Martha M. Murray
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Braden C. Fleming
Editors

The ACL Handbook

Knee Biology, Mechanics, and Treatment

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ISBN 978-1-4614-0759-1 ISBN 978-1-4614-0760-7 (eBook)
DOI 10.1007/978-1-4614-0760-7
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2013935492

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

To our patients, who motivate us every day to put our best effort toward finding improved treatments for ACL injury.

Preface

Emily was driving toward the basket, a move she had done a 1,000 times before. She went up for the shot, and upon landing, her knee gave way. She felt a shifting in her knee microseconds before she heard a "pop." After dropping to the court and grabbing her knee, she was carried off the court by her concerned coach and trainer. They wrapped her knee in ice packs and sent her to see her doctor in hopes it was not a devastating ACL injury. Her visit with me, an Orthopedic surgeon, would confirm a diagnosis of an ACL tear – a seeming death sentence for so many athletes.

Upon hearing her diagnosis, her questions were numerous and understandably so. "Will I play again?" "Will I be as fast as I was before my injury?" Her most pressing question was "When can I get back on the court?"

ACL injuries affect the lives of hundreds of thousands of people every year. The patients who sustain these injuries and the families, teammates, coaches, and healthcare providers, who care for these patients through their injury and recovery, all have questions related to this injury. Currently, we know the answers to some of the questions. For the most part, we know that patients can get back to sports after ACL injury, and with proper training, their performance can be where it was before the injury. Other questions we do not know the answers to, such as who will develop arthritis after their ACL injury and how that can be prevented.

Great work is going on in this field in an effort to prevent injury and to improve treatment options for our ACL-injured patients. In this book, we attempt to distill all of this information to make the science behind the treatment of ACL injuries more understandable. As you will see, much has been learned in this field, but there is substantial room for improvements.

Emily went on to have ACL reconstruction surgery and is back playing basketball. She underwent an extensive period of rehabilitation, and her mom feels she is playing even better now than before her injury. But still we wonder – can we someday return the joint to a more normal status? Can we prevent arthritis in these patients as they get older? Can we get the ACL to heal after a tear, rather than replacing it with a tendon graft? When given the right biological signal, could

ligament repair be a better long-term solution for Emily and individuals like her? These questions keep us, and many other doctors and researchers, working toward an improved understanding of the ACL and its response to injury. We hope you enjoy the material presented here, and we also hope the work in this area will lead us to better solutions for the treatment of ACL injuries, solutions which will involve repair and regeneration of this crucial ligament instead of its replacement.

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Part I
ACL Injury: The Clinical Problem

Chapter 1
ACL Injury Epidemiology

Patrick Vavken and Martha M. Murray

The book you are about to read focuses on ACL tears, why they do not heal, how to best treat them today, and what may be the best ways to treat them in the not too distant future. ACL tears have gained a fixed place in common knowledge, not only because of the devastating effects they have on the careers of sports idols but also because of the same effects they may have on friends and family members who may have suffered from such an injury (Fig. 1.1). But despite this "popularity" and the considerable amount of research done in this field, there are still a few very basic questions that deserve attention.

First and foremost, who is likely to tear his or her ACL? Identifying the risk factors for injury may help us focus our attention on individuals who are at risk, help them avoid high-risk situations, or direct them to preventative treatments. There are other important questions as well. For example, if a person has an ACL tear, is he or she more likely to tear the other ACL too? How many ACL tears are there in a given year? This will tell us how many knee surgeons we need and how many ACL injuries the medical system needs to be able to cope with. What happens after an ACL ruptures and how are ACL tears currently treated? Which procedures have the highest success rates? Which procedures do not work at all, and which people are at risk of re-tearing their ACL? All these questions, and many more, are of interest not only

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_1,

Fig. 1.1 High school women's lacrosse is one of the sports where athletes sustain ACL tears. Young women are at particular risk for this injury

for patients and their families but also for physicians, physical therapists, certified athletic trainers, insurers, and coaches.

Epidemiology is the study of the distribution and determinants of health and disease which enables us to answer many of the questions listed above. The most important tools for epidemiologists are surveys and large databases that collect data on a wide range of patients or subjects at risk to deduce effects from observed trends and commonalities.

Where Does the Epidemiology Data Come From?

