied specifically to the outer (upper) surface. Responses measured at deeper cell levels over a time course as a range of measures, from cell viability to specific gene expression or growth factor release and apoptosis. Penetration rates of drugs can be monitored fluorescently. In some cases it is important to confirm that a low permeability physiological epithelium has formed before estimating transcutaneous permeability. This is typically measured as a function of the transepithelial electrical resistance (TEER).

incorporate features to regulate complex integration into the recipient host site. In effect, they represent a 'lower bar' of application (i.e. achievable more easily and sooner), particularly in terms of the level of regulatory approval needed (Figure 2.6). They can be regarded commercially as the 'low hanging fruit' of tissue engineering. For example:

- Skin-equivalent models discussed already need to be sterile at the point of use (or they could not be used reliably for assay purposes). However, with professional handling, it is not as urgent as it is for implants to show they are free from human pathogens (e.g. viruses) or modified genes.
- They are not going to be rejected or provoke an inflammatory response, so they can be made using animal products with no difficulties. These present significant regulatory and safety problems for human therapeutic implants.
- A major regulatory hurdle is that therapeutic agents must be shown, in controlled trials, to be effective in exactly the manner (and to the limits) claimed. Clearly, if a model tissue did *not*

perform a useful function it would not be used, but this is not the same as facing a direct barrier to production.

2.5 Special needs for model tissues

2.5.1 Cell selection: constancy versus correctness

Sadly, there is no 'free lunch', and model tissues developed for testing purposes *do* have their own special needs and demands. These are inescapable consequences of their very specific 'function'. Function in this case is defined as: to give reliable, accurate reproducible responses which can be unambiguously interpreted in a way which is a reasonable reflection of the target tissue.

While an implant must provide a reliable benefit to the recipient patient, this will always contain a substantial degree of variability in its detail (e.g. rate of integration, strength, physical appearance). This is inevitable, not least as each recipient/patient is different from the next. The implant surgeon (e.g. plastic, orthopaedic, maxillo-facial) may well measure key patient performance indicators. This might be, say, the range of movement, pull strength and joint rotation angle after reconstruction of hand tendons. However, such measures of clinical success are commonly expressed as wide ranges of values, reflecting the spread of patient responses and injury type. In effect, it is *relative* improvement that is the key for patients. As long as the patient becomes substantially better than before surgery, the reconstruction was a success. Absolute or precise values for improving performance are hard to find and often not so appropriate.

This is not true for test-bed systems or a drug screening assay, including 3D model tissues. Here it is the norm to expect a numerical readout of the test response, expressed in absolute terms, or at least with very tight ranges, relative to a time-zero or zero concentration baseline. In short, this demands levels of reproducibility and precision which would not normally be expected of therapeutic systems. The most acute consequence of this pressure is evident in the type of cells selected to seed such 3D model constructs. "Where do we get our cells from?" could almost be considered the tissue engineer's mantra. In this case, for model tissue and implants the answer is swung around by 180° from that for clinical implants.

Therapeutically desirable cells tend to be synthetically active, non-immunogenic (or as close to the recipient as possible)and free from pathogens such as viruses. In contrast, for building an assay or test-bed based on a 3D construct, almost *none* of these previously indispensible requirements are particularly important. Viral agents and immunogenicity are marginal factors. The use of human cells is desirable, but not really essential. Interestingly, the gold standard aim for therapeutics, using cells from one particular human (i.e. autologous) becomes a 'no-no' for screening and test models, where pooled or 'average' cell responses are a benefit. Animal cells are quite acceptable. In testing, the *central* demand is 'Reproducibility, Reproducibility, Reproducibility'. But reproducibility is not the strong point of primary cells, freshly extracted from a tissue – especially human tissue.

