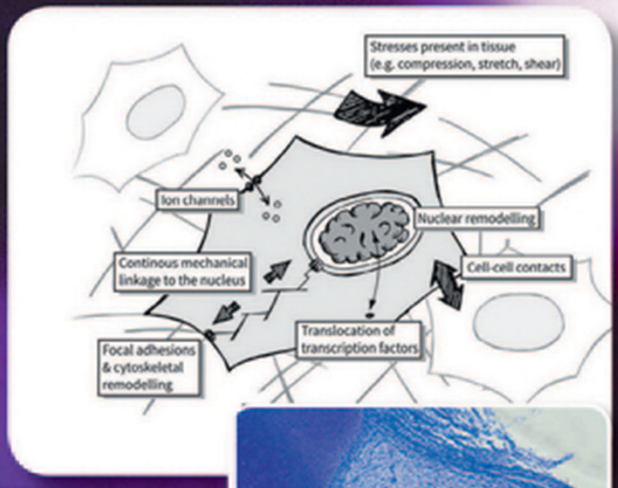
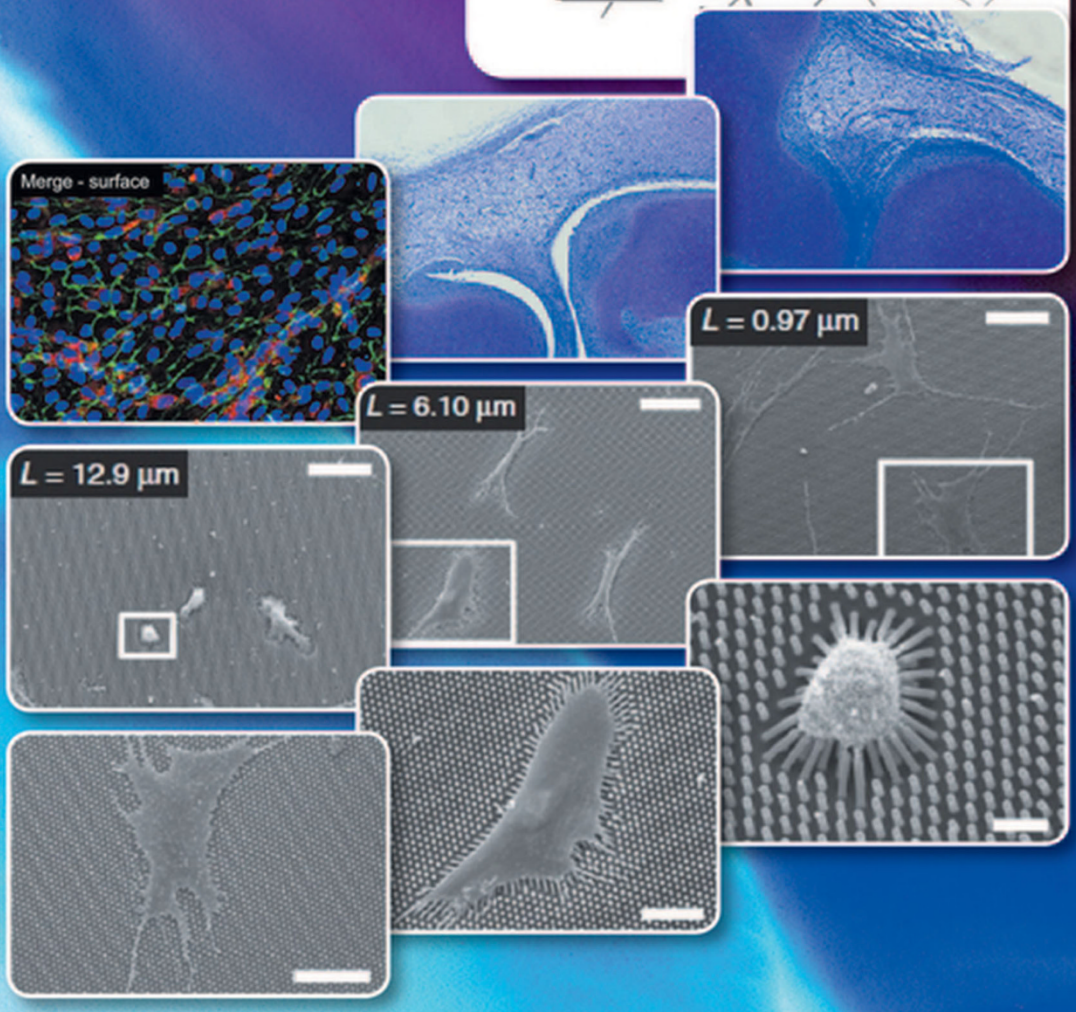
MECHANOBIOLOGY

Exploitation for Medical Benefit

Editor
Simon C. F. Rawlinson





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Edited by Simon C. F. Rawlinson

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Contents

List of Contributors

Kartik Balachandran
Department of Biomedical Engineering
University of Arkansas, Fayetteville
AR, USA

Christoph Ballestrem
Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences
University of Manchester
Manchester, UK

Charlie Campion
Institute of Bioengineering and School of Engineering and Materials Science
Queen Mary University of London
London, UK

Naomi C. Chesler
Department of Biomedical Engineering
University of Wisconsin–Madison
Madison, WI, USA

Yanan Du
Department of Biomedical Engineering
School of Medicine,
Tsinghua University
Beijing, China

Kian F. Eichholz
Trinity Centre for Bioengineering
Trinity Biomedical Sciences Institute and Department of Mechanical and Manufacturing Engineering
School of Engineering
Trinity College Dublin, Dublin
Ireland

Department of Mechanical, Aeronautical and Biomedical Engineering
Centre for Applied Biomedical Engineering Research
Materials and Surface Science Institute
University of Limerick, Limerick
Ireland

Alicia J. El Haj
Institute for Science and Technology in Medicine, University of Keele
Staffordshire, UK

Winston Elliott
Department of Mechanical Engineering
University of Colorado at Boulder
Boulder, CO, USA

Nicholas D. Evans
Centre for Human Development, Stem Cells and Regeneration, Institute for Life Sciences, University of Southampton
Faculty of Medicine, Institute for Developmental Sciences
University of Southampton
Southampton, UK
Bioengineering Sciences Group
Faculty of Engineering and the Environment, University of Southampton, Southampton, UK

Gabriel L. Galea
Developmental Biology of Birth Defects
UCL Great Ormond Street Institute of Child Health,
London, UK

Ulysse Gaspard
Department of Gynecology
Liège University Hospital
Liège, Belgium

Julien E. Gautrot
Institute of Bioengineering and School of Engineering and Materials Science
Queen Mary University of London
London, UK

Hamish T. J. Gilbert
Wellcome Trust Centre for Cell-Matrix Research, Faculty of Biology
Medicine and Health
University of Manchester
Manchester, UK

K. Jane Grande-Allen
Department of Bioengineering
Rice University, Houston, TX, USA

James R. Henstock
Institute for Ageing and Chronic Disease
University of Liverpool, Liverpool
UK

Karin A. Hing
Institute of Bioengineering and School of Engineering and Materials Science
Queen Mary University of London
London, UK

David A. Hoey
Trinity Centre for Bioengineering
Trinity Biomedical Sciences Institute and Department of Mechanical and Manufacturing Engineering
School of Engineering, Trinity College Dublin, Dublin, Ireland
Department of Mechanical, Aeronautical and Biomedical Engineering
Centre for Applied Biomedical Engineering Research, Materials and Surface Science Institute
University of Limerick, Limerick
Ireland

Chao-Kai Hsu
Department of Dermatology, National Cheng Kung University Hospital
College of Medicine, National Cheng Kung University, Tainan, Taiwan
Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
International Research Center of Wound Repair and Regeneration
National Cheng Kung University
Tainan, Taiwan

Chenyu Huang
Department of Plastic, Reconstructive, and Aesthetic Surgery, Beijing Tsinghua Changgung Hospital, Beijing, China
Medical Center, Tsinghua University
Beijing, China

Philippe Humbert
University of Franche-Comté, Besançon
France
Department of Dermatology, University Hospital Saint-Jacques, Besançon, France
Inserm Research Unit U645, IFR133
Besançon, France

Hanna Isaksson
Department of Biomedical Engineering
Lund University, Lund, Sweden

Amir Keshmiri
Engineering and Materials Research Centre, Manchester Metropolitan University, Manchester, UK
School of Mechanical, Aerospace and Civil Engineering, the University of Manchester, Manchester, UK

Hanifeh Khayyeri
Department of Biomedical Engineering
Lund University, Lund, Sweden

Franziska Lausecker
Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences
University of Manchester

Manchester, UK
Institute of Human Development
Faculty of Human Sciences
University of Manchester
Manchester, UK