Traditionally, epidemiologists have relied on the recollection or documentation of individual physicians or departments for data on ACL treatments and their outcomes, leading to broad statements such as "100,000–400,000 tears in the USA per year" or "one every 6 minutes in Germany." Naturally, such data come with a high chance of bias (an error consistent with a systematic deviation from the truth) and reports were frequently in conflict. Also, since it is not unusual that a year or more passes between a scientific study and its publication, such data are often outdated. Recently, there has been a major movement towards evidence-based outcome documentation with large prospective cohort studies and patient registries. Physicians at academic institutions in the United States have started multicenter studies to collect patient data independently and systematically, for example, the MOON (Multicenter Orthopedic Outcomes Network) [1, 2] and MARS (Multicenter ACL Revision

Study) [3, 4] cohort studies. The MOON group has studied over 2,700 patients undergoing ACL reconstruction, with 85 % follow-up at 6 years postoperatively.

Registries are more common in the Scandinavian countries [5–8]. With their socialized health-care system, all ACL treatments are recorded and can be linked to other patient data (age, gender, etc.) via social security numbers. To date, these registries have systematically collected data on tens of thousands of ACL patients. Similar endeavors have been implemented with success, for example, in Denmark or Italy. While these registries can enroll a large number of patients, one of the drawbacks has been difficulty in following the patients postoperatively because of the high rate of patients lost to follow-up. Nonetheless the registries are most useful for generating epidemiologic information about who is getting ACL tears at this time. In the United States, such registries are being set up but are run by insurances or health-care companies. For example, Kaiser Permanente has a registry of approximately 5,000 ACL patients. Naturally, conflict of interest may be an issue in a registry run by a commercial, for-profit company.

How Often Do ACL Tears Happen?

A good first question to start looking at the epidemiology of ACL injury is the frequency of ACL tears. In epidemiological studies, this is usually given as incidence, or the number of ACL tears per 1,000 people in 1 year (for all calculations, we assume the United States population is 320 million people). All additional risk factors set aside, current estimates are that 1–10 in 1,000 people tear their ACL per year. Assuming the current United States population is approximately 320 million, this translates into 32,000–320,000 ACL tears in the USA per year. Others have estimated this number to be as large as 400,000 per year [9]. For comparison, a year has roughly 10,000 h (8,736 h), which means there are roughly 3–40 ACL tears per hour in the United States. Official registries from other countries support these numbers: in Scandinavia (population = 25 million), there are two ACL tears every hour, and Germany (population = 82 million) and Switzerland (population = 8 million) each have one ACL tear every six minutes and every hour, respectively.

Who Tears an ACL?

ACL tears most frequently occur in situations of a valgus or anterior stress to the extended knee (Fig. 1.2) but can also occur during hyperextension or extreme internal rotation of the tibia. While this is usually associated with the image of a high-impact trauma, such as those seen in professional football or basketball, it is very important to realize that 80 % of all ACL injuries are “noncontact” injuries. These

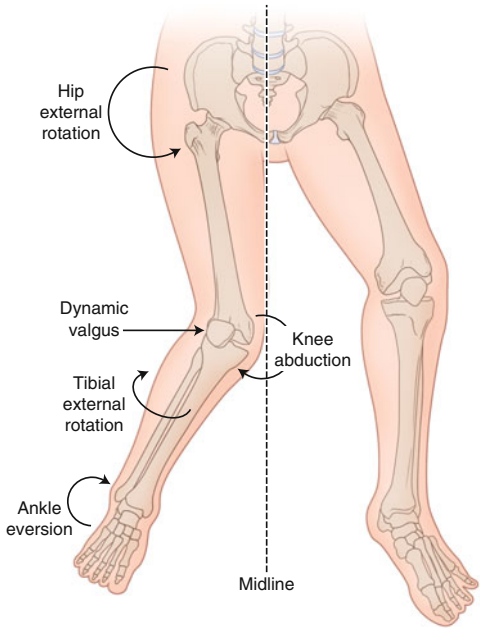

Fig. 1.2 Typical knee position for ACL tear. The ACL is most stressed in a position of combined valgus, adduction, and internal rotation – or a knock-kneed position with the foot apart and the toes turned in. Such a position is frequently seen during cutting or pivoting or when kicking a soccer ball

are injuries that occur when the player is not near another player, and they typically occur when the player is cutting, pivoting, or abruptly stopping. The exact mechanism of such noncontact ACL tears includes poor knee positioning (e.g., extension of the knee during landing) and a strong, unopposed quadriceps contraction. By identifying risk factors for ACL injury, it is hoped to develop individualized prevention programs that address muscle imbalances or poor positioning of the limb relative to the body to help minimize the incidence of ACL injury, especially for the noncontact-type injuries.