The key tension-balance underlying cell selection for clinical implants is around how close we can get to actually taking cells from the individual patient, economically and without causing extra harm. In complete contrast, for testing it is how much *reproducibility* we can afford to give up while still keeping the cells that are relevant to the system we want to test. This is because the most consistent, constant cells which would give the most reproducible cultures are transformed cell lines (similar to, and in some cases derived from, cancer cells). While these are, indeed, in wide use for conventional cell-based assays, they have frequently lost many of the properties of the parent native-tissue, or primary cells (Text Box 2.3).

To fall back on analogy, if the various forms and types of cell from a tissue are thought of as a cell 'family', then transformed cells would be, at best, the eccentric cousin who went to sea amid shady rumours. At worst they might be the mad uncle who has to be watched carefully on days out and has difficulty with everyday social interactions. On the

plus side, you know just where you are with these cells, and their behaviours are normally very well documented. As a result, we can just look them up and work out whether the properties they retain will do the job we have in mind for them.

The pragmatic tension or compromise, then, is between tolerating these cell eccentricities and enjoying the fact that they are constant and predictable. The trick is to ensure that the eccentricities do not interfere with the main parameters under test and that the madness *really is* as constant as we think. They should perform much the same month after month, passage after passage, without aging, developing into other cell types or differentiating new, imaginative features to surprise us. In some cases, cells with tumour-like properties should ring some alarm bells where we are designing test systems with a strong spatial element – spatial organisation and attachment is often not their strong point.

However, we are at a necessary, and still useful, staging position from where progressively more sophisticated gene modified cell lines may be produced. To paraphrase an old saying; no one is likely to produce this particular 'horse' (i.e. develop the 'ideal' cell line) before the demand rises for effective 3D test systems (i.e. the 'cart' for it to pull).

2.5.2 Support matrices – can synthetics fake it?

In later sections, there will be much comparison between the benefits and drawbacks of synthetic scaffolds versus native protein cell support materials for engineering of tissues. In fact, this forms one of the defining differences between tracks towards implantable and model tissues. In brief, synthetic polymers are used to support cell growth in 3D, with the aim that the polymer slowly degrades as the cells deposit a native extracellular matrix replacement. For clinical implants, this strategy has distinct advantages and a sound, long-term rationale. In effect, the early 'tissue' made of cells and synthetic polymer scaffold (plus optional, small amounts of native matrix) is implanted with the aim of maturing, *in the body*, to become a functional tissue. Host tissue in-growth, vascularization and local factors would help the transformation, often over a period

Text Box 2.3 Transformed cell lines

When cells are freshly isolated from a tissue, either by disrupting the tissue or by tempting its cells to migrate out onto a culture dish, this is called a ***primary culture***. Depending on where it is grown from and how, primary cultures can contain seriously mixed (heterogeneous) populations of cells. However, this is commonly considered to be a reasonable representation of cells in the original tissue.

As primary cultures expand, they are sub-cultured and this produces a cell line – those cells which survive on plastic and divide fastest. Individual cells can be can be selected out and cloned to give more homogeneous populations, but such cell lines tend to divide rather slowly and this rate reduces continuously with time and further sub-culturing. These are 'finite' cell lines, which gradually run out of proliferative steam.

However, such cell lines can give rise to continuous or transformed cell lines where growth and cell division continues rapidly in an unregulated, undiminished manner, rather like *in vitro* tumours. This can happen spontaneously in some cells, or due to the action of viruses, radiation, transfection or chemicals. Transformed cell lines can also be derived from tumours but, although they can share some features, normal transformed and malignant cells are not necessarily the same.

Clearly, the fast division rate and consistency of transformed cells is really handy, especially for routine testing. The downside, though, is that in acquiring such happy characteristics, some can seem to become the cell version of 'bonkers'. In losing their constraints on division, they also lose some of the basic properties and behaviours which made them typical of their tissue of origin. However, enough of these are retained to be useful, and a wide range of continuous cell lines are available with well described properties, including examples of epithelial, fibroblast and neural tissue properties.

Source: Freshney, R.I. (2005). *Culture of animal cells: a manual of basic technique*. Wiley-Liss, Hoboken, NJ. (ISBN 0471453293).

of months, whereby the artificial 3D 'matrix' is replaced by one which is natural.