Rachel Lennon
Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences
University of Manchester
Manchester, UK
Institute of Human Development
Faculty of Human Sciences
University of Manchester
Manchester, UK
Department of Paediatric Nephrology
Central Manchester University Hospitals NHS Foundation Trust (CMFT)
Manchester Academic Health Science Centre (MAHSC), Manchester, UK

Stefania Marcotti
Department of Materials Science and Engineering, INSIGNEO Institute for In Silico Medicine, Sheffield, UK

Lee B. Meakin
School of Veterinary Sciences
University of Bristol, Bristol, UK

Keiji Naruse
Department of Cardiovascular Physiology, Graduate School of Medicine Dentistry and Pharmaceutical Sciences
Okayama University, Okayama, Japan

Andromeda M. Nauli
Department of Pharmaceutical Sciences
Marshall B. Ketchum University, Fullerton
CA, USA

Surya M. Nauli
Department of Biomedical & Pharmaceutical Sciences
Chapman University, Irvine
CA, USA

Rei Ogawa
Department of Plastic, Reconstructive, and Aesthetic Surgery, Nippon Medical School, Tokyo, Japan

Hulin Piao
Department of Cardiovascular Physiology, Graduate School of Medicine Dentistry and Pharmaceutical Sciences
Okayama University, Okayama
Japan
Department of Cardiovascular Surgery
The Second Affiliated Hospital of Jilin University, Changchun, China

Gérald E. Piérard
Laboratory of Skin Bioengineering and Imaging (LABIC), Department of Clinical Sciences, University of Liège
Liège, Belgium
University of Franche-Comté, Besançon
France

Sébastien L. Piérard
Telecommunications and Imaging Laboratory INTELSIG, Montefiore Institute, University of Liège, Liège
Belgium

Claudine Piérard-Franchimont
Department of Dermatopathology
Unilab Lg, Liège University Hospital
Liège, Belgium
Department of Dermatology
Regional Hospital of Huy, Huy
Belgium

Andrew A. Pitsillides
Comparative Biomedical Sciences
The Royal Veterinary College
London, UK

Andrea S. Pollard
Comparative Biomedical Sciences
The Royal Veterinary College
London, UK

Patrick J. Prendergast
Trinity Centre for Bioengineering
School of Engineering, Trinity College
Dublin, Dublin, Ireland

Daniel Puperi
Department of Bioengineering
Rice University, Houston, TX, USA

Prashanth Ravishankar
Department of Biomedical Engineering
University of Arkansas, Fayetteville
AR, USA

Simon C. F. Rawlinson
Centre for Oral Growth and
Development, Institute of Dentistry
Barts and The London School of
Medicine and Dentistry
London, UK

Caretta J. Reese
Department of Biomedical &
Pharmaceutical Sciences
Chapman University, Irvine
CA, USA

Gwendolen C. Reilly
Department of Materials Science and
Engineering, INSIGNEO Institute for
In Silico Medicine, Sheffield, UK

Hitomi Sano
Department of Plastic, Reconstructive
and Aesthetic Surgery, Nippon Medical
School, Tokyo, Japan

Rinzhin T. Sherpa
Department of Biomedical &
Pharmaceutical Sciences
Chapman University, Irvine
CA, USA

Joe Swift
Wellcome Trust Centre for Cell-Matrix
Research, Faculty of Biology
Medicine and Health
University of Manchester
Manchester, UK

Ken Takahashi
Department of Cardiovascular
Physiology, Graduate School of Medicine
Dentistry and Pharmaceutical Sciences
Okayama University, Okayama
Japan

Wei Tan
Department of Mechanical Engineering
University of Colorado at Boulder
Boulder, CO, USA

Lian Tian
Department of Medicine, Queen's
University, Kingston, ON, Canada

Camelia G. Tusan
Centre for Human Development, Stem
Cells and Regeneration, Institute for Life
Sciences, University of Southampton
Faculty of Medicine, Institute for
Developmental Sciences
University of Southampton
Southampton, UK
Bioengineering Sciences Group
Faculty of Engineering and the
Environment, University of
Southampton, Southampton, UK

Zhijie Wang
Department of Biomedical Engineering
University of Wisconsin–Madison
Madison, WI, USA

Preface

Mechanobiology is the study of how tissues and cells interact with, and respond to, the physical environment, either through direct contact with a substrate via cell attachments or through cell-surface perturbation by a varying extracellular situation/climate.

The vast majority of cells are subjected to a fluctuating physical environment – and this is not restricted to the animal kingdom. In response to increased loading conditions (bending), the branches of trees compensate with new wood formation. Interestingly, though, conifers and hardwoods respond to this increased bending differently: conifers tend to produce "tension wood" on the upper part of the bough, whereas hardwoods produce "compression wood" on the lower surface – two distinct solutions to one problem.

This volume attempts to briefly introduce the topic of mechanobiology in humans to a broad audience, with the intention of making the phenomenon more widely recognized and demonstrating its relevance to medicine. It covers three broad topics: (i) recognition of the mechanical environment by extracellular matrix (ECM) and primary cilium, (ii) selected tissue types, and (iii) physical, computational/substrate models and the use of such findings in practice.

Obviously, the list of chapters for each topic is not exhaustive – there are too many examples, and this volume therefore can only be an introduction. The tissue types discussed are some of the more immediately recognizable as being subjected to mechanical forces, though a few are less obvious.

One important question is, given that most biology is subjected to the mechanical environment, how can we best reproduce that in experimental conditions? Would the effect of a compound be influenced if the tissue/cells were subjected to their normal physiological environment at the time of application? Such questions need to be at least acknowledged, if not accommodated within experimental design.

I hope the volume generates interest in, and appreciation of, this emerging field with those considering a career in science or medicine.

Finally, I would like to thank all the contributing authors to this manuscript. They have all devoted their time to writing their chapters and have focused on presenting their ideas clearly and logically to the target audience.

Simon C. F. Rawlinson

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Extracellular Matrix Structure and Stem Cell Mechanosensing

Nicholas D. Evans and Camelia G. Tusan

Centre for Human Development, Stem Cells and Regeneration, Institute for Life Sciences, University of Southampton, Faculty of Medicine, Institute for Developmental Sciences, University of Southampton, Southampton, UK
Bioengineering Sciences Group, Faculty of Engineering and the Environment, University of Southampton, Southampton, UK

1.1 Mechanobiology

An ability to sense the external environment is a fundamental property of life. All organisms must be able to interpret their surroundings and respond in a way that helps them survive – for example, by feeding, moving, and reproducing. The ability to sense also allows organisms to communicate with one another. Communication and cooperation were the primary driving forces that led to the evolution of complex multicellular organisms from simpler unicellular organisms. Evidence of this remains in many of the signaling pathways found in mammals that promote cell arrangements during development, which evolved from primordial chemical signals that unicellular organisms used to communicate with one another (King et al. 2003). Cells in our mammalian bodies are experts at communicating with one another using chemicals, and our physiology is completely dependent on this, from the precisely orchestrated cascades of growth factors during development to the hormones necessary for homeostasis and the immune mechanisms fundamental to repelling microbes.

Cells can also interact with one another by direct contact. Cells express characteristic surface proteins of various types, most prominently the cadherins, which allow them to determine whether they have a close neighbor.

Organisms are not just aggregates of cells – cells also make materials that provide structural support and knit groups of cells together. This material is called "extracellular matrix" (ECM). Again, the ECM is rich in chemical information for cells, provided in the three-dimensional information encoded in the myriad proteins that may be deposited there. In this way, cells can communicate with one another not only in space, but also over relatively long periods of time, with insoluble ECM having a much longer half-life that secreted soluble cues (Damon et al. 1989).

But this is not the whole story. The environment is not solely open to sensing by chemical means. Consider what we think of as our own senses: sight, sound, smell, taste, and touch. Smell and taste are perhaps the most analogous to the cellular sensing mechanisms just

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described, while sight is a somewhat more specialized form of sensing, based on the ability of certain cellular molecules to become altered by the absorption of electromagnetic radiation. Sound and touch are also fundamental sensations, the former a specialized type of the latter, based on our ability to detect the mechanical force of the interaction of matter with our bodies. This property is generally referred to as "mechanosensitivity," the study of which is known as "mechanobiology." But despite the importance of these senses, for many years they remained relatively under-researched in the field of biological sciences, and were limited to some fascinating, specialist examples. One such example is the hair cells of the inner ear, which transduce movement into neural signals that can be interpreted by the central nervous system (CNS) (Lumpkin et al. 2010). These cells not only detect vibrations in materials of particular wavelengths that we understand as sound, but are also able to act as accelerometers – detecting acceleration due to physical movement or the continuous acceleration resulting from the earth's gravity. In addition, a similar system is thought to be present in the skeleton. Astronauts who experience long periods of reduced acceleration in the microgravity of the earth's orbit suffer from a reduced bone mass on return to earth (Sibonga et al. 2007). A prevailing hypothesis (yet to be universally accepted) is that osteocytes within the bone matrix, like the hair cells of the inner ear, are able to detect and respond to acceleration (Klein-Nulend et al. 1995). Evidence for this comes from the observation that bones remodel in response to mechanical stress, tending to increase in density (and strength) in regions where the applied stress is the greatest, an effect unambiguously demonstrated in the forearms of professional tennis players (Figure 1.1), where bone thickness is greater in the dominant arm (Ducher et al. 2005).