ACL Tears in Female Athletes

There is a higher risk of ACL injuries in females than in males. Depending on additional risk factors, which will be discussed later in this chapter, the female to male ratio of ACL tears has been reported to range from 2:1 to 8:1, although most evidence suggests this ratio to be closer to 3:1 (Fig. 1.3) [11]. A large number of gender-specific risk factors have been proposed and assessed to explain this difference. Proposed risk factors have included (1) decreased room for the ACL at the end of

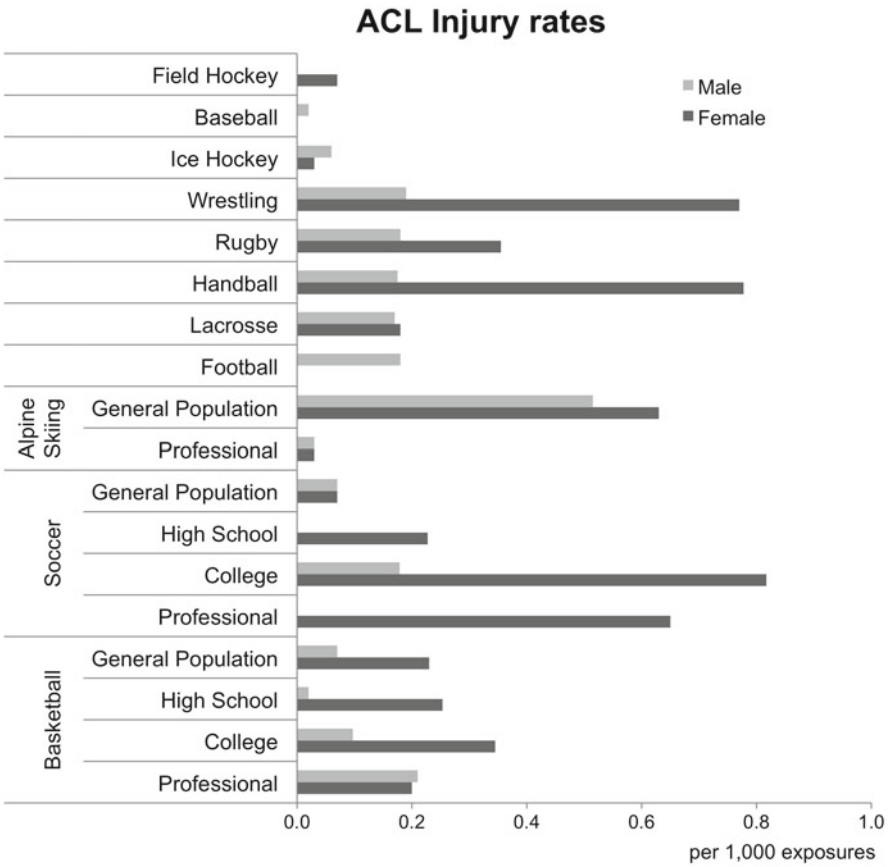

Fig. 1.3 Male–female ratio. This figure shows the ACL injury rates (per 1,000 exposures) for men and women for specific sports. Typically, the rates of injury in women are higher than the male rates at a ratio of 3:1–8:1, depending on the sport participated in (Reproduced from Renstrom et al. [10], with permission from BMJ Publishing Group Ltd.)

the thigh bone (decreased intercondylar notch size), (2) influence of the menstrual cycle (although multiple studies have shown different phases to be at risk, so this is not clear), and, most importantly, (3) development of knee valgus during impact on landing (the knees drop toward each other – Fig. 1.4). Injury prevention programs have been shown to decrease the risk of ACL injuries, particularly for women athletes, by teaching them to 1) land in a safer, non-valgus position, 2) to focus on keeping the knees over the toes when landing, 3) to land softly on the toes rather than the whole foot, and 4) to land on two feet when possible (see prevention section on page 10 for links to specific programs).