While this is plausible for a patient, it is not feasible for mass production of model tissues for screening pharmacological candidates.

Present synthetic polymer supports effectively fall between two adjacent hard places! First, they are not particularly biomimetic in composition, patterns of biodegradation or (often) 3D μ-structure, so they make a poor, even negative contribution to the modelling of real tissues, especially those with matrix (i.e. all connective and many cell-rich tissues). On the other hand, waiting weeks in culture for the synthetic-natural transformation to occur is not practical or economic, even if the cells used are capable of that transformation.

Consequently, engineered constructs based on the most widely used synthetic polymer supports are generally poor candidates for model test tissues. New forms of synthetic, biomimetic support materials may change this by using components which are sufficiently biomimetic, *without cell action*, to act immediately as model 3D matrices. These are the hybrid matrix types which will be analyzed in Chapter 4. This dual problem does not normally apply to support materials made from natural proteins, as these can be rapidly fabricated in forms which mimic natural extracellular matrix, from the start and without cell action.

This, then, is another version/example of the tension we must balance between building in too much and too little biomimesis, and at what stage. For tissue models, the biomimetic bar can be low – but it should be reached very quickly.

2.5.3 Tissue dimensions: when size does *matter!*

Another significant difference between the inherent aims of engineered tissues for clinical versus test uses is size. Certainly for the development of mass testing and screening, it is a high priority that the test constructs are small and plentiful, to satisfy the need for many tests, many replicates and small volumes. Presently this is envisaged as systems which use 12, 24 or 96 well plates, maintained in conventional culture. This is important, as it favours rapid test times and low reagent consumption, some of which can be very costly. It is fortunate, then, that many existing output measures – molecular, optical and electrical – are minimally invasive and collect data rapidly from low tissue volumes. In contrast, clinical implants commonly need to be of much larger dimensions.

On the whole, tissue defects of the size envisaged for a 96-well plate (few mm) heal themselves reasonable well. Many surgical applications need much larger grafts, in the multi-gram to kilogram range. While small constructs with μ-scale structure can be challenging to fabricate, because of the scale of structures and the range of hierarchies involved, fabricating a mass of tissue and keeping it alive and functional is a separate problem.

Again, we see that there can be a clear segregation of options which is implicit in our choice to engineer either model tissues or clinical implants.

2.6 Opportunities and sub-divisions for engineering clinical implant tissues

So, what sort of tissue-making opportunities are out there? Actually a great deal more than we might first imagine – even within the overall groupings of *models* and *implants*.

It is not really necessary to go into detail at this stage, but it is important to understand at least some of the general options and directions. In fact, new approaches and imaginative forms of target tissue-functions are still appearing as our understanding and technologies grow. Some of the general families and groupings are listed below.

1. Implantable, physiological tissue copies:
   (a) MATURE
   (b) IMMATURE/temporary, repair templates.
2. Model tissues or copies of:
   (i) NORMAL tissues
   (ii) ABNORMAL/pathological tissues
   (iii) biological PROCESSES (e.g. integration).

Within these general groupings, we can distinguish opposing categories:

- Large (>mm scale), versus small tissues (e.g. 10s–100s of μm in scale).
- **Matrix-rich** (commonly connective) tissues versus **cell-rich** tissues (organs or epithelia).
- Hard versus soft.
- Random, symmetric (non-directional) and asymmetric or anisotropic tissues.
- Vascular versus avascular tissues.
- Mechanically fixed interface tissues versus gliding interfaces.
- Biologically active versus bio-inert.
- Permeable versus barrier.
- Tissues which operate as defined mechanical units versus those which operate as metabolic units (e.g. glands producing hormones; filtration organs).
- Aphysiological tissues, i.e. copies of natural tissue, but used in new ways or unnatural locations. These include constructs which copy tissue function for non-natural reasons (e.g. controlled drug release, cell carrier devices).