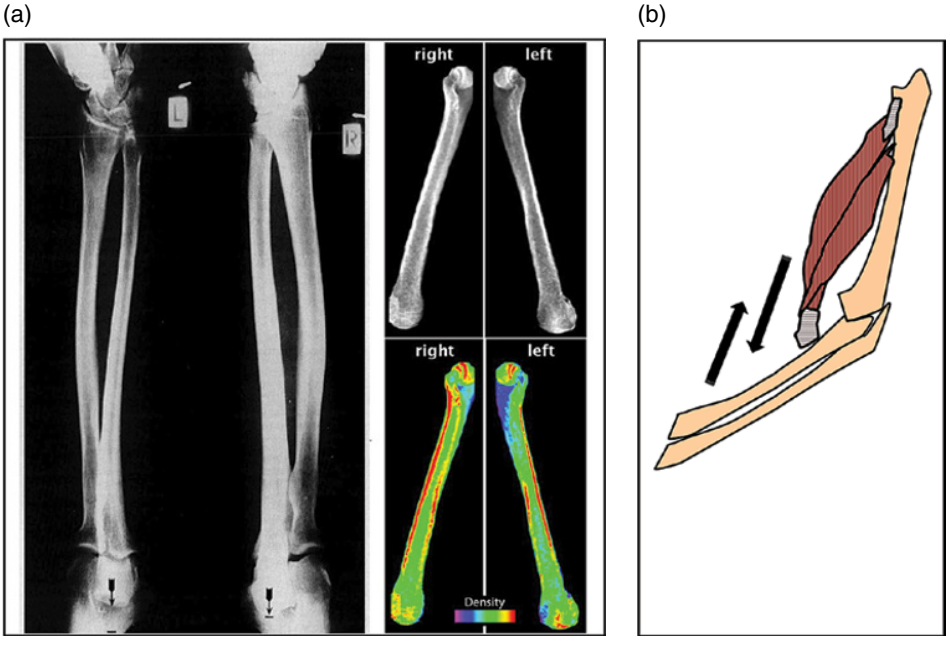

Figure 1.1 Bone growth and development are affected by mechanical stress. (a) The response of tissues to mechanical stimulation can clearly be seen in the arms of a professional tennis player. The bone thickness and density are greater in the dominant right arm. (b) On hitting the ball with the racket, the skeletal muscle pulls against the bones, causing them to rebuild and become denser. *Source:* x-ray images reproduced from Krahl et al. (1994) and Taylor et al. (2000).

Aside from these specific examples of mechanosensing, it is increasingly evident that all cells retain intrinsic mechanisms for sensing the mechanical properties of the environment around them. And this property has fundamental repercussions in almost all aspects of physiology and disease. In the context of human health and well-being, one aspect of mechanobiology that continues to receive special attention is its effect on stem cells.

1.2 Stem Cells

Stem cells are cells that can divide to make more copies of themselves, or which can differentiate into two or more specialized cell types. The concept of the stem cell emerged from ideas about both evolutionary and developmental biology in the late 19th century, generally with the notion that cell lineages, either throughout evolution or in the development of an organism, followed a family tree-like pattern of descent, with the putative stem cell at the top (Maehle 2011). This concept was brought into sharp focus in the mid-20th century with the work of a succession of experimental biologists who characterized "haematopoetic stem cells." These cells were shown to have enormous plasticity and replicative power, and to completely reconstitute the immune systems of animals lacking a working one (the immune systems of these animals had been destroyed with radiation), supporting the early ideas of proponents of the stem cell hypothesis, such as Pappeheim (Figure 1.2a) (Ramalho-Santos and Willenbring 2007). Today, the concept of the stem cell has spread throughout organismal biology, with stem cells identified in most if not all organs and tissues of the mammalian body. Some are amenable to extraction and culture in *in vitro* or *ex vivo* conditions and can be studied relatively easily, but some must be studied *in situ*. In the latter case, stem cells are known to occupy specific locations where they retain their stem-like properties. There, they have the correct provision of extracellular signals necessary to keep them in a state primed to divide and produce more functional descendants in normal homeostasis or in case of disease or injury. Such regions are called stem cell "niches," and the characteristics of such niches are vital to understanding how stem cells are regulated in normal and disease processes (Figure 1.2b).

Of particular interest is the pluripotent stem cell – so called because it has the ability to generate all of the cell types found in the adult organism. These cells, like cancer cells, divide indefinitely, making them a highly attractive source for cell replacement therapy, for example in diseases where the loss of a particular cell or tissue causes the severe effect of the disease. Originally, pluripotent stem cells were synonymous with embryonic stem cells (ESCs), but now it is known that cells with such properties can be artificially engineered from many adult somatic cell types – these are called "induced pluripotent stem cells" (iPSCs) (Takahashi and Yamanaka 2006). ESCs, which exist only transiently in development, can be extracted from the early blastocyst of the developing embryo and kept in an undifferentiated, developmentally frozen state by growing them in a precisely defined medium containing a cocktail of chemicals (Evans and Kaufman 1981; Thomson 1998). Similar conditions are required for iPSCs. On exposure to the right chemicals, at the correct concentrations, and at the appropriate time, such cells can be directed to differentiate to various lineages (e.g., pancreatic β cells, dopaminergic neurons, and hepatocytes). Controlling this is, of course, key to the utility of iPSCs in medicine – producing an adequate number of functional cells is necessary if they are to fulfill their intended medical use.