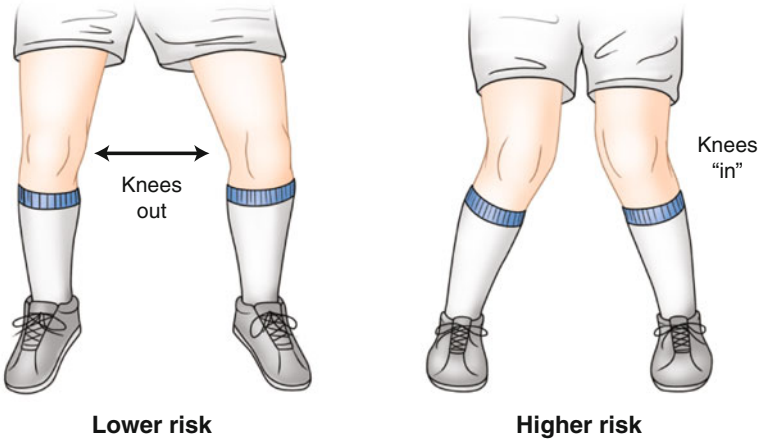

Fig. 1.4 Landing mechanics. One reason for the higher rate of ACL tears in females compared to males is the landing mechanism after jumping. Males usually land with the knees apart and above the feet ("lower risk" position), whereas females land in a "knock-kneed" or valgus position close to full extension leading to excessive stress on the ACL ("higher risk" position). Improving landing position and mechanics is a very simple yet effective way to prevent noncontact ACL injuries

Sports-Specific Risks of ACL Tears

Another important group of risk factors is professional or recreational exposure to different types of sports. Higher-risk activities include those that involve high running speeds, abrupt changes of speed and/or direction, and jumping and landing. The combination of these higher-risk sports with other risk factors such as lack of experience, lack of education for proper technique, and inadequate equipment results in fairly specific risk patterns for a number of sports (see Fig. 1.3).

The four sports that are most noted for the danger they pose to the ACL are alpine skiing, soccer, basketball, and football. Alpine skiing is an excellent example for the role of experience in the risk of ACL injury. Recreational skiers have one of the highest risks for ACL injury, but the risk for "expert" skier is 16 times lower and actually the lowest in the high-risk sports even though the expert skier spends more time on the slopes (i.e., higher exposure). Technical skill and a better general fitness condition are most often quoted as the reasons for this. Interestingly, skiing, when done by experts, represents the rare occasion in which no gender differences are seen [12]. The only other sport to have shown no gender difference in ACL injury is lacrosse [13].

Large studies have been conducted on ACL injuries in soccer and basketball, showing ACL injury rates of 2.8–3.3 % for females and of 0.7–1.2 % males, consistent with the threefold higher rate in females mentioned above [13, 14]. As opposed to skiing, studies showed that the risk of sustaining an ACL tear is higher for professional athletes than it is for recreational athletes in soccer and basketball. Further analysis of ACL injuries in soccer and basketball revealed a plethora of potential risk factors, including the above-mentioned motion patterns, but also the type of turf, the type of floor, or the type of shoes worn.

Natural grass fields are associated with lower rates of knee, ankle, and foot injuries than artificial turf. A number of studies have suggested that shoe design could influence ACL injury risk. The "release coefficient," which is the force-to-weight ratio of the shoe and surface interaction, has recently gained attention. Briefly, an optimal shoe design will have minimal rotational friction – which would reduce rotational stress on the leg – and maximal translational friction, to allow safer stopping and subsequently fewer ACL injuries. Heidt et al. tested 15 different types of shoes and found that 73 % were "unsafe" or "probably unsafe" [15–17]. Unfortunately no specific shoewear recommendation could be deducted from these studies.

Age-Specific Risks of ACL Tears

One issue that deserves special attention is the question whether age, specifically young age, is a risk factor for ACL tears. Over the past few years, there has been an approximately 400 % increase in ACL injuries in children and adolescents, and it is currently estimated that 50 % of all patients with an ACL tear are between the ages of 15 and 25 (Fig. 1.5). In women, the peak incidence of ACL injuries occur in the 15- to 19-year-old age group [18, 19]. Many consider this counterintuitive, particularly given the old adage that young bones are more flexible than older ones. There has been much stipulation and research as to the causes for this dramatic rise in pediatric ACL injuries. Some suggest a heightened attention to this type of injury has led to a higher rate of detection, with no real change in injury rates.