2.6.1 Making physiological implants: spare parts or complete replacement?

This is a distinction between tissue targets where, on the one hand, the surgeon aims to plug in the whole functional component (like a garage might fit your car with a complete new engine) or where, in some cases, the preferred surgical approach is just to repair a key defective component using spare parts and surgical skill (back to the garage, a skilled mechanic might make your engine as good as new by replacing its pistons and valves).

The 'making a whole heart' approach mirrors conventional cadaveric transplantation logic (but without the cadaver). Spare part engineering in the same area might be seen as engineering heart valves, chordae tendineae (so called 'heart strings') or micro-vascular patches. Clearly, engineering the whole functional unit is a big call for the tissue engineer, but easier for the surgeon. Spare part tissue engineering inverts this approach, requiring simpler tissue implants but placing greater demands on the surgeon: another tissue-engineering-tension.

2.6.2 Making pathological and aphysiological constructs: inventing new parts and new uses

Making pathological tissues sounds like a contradiction in terms until we think of the many uses of model (non-therapeutic) constructs (Text Box 2.4). In this case, once we have normal tissue mimics for measuring drugs effects (or the poisonous potential of this wonder-fertilizer or that baby shampoo), it is logical to want to make them go wrong! When we can make a model cartilage-bone tissue fibrillate and break down, perhaps we will also understand why it happens in osteoarthritis. If we can engineer a replica kidney, perhaps it will be possible to injure it and understand what causes it to fail – and perhaps to screen a candidate drug to reverse the pathology.

Aphysiological tissue targets provide us with a very different and fascinating view of tissue engineering logic. There are a number of ways of viewing this concept. One illustration builds on the idea that some therapeutically useful tissues really never existed, even though we *can* make them. For example, consider the need to improve the quality of life for spinal injury patients. The tissues and organs down-stream of the spinal lesion are, of course, initially fine (though muscle function gradually degrades), but they are no longer under the patient's control. Restoration of a few key functions can be tantalizingly close. In this case, if we were able to engineer nerve conduit tissues to guide nerve regrowth efficiently over significant distances, it might be possible to re-innervate critical muscles *below* the injury. In such patients, it is clear that the ability to cough is surprisingly important and can be a significant functional loss. Nerve redirection from above the spinal lesion to the diaphragm muscle responsible for this function would be a major benefit. No such nerve exists naturally in the human body yet, were it to be achieved, it would behave as a 'natural' nerve, but with an aphysiological anatomy.

Text Box 2.4 Examples of 3D model tissues

Engineered tissue models can also be used in *research* to define new elements of well known pathologies (or disease processes), or to identify the basic elements of normal tissue physiology (especially cell responses in systems which are otherwise too complex to dissect apart). Examples include:

(i) 3D models of articular cartilage. These include chondrocytes embedded and cultured within weak agarose gels and:
  (a) provided with growth factors to alter cell-matrix metabolism, making it possible to understand how cartilage matrix is/is not rebuilt after damage or degeneration, for example in osteoarthritis[1];
  (b) monitored under confocal microscopy while applying controlled compressive loading, to understand how cells and cell nuclei are distorted and so help explain common pathways in cartilage mechano-biology[2].

(ii) 3D models to test the mechanisms underlying hypoxia-driven angiogenesis (new sprouting and growth of blood vessels from existing capillaries towards sites of low oxygen). This involves growth of various cell types (fibroblasts, vascular smooth muscle or bone marrow stromal stem cells) in large-diameter 3D dense collagen matrices with an embedded (core) oxygen monitoring probe. This has allowed direct correlation of the dynamics of cell-induced hypoxia on the production and release of growth factors stimulating angiogenesis. Key to this is that, unlike previous *in vivo* systems, the key determining factors of O_2 consumption – diffusion transport through the matrix and delivery (via vessels) – can be modelled, calculated and correlated with actual O_2 levels[3].