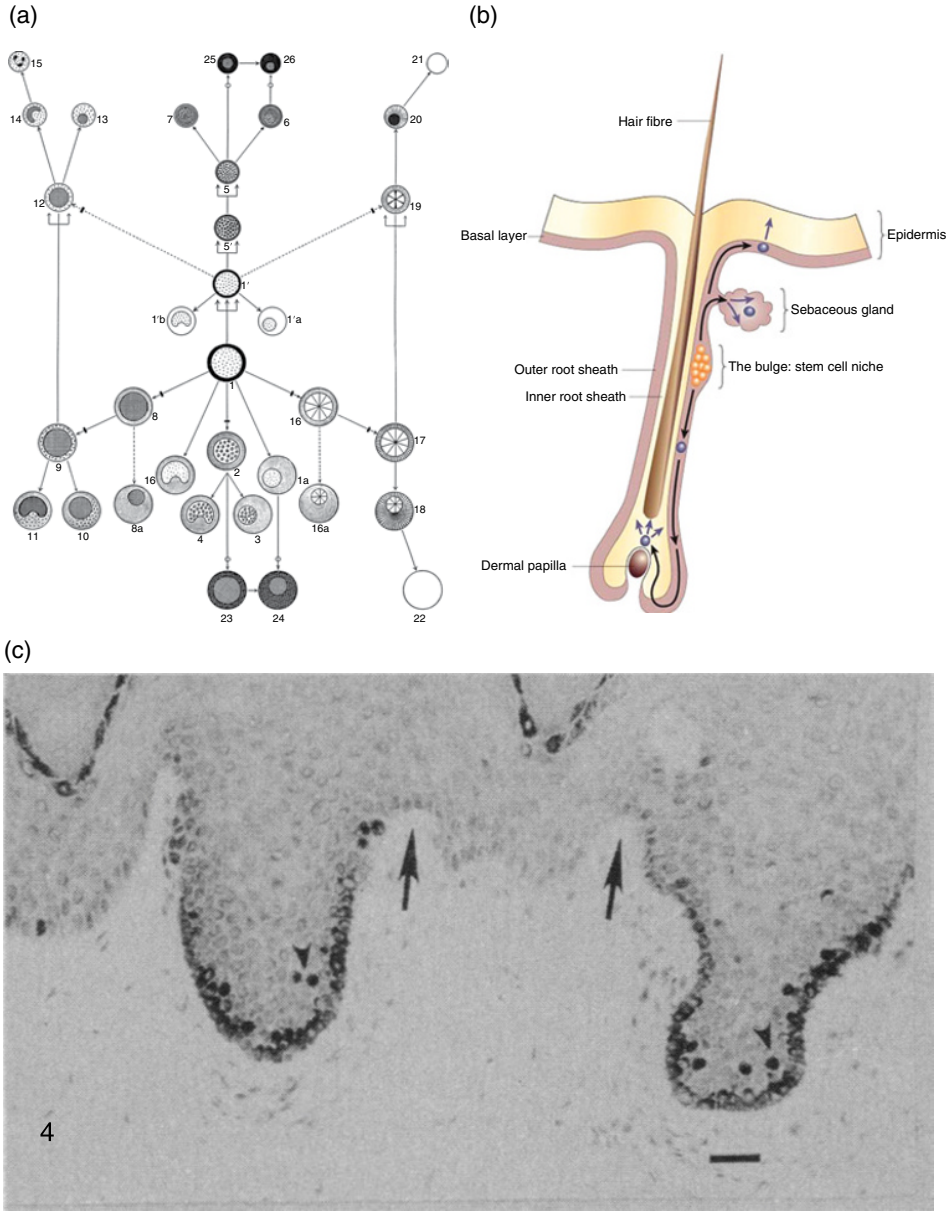

Figure 1.2 Stem cells and their niches. (a) Artur Pappenheim's hypothesis of hematopoiesis from 1905. The center cell, designated a "stem cell," represents the common progenitor of the entire blood system. (b) Stem cells exist in "niches" throughout the body, one of the best characterized being the bulge of the hair follicle. They become active during the anagen phase of the hair follicle cycle, replenishing many of the cell types that contribute to the follicle. Mechanical microenvironments such as topography may provide specific extracellular signals vital for keeping the stem cell in normal homeostasis. (c) Skin stem cells have been postulated to inhabit the rete ridge regions of the basal layer of the epidermis, formed by the epithelial morphology. *Source:* (a) reproduced from Ramalho-Santos and Willenbring (2007); (b) reproduced from Reya and Clevers (2005). Reproduced with permission of Nature Publishing Group; (c) reproduced from Lavker and Sun (1982).

As implied earlier, the provision of chemical signaling is a very well-explored concept in stem cell biology, in the context of both understanding adult stem cell niches and controlling (or not) the differentiation of pluripotent cells, but it is not the whole story. An increasing body of work now indicates that mechanobiological properties of the stem cell microenvironment – particularly the stiffness of the growth substratum – may be of fundamental importance in stem cell biology and regulation.

1.3 Substrate Stiffness in Cell Behavior

We saw earlier that certain cells have evolved to be able to detect externally applied force. However, virtually all mammalian cells need to apply force to their environment. This is seen perhaps most clearly in the "contact dependence" of most adult somatic cell types, where they must interact with a solid extracellular support in order to survive, grow, and divide. In the absence of such attachment, most cells – be they skin cells, muscle cells, brain cells, or otherwise – undergo a specialized form of controlled cell death called anoikis (Frisch and Screaton 2001). (Note that certain cell types, such as cells of the blood, do not share this feature, for obvious reasons.) So, what then is the signal that enables a cell to determine whether it is attached to a solid support? It all comes down to the cytoskeleton of the cell.

A cell's cytoskeleton is a complex arrangement of different polymer filaments that fulfil a number of vital functions – trafficking of organelles (such as endosomes and mitochondria), chromatid separation at mitosis, and motility. Cell motility depends on the interaction of a cell with its external environment, requiring the cell to move in relation to an external frame of reference. In the case of a contact-dependent cell type, this must be a solid support. By simple Newtonian mechanics, if a cell is to move in relation to such a solid support, it must exert a force on it. And if the cell is to gain any purchase on a material, the material must be able to accommodate and resist the force that the cell exerts. For this to occur, the cell must be able to generate tensile force within its cytoskeleton and do work.

1.3.1 A Historical Perspective on Stiffness Sensing

The notion of cells being able to probe the mechanical context of their environment has been appreciated for many years. Work in the 1970s showed that epithelial cells have markedly different morphologies and behaviors depending on whether they are grown on floating collagen gels or on hard growth substrata. Emerman et al. (1977) inferred that, aside from the different access to nutrients and different properties of gas exchange present in floating collagen substrates, the flexibility of the material could be affecting the shape of the cells by a postulated mechanical feedback. In later work (Shannon and Pitelka 1981), the same authors, quite directly, were able to conclude that stiffness (referred to as "flexibility" in their publications) was directly responsible for the functional phenotype of mammary cells on floating gels: while cells cultured as monolayers on floating collagen gels maintained a cuboidal secretory phenotype, cells cultured on the same collagen gels artificially stiffened by gluteraldehyde crosslinking appeared flattened and did not form the mature, secretory phenotype. Concurrently, other groups provided evidence for the accepted idea that cells exert force on the material on which they grow.

By developing a method of producing very thin membranes of silicone rubber, Harris et al. (1980) showed in striking visual images the degree to which silicone-adherent cells were able to deform the surface on which they grew. Most of these early studies did not further explore the biomechanical properties of such ECMs, but interpreted the key findings as being due to cell shape.

At around the same time, Folkman and Moscana (1978) were able to demonstrate experimentally (by reducing the adhesiveness of cell culture substrata) that there was a clear correlation between cell spreading and cell proliferation. This idea had been predicted by other researchers (e.g., Dulbecco 1970), who observed a higher mitotic index in cells given space to spread at the periphery of an artificially created *in vitro* "wound." Nevertheless, Folkman and Moscana (1978) were first to show direct evidence of a dependence of cell division on cell spreading, independent of the effects of (for example) cell–cell contact or cell density. These experiments were extended by Ingber and Jamieson (1985), who developed the idea of the "tensegrity" model of the cell's cytoskeleton – that is to say, that cell phenotype and tissue formation could be regulated by the mechanical phenomena occurring in the cytoskeleton. This led Inger and Folkman (1989) to show the importance of matrix "malleability" in the control of *in vitro*-simulated angiogenesis.

As techniques in bioengineering developed, other groups confirmed the dependence of cell shape and spreading on other cell functions besides division. For example, Watt et al. (1988) developed a method of depositing adhesive ECM islands of areas of between 500 and 2000 μm^2. Primary keratinocytes, plated on and confined to these islands, showed clear phenotypic differences depending on the degree to which they spread. In general, cells on larger islands (which had more space to spread out) synthesized more DNA than those on smaller islands, and the former remained undifferentiated while the latter did not. This idea was investigated several years later by Chen et al. (1997), who demonstrated via experiments based on the principle of depositing defined patterns of ECM on otherwise nonadhesive surfaces that cell spreading, rather than ECM contact area *per se*, influenced cell behavior, including apoptosis and cell proliferation.

Despite a great deal of evidence from the late 1970s and 1980s that the "malleability" or "flexibility" of ECMs could influence how cells behaved, including ideas about how intracellular tension might translate into biochemical signals, it was not until 1997 that the first formal test of how matrix stiffness affects cell behavior was conducted. Pelham and Wang (1997) employed a commonly used laboratory material – polyacrylamide – and varied the ratio of the monomer backbone of the polymer to its crosslinker to produce materials with a range of defined stiffnesses, which they measured simply by hanging weights from the polymer and measuring the extension (many will be familiar with the equivalent school-lab test of Hooke's law). Importantly, they attached thin films of these gels to a solid (glass support) and were able to covalently attach a matrix protein to the surface using polyacrylamide, converting the polymer into a material that could support the culture of a range of mammalian cells. Pelham and Wang were able to show that cells on stiffer substrates exhibited more stable focal adhesions than those on softer surfaces, which were more irregularly shaped and dynamic. The development of this (seemingly simple) technology was timely for those interested in cell traction dynamics, who had been inspired by Harris et al.'s (1980) work on substrate wrinkling. For example, Jacobson and colleagues had previously attempted to extend Harris' work to quantify the tractions that cells exerted on surfaces by using rubber substratum under tension

(Oliver et al. 1995; Dembo et al. 1996). However, these techniques were never optimized for use with mammalian cells. Subsequent to Pelham and Wang's publication, however, Wang teamed up with Micah Dembo to use the polyacrylamide method, combined with the introduction of fiduciary particles incorporated within the gels, to directly measure traction forces (Dembo and Wang 1999). This technique is now called "traction force microscopy" and is an established technique in a number of research fields, with more than 400 publications recorded in PubMed to date (e.g., Plotnikov et al. 2014). In addition, polyacrylamide surfaces also enabled the direct study of empirically defined ECM stiffnesses on a range of cell types. For example, in an echo of Inger and Folkman (1989), Deroanne et al. (2001) showed that a reduced substrate stiffness promoted tubulogenesis in endothelial cells, while Wang's group extended its earlier findings by showing that substrate stiffness could affect the motility of cells (Lo et al. 2000) and was a more important factor in the behavior of normal cells than were transformed cell lines (Wang et al. 2000).