Another possible reason for this increase is a change in leisure-time activities for middle and high school students. Currently, about 45 million children are participating in organized teams in competitive sports, starting at ages as young as 6 years. Making the soccer or football team is associated with a considerable training load during a vulnerable time in musculoskeletal growth. While participation in sports has many benefits for children and adolescents, some practices involve repetition of fairly narrow groups of motions, leading to an imbalance in the development of muscle strength and joint flexibility. These imbalances lead to excessive strain on ligaments such as the ACL and predispose them to injury.

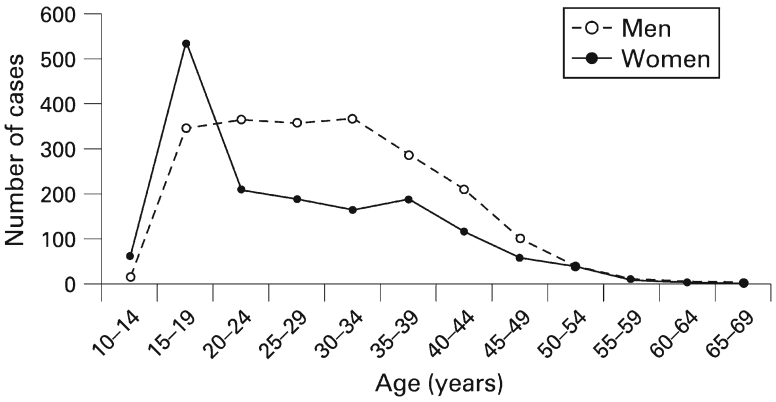

Fig. 1.5 The effect of age and gender on ACL injury rates. This graph shows the distribution of patients in the Norwegian National Knee Ligament Registry by age and gender (Reproduced from Renstrom et al. [10], with permission from BMJ Publishing Group Ltd.)

Knowing the Risk Helps in the Prevention of Injury

Describing these risk factors allows us to identify individuals at increased risk of ACL tears. Risk factors can be reliably used by doctors, parents, and coaches to identify players who might be more at risk for an ACL tear because of their muscle development or limb alignment. The benefit of identifying high-risk individuals is that a number of simple exercises have been developed and assembled to create highly effective ACL injury prevention programs (Fig. 1.6). Such programs consist of simple balance board and postural training that typically require 30 min twice a week, but recent evidence has shown that for every 40 high school students enrolled in such programs, 1 ACL tear can be prevented. And after all, the most effective treatment for any disease is its prevention.

Excellent descriptions of such programs, including pictures, background information, and physician information, can be found online at the Children's Hospital Boston webpage (http://childrenshospital.org/cfapps/research/data_admin/Site2226/mainpageS2226P9.html) or the PEP program website (http://smsmf.org). The International Federation of Football Association, FIFA (Federation Internationale de Football Association), has endorsed a warm-up and exercise program to reduce ACL injury, called FIFA 11+ (see Fig. 1.6). The program can be downloaded from the Internet on the FIFA website (http://f-marc.com/11plus/) and has been shown to be effective in reducing ACL tears [20, 21] even in sports other than soccer, for example, in male, elite basketball players [22].

Fig. 1.6 ACL prevention programs – FIFA 11+. The International Federation of Football Association published this training program to prevent noncontact ACL injury (http://f-marc.com/11plus/). Scientific studies have proven its effectiveness for soccer players and in other sports such as elite basketball (see text for details)

How Many Surgeries Are Done Each Year for ACL Injuries?

How about those ACL injuries that cannot be prevented? For the active patient, the current gold standard of treatment is ACL reconstruction. Current estimates are that about 100,000–400,000 ACL surgeries are done per year in the USA alone [18, 19]. The Statewide Planning and Research Cooperative System (SPARCS, www.health.ny.gov/statistics/sparcs/) database from the New York State Department of Health lists approximately 7,000 surgical ACL surgeries per year in the state of New York, which corresponds to 35 ACL surgeries per 100,000 people in the state. If this rate were similar across the USA, this would correspond to 112,000 surgeries in the USA each year. The national registries from Scandinavia show similar numbers with ACL reconstructions of 34 per 100,000 inhabitants in Norway, 38 per 100,000 in Denmark, and 32 per 100,000 in Sweden.