Sources:

1. Jenniskens, Y. M., Koevoet, W., de Bart, A. C., Weinans, H., Jahr, H., Verhaar, J. A., DeGroot, J. & van Osch, G. J. (2006). Biochemical and functional modulation of the cartilage collagen network by IGF1, TGFbeta2 and FGF2. *Osteoarthritis and Cartilage* **14**, 1136–1146.
2. Knight, M. M., Toyoda, T., Lee, D. A. & Bader, D. L. (2006). Mechanical compression and hydrostatic pressure induce reversible changes in actin cytoskeletal organisation in chondrocytes in agarose. *Journal of Biomechanics* **39**, 1547–1551.
3. Hadjipanayi, E., Cheema, U., Mudera, V., Deng, D., Liu, W. & Brown, R. A. (2011). First implantable device for hypoxia-mediated angiogenic induction. *Journal of Controlled Release* **153**, 217–224.

By taking the same idea in another direction, we can move towards tissue engineering of controlled release devices. It is true that if we aim to make, say, pancreatic islet glands for diabetics or adrenal glands, these would, at the same time, be conventional engineered tissues. They also, incidentally, teach us a great deal about the special controlled perfusion properties needed if we aspire to make implantable controlled-release depots. Many such applications are under development, normally towards achieving prolonged or controlled rates of release of entirely unnatural drug agents or therapeutic proteins as they have never been delivered before. These, then, can be considered as forms of aphysiological construct, performing non-native jobs but using physiological mechanisms and tissue-mimetic structures.

2.6.3 Learning to use the plethora of tissue requirements as an opportunity

Having visited a few of the target threads of tissue engineering, the reader might now recognize the huge radiation of possibilities that has evolved in tissue engineering logic. As each tissue brings its own opportunities and demands, so these are multiplied by the different possible implant locations and the ways in which they can be degraded. This spreads further where our vision takes us beyond 'normal' physiology. Imagine for a moment designing a tissue engineering approach to enhance 'repair' of joint articular cartilage (which does not normally repair much at all) by controlled growth factor delivery. This would involve controlled release, chronological delivery and diffusion gradients, under controlled and incremental compression-shear loading. How

different these demands are to those required for a blood vessel construct, using a bio-resorbable polymer scaffold, seeded with cells and growth factors. The neo-vessel must produce an appropriately elastic extracellular matrix and host cell in-growth over the lumen to prevent blood clot formation, while resisting peeling off under fluid shear.

This is tissue engineering. It aspires to enhance and improve on natural tissue repair, but through a huge variety of routes applied to almost any tissue, situation and failure state. The variants are almost infinite. Not to be daunted, this means that opportunities for adaptation of our available technologies, are also limitless. However, to take advantage of this huge opportunity we must accept the responsibility to be:

- selective in the extreme;
- strategically imaginative; and
- logically robust, in the tissues and targets we choose.

This will be a recurrent theme in later chapters. Interestingly, this series of traits was thin on the ground in the early stages of (not-so-extreme) tissue engineering. The joy of 20:20 hindsight allows us to understand how '*high intensity, low attention-span*' commercial support for research dictated the selection of tissue and application targets. It is now quite clear that some of the tissue applications are *not* such 'low-hanging fruit' as industry initially imagined. In view of our current learning, it will be useful to identify where the fruit was:

- not as low as we imagined,
- not a desirable fruit at all,
- or just an example of boardroom-wordsmanship.

What is now clear is that the need to solve society's greatest injuries 'in a single bound', using untried technologies from poorly connected disciplines, was a Superman quest.

2.7 Overall summary

Given the view of this treasure chest of opportunities which hindsight supplies us, we might also suspect that our problem has been one of *aiming low and still missing*. But perhaps the first target we *really* had to hit was simply to generate the motivation for the biological-, engineering-, surgical- and materials-tribes to meet up, talk and work together, so that we could properly understand the problem. This, for sure, was successful and worthwhile.

Further reading

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4. Holmes, A., Shaksheff, K. & Brown, R. A. (2009). Engineering tissue alternatives to animals: applying tissue engineering to basic research and safety testing. *Regenerative Medicine* **4**, 579–92.
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