Other groups began to take interest. In 2004, a group led by Dennis Discher showed that ECM stiffness was particularly important in the growth and differentiation of muscle cells (Engler et al. 2004). It demonstrated that while the formation of myotubes from myoblasts was unaffected by the stiffness of the ECM (though the subsequent phenotypic differentiation was affected), only those myotubes on ECMs with a stiffness corresponding to the stiffness of the tissue found *in vivo* formed striations. Together with the earlier observations, this study brought into sharp focus some of the disadvantages of the accepted methods of cultivating cells on rigid materials (glass or plastic). To date, most groups still work with rigid growth materials, but it is notable that there is a keen drive to provide more realistic methods of organ/tissue culture for drug testing (Feng et al. 2013), and several companies now make a business from selling growth substrata of defined stiffness (e.g., Matrigen, www.matrigen.com).

Subsequently, Discher's group highlighted the importance of mechanosensing in tissue cells (Discher et al. 2005), before publishing a seminal research paper showing that matrix elasticity alone can direct the differentiation of stem cells (Engler et al. 2006). The influence of this latter publication is reflected in the number of citations it has received (>5000) and the increase in the popularity of research on stem cell mechanotransduction.

1.4 Stem Cells and Substrate Stiffness

Discher et al. (2005) showed that a population of stem cells isolated from the bone marrow – mesenchymal stem cells (MSCs) (note that this term is somewhat controversial: the cells they studied may be more accurately referred to as "marrow stromal cells," a mixed population of primary cells likely to contain populations of stem and progenitor cells (Bianco et al. 2013)) – assumed different morphologies as a function of substrate stiffness. Moreover, over a period of several days, cells adherent to soft matrices (<1 kPa) began to express proteins specific to neuronal lineages, those on intermediate stiffnesses (~10 kPa) began to express markers of muscle differentiation, and those on stiffer surfaces began to express markers of bone cell differentiation (~30 kPa). This was tentatively shown not to be due merely to ECM surfaces preferentially selecting the adherence of one progenitor over another, as the authors could show transdifferentiation of cells

over a period of time. These data reflect earlier work by McBeath et al. (2004), who showed adipogenic differentiation of MSCs confined to small islands and osteogenic differentiation on large islands (using a similar strategy to that employed by Watt et al. 1988). One might infer from these data that it is the stiffness-mediated change in cell shape that controls the phenotypic response, but Tee et al. (2011) have shown that when cell spreading is controlled and equalized on substrates of differing stiffnesses, cells remain able to modulate their cytoskeletal properties based on the stiffness, independent of the degree of spreading.

1.4.1 ESCs and Substrate Stiffness

What about other stem cells? Li et al. (2006) have shown that human ESCs can be maintained in an undifferentiated state on polymeric substrates with tunable stiffnesses. Later, Evans et al. (2009) showed that substrate elastic modulus can affect the initial differentiation behavior of murine ESCs, with stiffer substrates promoting mesendodermal differentiation and softer surfaces promoting ectodermal differentiation. This led to a greater differentiation of these stem cells to the osteogenic lineage. In the same study, collagen-functionalized polydimethylsiloxane (PDMS) was used as an ECM material, with stiffnesses ranging from 40 kPa to several megapascals, rather than the 0.1–50.0 kPa range that is investigated using polyacrylamide (Evans et al. 2009). In a series of papers later published by Ning Wang's group (Chowdhury et al. 2010a, 2010b; Poh et al. 2010), it was found that in contrast to many other mammalian cell types, murine ESCs are not sensitive to the modulus of their substrate and do not spread when in an undifferentiated state, even on stiff surfaces. In addition, cultivating cells on soft substrates could promote sustained self-renewal even in the absence of chemical factors normally required for self-renewal (leukemia inhibitory factor, LIF). Finally, mechanical stimulation of ESCs by exertion of torsional forces at the cell surface using arginylglycylaspartic acid (RGD)-conjugated beads could induce differentiation. This highly interesting work suggests that murine ESCs are an example of a cell type that does not have the ability to probe and sense ECM stiffness, but does however have the ability to detect applied force. Reflecting this, it has been shown that murine ESCs are unusual among mammalian cells in not being dependent on adherence to a surface for survival – they can be grown in suspension when cell aggregation is prevented. Recent work has shown that murine ESCs can be maintained in suspension in spinner flasks when an antibody against E-cadherin is added to the growth medium (Mohamet et al. 2010). This may also explain the requirement for the widespread use of gelatin coating as a substrate for murine ESC culture. Though one might expect gelatin to promote cell adhesion, in some cases it is used as an additive to prevent protein adsorption to surfaces and cell attachment (Milne et al. 2005). While this has never been tested, it may be speculated that gelatin facilitates self-renewal of murine ESC by allowing the growth of loosely adherent colonies while preventing the growth of more adherent, differentiated cells that arise spontaneously during cultivation. In an interesting discussion, Chowdhury et al. (2010b) speculated that early single-celled eukaryotes may have been subject to an evolutionary advantage that made them stiffer, enabling them to engage in mechanical functions such as invasion and crawling around the earth's primitive ocean floors, and that the mechanical state of ESCs is an echo of the early origins of multicellular life. It is therefore probable that in work investigating the effect of stiffness on murine ESC

differentiation, the true effect of matrix stiffness is on the differentiation or selection of progenitor cells that arise stochastically during the very early stages of ESC commitment. Despite this, matrix rigidity or stiffness has been shown to affect the differentiation of murine ESCs into a range of different cell types, including cardiomyocytes (Shkumatov et al. 2014), pancreatic β cells (Candiello et al. 2013), endoderm (Jaramillo et al. 2012), and neurons (Keung et al. 2012).

Human ESCs are strikingly different from their murine counterparts. Whereas the latter form compact, sometimes multilayered, domelike colonies *in vitro*, the former grow as tightly packed epithelial sheets (Figure 1.3). In fact, the survival of human ESCs is linked to their cell–cell adhesive properties, and propagation efficiency decreases markedly on cell dissociation (in direct contrast to murine ESCs). It has been shown that this can be mitigated by the inclusion of a rho-associated protein kinase (ROCK) inhibitor (Y-27632) in the growth medium, which is thought to act by inhibiting cell contractility (Watanabe et al. 2007). An increase in the activity of the actin–myosin system is thought to be the reason for this apoptosis, which is usually prevented when the cells are adherent to one another and cytoskeleton tension is optimal (Ohgushi et al. 2010). Some have speculated that this reflects the embryonic origin of human ESCs as compared to murine ESCs. Human ESCs are similar to cells of the epiblast – a polarized epithelium that arises in the blastocyst – while murine ESCs are similar to the inner cell mass, which has no obvious polarity (Figure 1.3). Correct development of

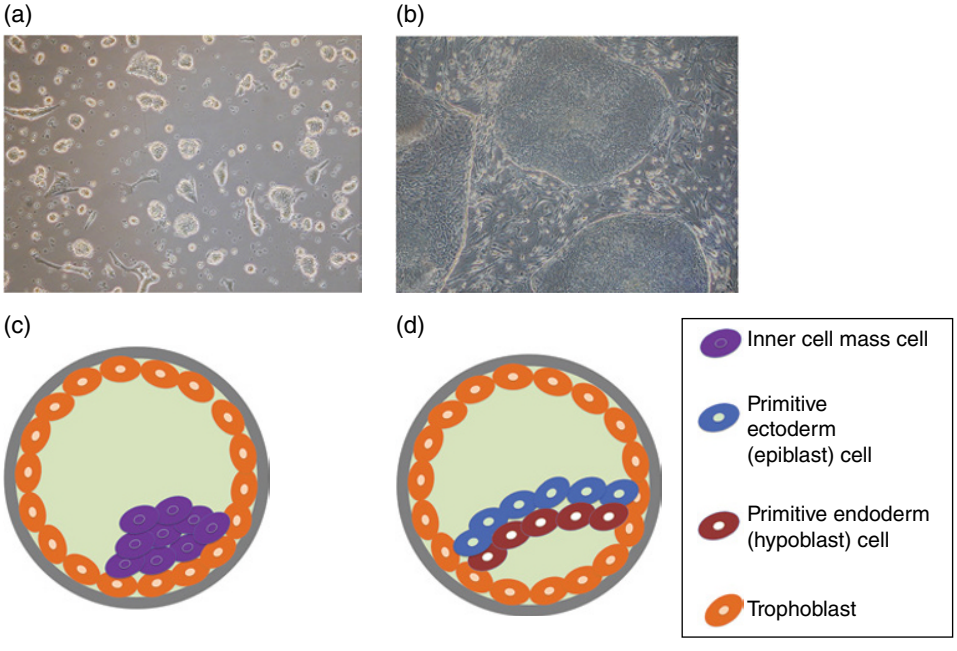

Figure 1.3 ESCs: differences in origin. (a) Murine ESCs form domelike, rounded colonies several cell layers thick, whereas (b) human ESCs form flattened, epithelial colonies. This may reflect differences in their origins. (c) Murine ESCs are thought to be analogous to cells of the inner cell mass of the embryo, which has no obvious polarity. (d) On the other hand, human ESCs (and murine EpiSCs) are likely to be more closely related to cells of the epiblast of the blastocyst. This structure is a polarized epithelium covering a basement membrane on the surface of the primitive endoderm (hypoblast). (*See insert for color representation of the figure.*)

the primitive ectoderm from the epiblast relies on appropriate patterning of cells, and it may be that cells that lose epithelial integrity and become detached from the epiblast sense the change in their mechanical microenvironment and are programmed to die by apoptosis (Ohgushi and Sasai 2011). Note that murine epiblast stem cells (EpiSCs) – which have many of the characteristics of human ESCs – can now be isolated from murine blastocysts at later time points compared to the original ESCs researched since Evans and Kaufman's 1981 paper (Brons et al. 2007), indicating that murine and human ESCs as commonly studied reflect mammalian tissues at two distinct developmental time points.

These data imply that matrix attachment and control of cell contractility in human ESCs within colonies may be more critical for the early differentiation of these cells than for murine ESCs. But in contrast to the large literature on murine ESCs and adult stem cells, research on the effect of matrix mechanical properties on human ESCs is poorly represented. Work from Healy's group demonstrated that human ESCs could be grown on materials with tunable stiffnesses (Li et al. 2006), but a PubMed search of "('human embryonic stem cell' OR 'human embryonic stem cells') AND ('stiffness' OR 'elasticity' OR 'rigidity')" at the time of writing yields fewer than 30 publications, many of which focus on the rheological properties of the cells themselves, rather than specific effects on their differentiation or self-renewal. In an example of the later, direct approach, Sun et al. (2012) investigated the effects of stiffness on human ESCs of using bendable PDMS pillar arrays, which they contended could be used to approximate "effective" stiffnesses of between ~2 and >1000 kPa, and measured self-renewal markers and E-cadherin expression in single cells and small aggregates of cells. They found higher expression levels of OCT4 in cells on matrices with higher effective stiffnesses, reflecting the fact that these cells are mechanosensitive and exhibit the correct phenotypic responses only when adherent to a surface with the optimal stiffness. Narayanan et al. (2014) produced growth substrates exhibiting a range of stiffnesses by decellularizing native ECMs and were able to show lineage-specific differentiation, though note here that because the chemical and physical properties of the ECMs were adjusted together with stiffness, it is not possible to judge any independent stiffness effect. Finally, Arshi et al. (2013) used a PDMS system similar to that of Evans et al. (2009) and found a preference for ESCs (initially differentiated in suspension culture as embryoid bodies) to differentiate into cardiomyocytes on surfaces of a higher stiffness.

One possible reason why the literature on the effect of matrix stiffness is limited in the case of human ESCs is their rather fickle growth conditions. Though the culture and isolation of human ESCs was first reported in 1998, it remains technically challenging and labor-intensive to grow these cells. Today, in most labs, human ESCs are grown on a feeder layer of murine embryonic fibroblasts. These cells provide a host of insoluble and soluble chemical cues to facilitate self-renewal. Otherwise, human ESCs are routinely grown on a propriety complex matrix preparation called Matrigel in the presence of medium conditioned by embryonic fibroblasts. Discovering a matrix that allows a more convenient method of propagating cells is currently a priority in the field, and matrices based on laminin isoforms are a particularly active area of research (Rodin et al. 2014).

To try and facilitate growth of human ESCs on polyacrylamide substrates, Weaver's group has published a methods paper demonstrating crosslinking of Matrigel to polyacrylamide surfaces (Lakins et al. 2012). This group fabricated polyacrylamide gels of ~100 μm depth and crosslinked Matrigel to the hydrogel via an ultraviolet (UV)-catalyzed

conjugation of a protein-reactive N-succidimidyl ester to the gel surface. Interestingly, the group found clear morphological differences at different stiffnesses. Colonies on soft materials formed epithelial layers that were more columnar in nature than those on stiffer substrates, with a higher aspect ratio, basally displaced nuclei, and better developed E-cadherin staining at adherens junctions between cells. Very recently, the same group published another paper advancing this method and incorporating traction force microscopy to measure matrix deformations beneath colonies (Przybyla et al. 2016). These two papers are particularly exciting as they begin to address the often overlooked question of how groups of cells perceive stiffness, as compared to individual cells. In addition, they provide a methodological framework for probing the effect of the mechanical microenvironment in very early developmental events, by determining how local changes in, for instance, tension in epithelia map to changes in the phenotypic behavior of cells. This avenue of research is likely to yield some very interesting data over the coming years.

1.4.2 Collective Cell Behavior in Substrate Stiffness Sensing

One key question that is beginning to gain recognition is how cells might behave collectively to probe the stiffness properties of the extracellular environment that they inhabit. It is certainly true that many cells behave individually when probing the mechanical properties of their environment (e.g., mesenchymal cells, such as fibroblasts and macrophages). A far greater number of cell types, however, rely on cell–cell contact. This is particularly true in epithelia, where tight adherens junctions ensure barrier function and the integrity of the tissue as a whole (e.g., in skin and gut, and of course in embryonic tissues, as already discussed). In this way, the mechanobiology of the growth environment may be probed collectively at the tissue level rather than at the single cell level (which is the level most often studied in the literature). By acting together in this way, such cell collectives may be able to gain physical and mechanical information about their environment that would be unobtainable if they were to act as individual cells. As a rather crude analogy, consider a long line of people linked arm to arm. If a trap door were to open somewhere along the line, the force required to prevent the unfortunate people previously standing on it would have to be borne by others in the line. Someone standing a significant distance away from the trapdoor would understand that an event had happened, know that it had happened in a particular direction, and have to change their behavior according (perhaps by leaning to one side). There is plenty of evidence that cells behave in the same way, *in vivo* and *in vitro*. For example, groups led by Fredberg and Trepat have shown long-range force propagation in epithelia, in response to both pushing and pulling. When epithelial layers are disrupted, or when a gap in an epithelium is engineered by allowing cells to grow around a post and then removing it, cells migrate and divide to reoccupy the empty space (Tambe et al. 2011; Anon et al. 2012). Trepat and Fredberg (2011) use the analogy of a mosh pit to help explain how this happens. Cells are constantly migrating, but in epithelia they are closely packed and therefore constrained. Following the formation of a wound edge, cells make net movements into the space, dragging (and being constrained by) cells behind them, to which they are attached. This leads to the formation of more space in areas distal to the "wound," allowing other cells the freedom to migrate in the direction of the space, or to stimulate their division.

These types of "long-range" force transmission may become particularly important in the mechanisms through which groups of cells probe substrate stiffness. In the early experiments on floating collagen gels, Emerman et al. (1977) found that mammary epithelial cells grown on unconstrained, floating soft collagen gels were able to contract the gels to around one-quarter of their original size, illustrating that groups of epithelial cells can exert significant force at their basal surfaces. Later, Trepat (2009) found that colonies of Madin–Darby canine kidney (MDCK) epithelial cells grown on basally adhered polyacrylamide gels (<100 μm in thickness) were insensitive to their substrate stiffness when grown as colonies but not when grown as single cells. These observations are important because they suggest, first, that groups of cells acting in concert are able to significant deform soft ECM materials and, second, that constraining a colony of cells may render the cells incapable of detecting the modulus of the material on which they grow. To understand why this might be the case, we must consider a number of reported studies on cells grown on substrates that vary in thickness.

Buxboim et al. (2010) developed a technique for casting polyacrylamide gels of various thicknesses adhered to an underlying glass support (in a manner similar to Pelham and Wang 1997). They found that even at very low elastic moduli (<1 kPa), at certain depths, cells started to behave as if they were on much stiffer gels (when the thickness was decreased to <10 μm). Lin et al. (2010) have provided a theoretical explanation for this phenomenon, which relates this "critical depth" to a value approximate to the lateral dimension of the adherent cell. In simple terms, the reason that cells can detect substrate depth in this way is because of the manner in which a cell probes the stiffness of its substrate. As a cell makes focal adhesions, it begins to contract, exerting a shear force on the ECM and detecting the stiffness of the material by monitoring the resistance to this force. However, in this case the force required to deform the surface of the ECM a given distance is dependent not only on the Young's modulus of the material, but also on the thickness. One can understand this more clearly by using an analogy: it is much easier to shear the surface of a deep plate of jello (referred to as jelly in the UK) than that of a very thin one, even though the Young's modulus of the material remains constant (see Evans and Gentleman 2014). As there is less gel, and because it is prevented from moving at its basal surface, a lateral shear deformation of a given magnitude will impart a much greater strain on the thin gel than on the thick one (Figure 1.4).

What are the consequences of this effect for groups of cells? Epithelial cells foster very tight intracellular junctions, and are able to exert significant force on ECMs, and therefore significant strains. Recent data from Zarkoob et al. (2015) support this. This group found average surface matrix deformations of ~4.2 μm for single keratinocytes, compared with 19.4 μm for groups of around eight cells, with some deformations reaching more than 100 μm. Similarly, Mertz et al. (2012) found that contractile forces scaled with the size of the colony. Though not tested, these deformations must penetrate significant depths into hydrogel substrates. Taking into account theoretical considerations of depth sensing (Lin et al. 2010; Edwards and Schwarz 2011; Banerjee and Marchetti 2012), this may provide an explanation for why Trepat (2009) found no effect of substrate on large colonies (millimeter-size) of MDCK cells: by acting collectively, cell-comprising colonies measure a greater stiffness on fixed elastic substrata than their Young's modulus would suggest. How deeply might colonies of cell sheets feel? This remains unknown, but based on the theoretical work and on Emerman's observations in the 1970s, it might

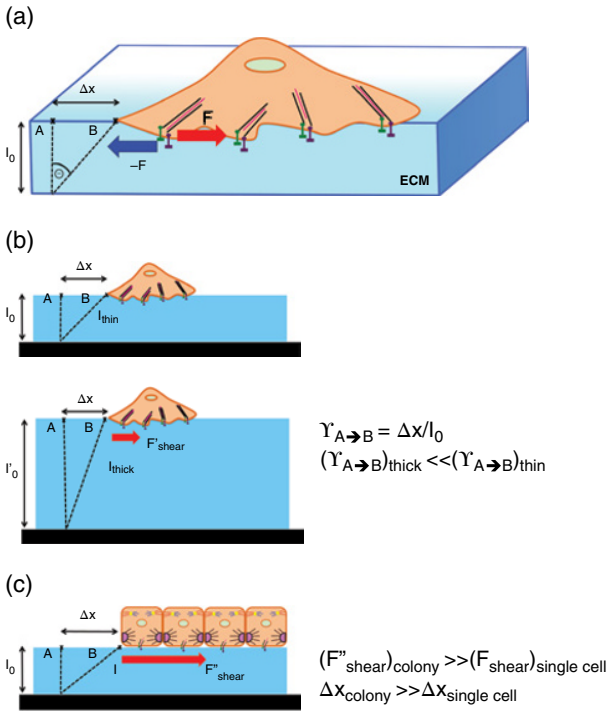

Figure 1.4 Cellular mechanosensing of substrate thickness. (a) In order to contract a gel from A to B for a distance Δx, a cell needs to form focal adhesions on a solid support, as shown in the cartoon schematic (the cones represent the integrin connections), and then exert a force (right arrow). During this process, a tensile force (skewed lines represent the actin–myosin dependent contraction) is generated in its cytoskeleton. The material has to be able to resist and accommodate to the force that the cell applies, in this case a shear force. (b) and (c) illustrate in a simplified way the difference in force that a cell must exert in order to contract a thick versus a thin gel of equal shear modulus. (b) The shear strain is measured as the ratio between the transverse displacement of the gel (Δx) and its initial length (l). An attached cell exerts a shear force on the gel (top left) and deforms it to a distance Δx. An equal deformation Δx in the direction A → B requires a greater shear strain ($\Upsilon_{A\rightarrow B}$) on thin gel compared with thick. Even if the shear modulus of the material is the same, the shear stress required to deform the thin gel is greater than that required for the thick gel. As a consequence, the tension generated in the cytoskeleton may reach a critical threshold on thin gels, causing the cell to spread more; on thick gels, the cell may be unable to generate the same tension, and thus remains rounded. (c) For colonies of cells, the transverse displacement may be greater than that for a single cell. This may be a collective behavior mediated by tight intracellular interactions. Note that this figure is for explanatory purposes only and ignores many variables.

be related to the size of the sheet: many hundreds of microns. Furthermore, epithelia would be able to detect not only uniform changes in substrate thickness, but also regional changes determined by stiff objects or heterogeneities deep within the hydrogel.

Collective stiffness sensing may have important implications in many areas of biology. One example is in skin wound-healing. To facilitate wound coverage and healing, a new layer of epithelium must migrate out over the surface of the underlying granulation tissue. The stiffness of this material and its heterogeneity may play an important role in

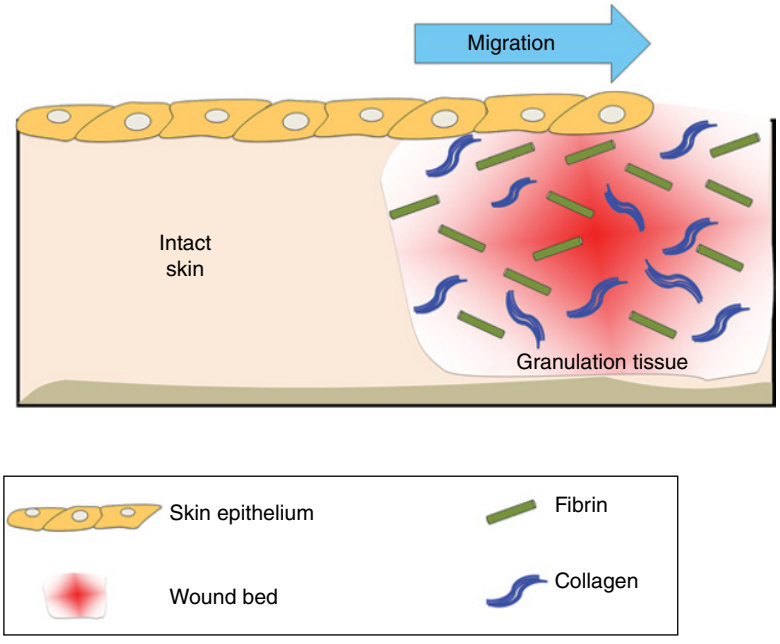

Figure 1.5 Mechanosensing of the stiffness and heterogeneity of the wound granulation tissue is important in wound-healing. A new layer of epithelium has to migrate from the intact skin over the granulation tissue. Granulation tissue is formed in the wound bed after skin injury. It is a heterogeneous material, manly formed of fibrin and type III collagen. During the healing process, its stiffness varies, and this might influence skin repair.

the rate of cell migration and in cell patterning and differentiation (Figure 1.5). Some data support this already (Wang et al. 2012). Further, embryonic development involves the rapid movement of a variety of different epithelia. Such epithelial tissues may be influenced not only by chemical signaling gradients, but also by the material characteristics of structures in other parts of the embryo, which they detect as mechanical gradients. Mechanically informed patterning may be a crucial but overlooked mechanism of mammalian development.

1.5 Material Structure and Future Perspectives in Stem Cell Mechanobiology

Artificial ECMs are useful for reductionist experiments aimed at determining the effect of a limited number of parameters on cell behavior, but they do not approach the complexity of ECMs *in vivo*. Those that have been investigated *in vitro* include (but are not limited to) synthetic materials such as polyacrylamide, PDMS, polyethylene glycol (PEG), and naturally derived materials such as alginate, collagen, and hyaluronic acid. These materials are discussed in a recent review (Evans and Gentleman 2014). Polyacrylamide has been the most popular, probably due to its ease of fabrication, linear elastic behavior, nontoxicity, and low cost. However, like all materials used in such experiments, it does have some limitations. First, though it is generally accepted that it

behaves in an elastically linear fashion (Style et al. 2014), at greater deformations (such as those that might be exerted by groups of cells) this approximation does not hold (Boudou et al. 2009). Second, it is nonadsorbing, so matrix proteins must be chemically crossslinked to its surface to enable cell attachment. In principle, this could be any ECM protein with a reactive side group, but type I collagen is most often used. Third, and perhaps most importantly, it is difficult to modulate its stiffness without affecting the degree of crosslinking and porosity of the gel. This point was illustrated recently by Trappmann et al. (2012), who provided evidence to show it is the porosity of polyacrylamide, rather than the bulk stiffness, that is responsible for the cell behavior seen at low stiffnesses. They showed that porous gels that had been artificially stiffened had very similar effects to their unstiffened counterparts: cells remained rounded on both. They attributed this to the difference in ligand tethering on soft (large pores) versus stiff (small pores) gels: on the latter, the anchoring points between the collagen ECM are close together, while on the former they are wide apart. The authors suggested that large ligand spacing leads to loosely attached, floppy collagen fibers, which are perceived by the cell as a lower gel stiffness, regardless of the bulk modulus. It is somewhat difficult to reconcile these data with the large body of literature on traction force microscopy, however, which relies upon the displacement of fiduciary markers *within* the hydrogel for the calculation of cellular contractile forces. And if this theory were correct, it would predict that there are no depth-dependent effects on detection of substrate elasticity: surface ligand chemistry remains constant *regardless* of the thickness of the gel.

Despite this, most natural ECMs do not behave in a predictable, linear fashion. For example, collagen has a hierarchical structure, consisting of interlocking fibrils that may be elongated or coiled. It has been shown to exhibit viscoelastic behavior, its stiffness changing over time and with the rate and magnitude of applied force (Knapp 1997). These nonlinear effects can be envisaged by thinking of the collagen as a tangled ball of fibers: under a given applied force, some of the fibers will resist under tension, while others will be under shear or compression. Illustrating this, it was found in studies of cells growing on collagen substrata of various thicknesses that fibroblasts began to "depth-sense" the underlying glass support at much greater depths than they did on polyacrylamide (~65 versus ~5 μm) (Rudnicki et al. 2013). This was attributed to the effect of fiber alignment, which becomes more prominent at smaller scales. In effect, as the thickness decreases, cells begin to measure the stiffness of collagen fiber bundles held taut from their anchor point at the underlying glass surface to the surface of the gel at the cells' focal adhesions, rather than the stiffness of a randomly arranged network of interconnected fibers. It is likely that many other ECMs found *in vivo*, such as those composed of other collagens or of composites of collagens and other natural materials, will display similar behavior.

1.6 Conclusion

Stem cell mechanobiology is a fascinating topic that will continue to occupy the efforts of a diverse range of researchers over the years to come. This research will require the input and collaboration of experts from a great variety of backgrounds, from physicists and engineers to medical scientists and developmental biologists. Understanding how our bodies develop and respond to disease and infection is essential to human health and well-being, and mechanobiology is now appreciated as a key player in this field

(e.g., in cancer (Butcher et al. 2009) and in scarring (Engler et al. 2008)). In biomedical engineering, materials and devices must be designed to interface closely with body tissues, and an appreciation of the mechanical effects of this will be important. In regenerative medicine, materials are designed to encourage the restoration of tissue function, which depends largely on the behavior of stem cells; answering fundamental questions about the response of stem cells to ECM mechanics, in parallel to the cohesive tissues in which they reside, will be key to the success of these strategies in the future.

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2

Molecular Pathways of Mechanotransduction

From Extracellular Matrix to Nucleus

Hamish T. J. Gilbert and Joe Swift

Wellcome Trust Centre for Cell-Matrix Research, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK

2.1 Introduction: Mechanically Influenced Cellular Behavior

A broad range of cellular phenomena are responsive to the mechanical properties of the local environment. These include alterations to: cell morphology (Pelham and Wang 1997) and contractility (Discher et al. 2005), often manifested in changes to cell spread area and the "focal adhesion" (FA) complexes that interface the cellular cytoskeleton and substrate; cell motility or "durotaxis" – movement directed by a gradient of matrix stiffness (Lo et al. 2000; Winer et al. 2009; Hadjipanayi et al. 2009b; Raab et al. 2012); cell proliferation rates (Klein et al. 2009; Hadjipanayi et al. 2009a); and apoptosis (Wang et al. 2000). One of the most exciting effects of environmental mechanics is on stem cell fate, leading them either to remain quiescent or to divide asymmetrically to facilitate commitment to lineage. The "stem cell niche" is broadly defined as the set of local environmental influences that can affect stem cell behavior, and combines chemical (e.g., soluble factor) and mechanical (e.g., substrate stiffness) inputs, both of which include contributions from the extracellular matrix (ECM) and surrounding cells (Schofield 1978). Mesenchymal stem cells (MSCs) have often been used in studies of mechanobiological processes, with early reports noting sensitivity to mechanical stimulation (Pittenger et al. 1999). More recent work has shown MSCs to have increased tendency toward soft-tissue lineages, such as fat, when cultured on soft substrate, and toward stiff-tissue lineages, such as bone, when on stiff substrate (Engler et al. 2006). The mechanical inputs that cells interpret are a combination of force and geometry over length scales of nano- to micrometers (Vogel and Sheetz 2006), but in all cases signals are eventually transduced through to changes at a molecular level. To give the required specificity of action, for example in turning a genetic program on or off, these molecular-scale signals must be regulated with exquisite spatial and temporal accuracy.

This chapter discusses each of the primary modes of molecular mechanosensing, starting outside the cell in the matrix and working into the nucleus (see Figure 2.1). Though a number of specific signaling modes are discussed and exemplified, spatial and temporal control of signaling is achieved through two recurring motifs: (i) *force-mediated regulation of activity through chemical modification*; and (ii) *force-mediated regulation*

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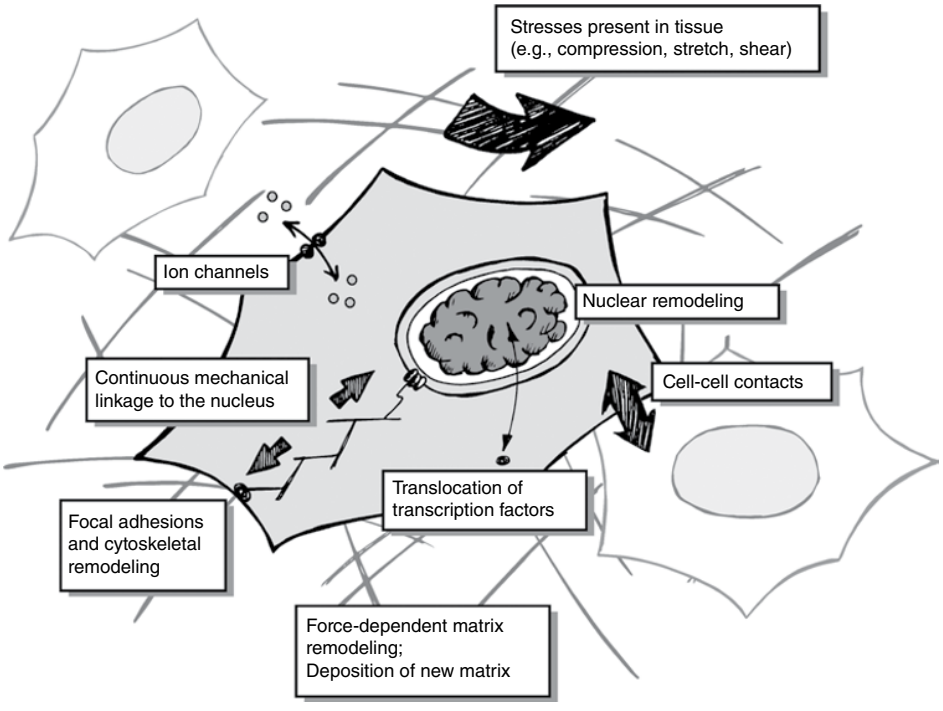

Figure 2.1 Overview of mechanotransduction pathways. Cells in tissue are subjected to stresses in the forms of compression, stretch, and shear; these perturbations may reach a cell through contact with matrix and extracellular fluids, or through cell–cell interfaces. Forces are passed through FAs at the cell surface, via a network of continuous mechanical linkages in the cytoskeleton, and into the nucleus. Structures within the matrix, cellular membrane, cytoskeleton, and nucleus are continuously remodeled in response to mechanical perturbations, and changes are transduced into molecular signaling pathways, such as through activation of ion channels or transcription factors (TFs). These signals are ultimately interpreted to affect cellular behavior.

of activity through change in the distribution, localization, or conformation of molecules. In many cases, these processes occur in concert; for example, a post-translational modification such as phosphorylation may alter the mobility of a protein, or a change in protein conformation may regulate its susceptibility to modification. We will also address some of the technological advances that have made the study of mechanotransduction pathways feasible, in the development of increasingly sophisticated *in vitro* models of tissue, as well as analytical methods that have allowed detailed study of mechanical properties, morphology, and composition.

2.2 Mechanosensitive Molecular Mechanisms

2.2.1 Continuous Mechanical Linkages from Outside the Cell to the Nucleus

The structure of a cell is maintained as it pulls against the matrix and cells that surround it, in a system of permanent stress caused by the cytoskeleton and myosin molecular motors (Ingber 2006). Cell–matrix contacts are mediated by membrane-spanning