The severity of an ACL tear is further illustrated by the high number of concomitant injuries. Only one-third of the ACL treatments in New York found an isolated ACL tear, while the other two-thirds of patients had concomitant injuries to the same knee. Thirty-two percent of all patients also required treatment of a meniscal injury. Nineteen percent had meniscus damage combined with further problems, such as collateral ligament sprains or cartilage impact damage [23, 24]. In Denmark, 40 % of the roughly 2,000 patients treated annually for ACL tears had a concomitant meniscus injury, and 55 % needed treatment of a cartilage injury [23, 24]. Similar numbers are reported for Sweden, Norway, Germany, and Switzerland [23, 24].

If I Have Had One ACL Tear, What Is My Risk of Getting a Second ACL Tear?

If you have had an ACL tear, are you more at risk for getting another ACL tear than someone who has never had a tear? The short answer is "yes." As we have seen in the section above, the baseline risk for an initial ACL tear is 35 out of 100,000, or 0.035 %. However, once you have had an ACL tear, the risk of tearing your other ACL within the next 2 years is reportedly between 3 and 23 % [2, 25]. Since this range is quite extensive, one large, high-quality study assessed the risk of contralateral ACL injury over 5 years after an ACL tear and reported that the risk was in the range of 8–16 % [26].

All these values are much higher than the risk for the first ACL tear, suggesting that those individuals who tore their ACL once are at a higher risk of tearing the other one, too. This may be due, in part, to a return to high-intensity, high-risk activities such as participation in cutting and pivoting sports seems to predispose the contralateral ACL to injury. This is also well illustrated by the fact that in those patients whose ACL tears are treated conservatively, that is, those advised to avoid contact or high-risk sports, the contralateral ACL tear risk is only around 1 %.

Other risk factors, including major differences in limb strength at return to sport, are also likely to play a role. A number of additional risk factors, including anatomy, early return to sport, and even familial factors, have been examined, but none have been directly associated with the risk of tearing the contralateral ACL after a primary ACL rupture. Even gender was not shown to have an association with a heightened risk.

The risk of re-rupturing a surgically treated ACL is about 6 % (2–8 %), that is, only half the risk of tearing the "other," contralateral ACL after ACL treatment [2]. Most of these graft ruptures occur within 12 months after surgical reconstruction. Thus, during this first year, the risk of re-rupture can be as high as the risk of a contralateral injury (12 %) [2]. One study looking at 612 patients with torn ACL grafts found that, unlike the first ACL tear, only 5 % were due to a noncontact injury [27].

Proposed risk factors for ACL re-ruptures include neuromuscular factors, the biochemical environment, age, and the level of activity postoperatively. As far as neuromuscular factors are concerned, since the standard ACL reconstruction replaces the torn ACL with a tendon graft, there is no functioning intrinsic innervation and therefore no dynamic feedback loops. One of these feedback loops, the reflective activation of the hamstrings to prevent forward translation of the tibia, has been shown to protect the ACL [28]. If this dynamic feedback loop is interrupted, the hamstrings are no longer signaled to help the ACL when it is stressed, and thus, the risk of ACL injury is increased. For the biochemical environment, an earlier ACL injury and surgery also alters the biochemical balance of the knee joint through inflammation, which has been suggested to affect ACL graft healing and longevity.

Both age and graft selection have also been shown to affect rates of ACL tears, with younger patients having higher graft failure rates and those patients having an allograft reconstruction also having higher rates of graft failure [29]. These factors have been found to be multiplicative. For example, a 14-year-old with an allograft (i.e., tendon obtained from cadaveric donor) ACL reconstruction has a 22 % chance of tearing his/her graft, while the same patient has only a 6.6 % chance of tearing the graft if it is an autologous graft (i.e., tendon obtained from self). In contrast, a 40-year-old patient has only a 2.6 % chance of tearing his/her allograft and only a 0.6 % to 1 % chance of tearing the autograft (Fig. 1.7) [29].

The Cost of ACL Injury

During recent years the scope of epidemiology has expanded to include the economics of health care. The days of abundant financial resources for health care, if they ever existed, are long gone. Costs and cost-effectiveness have become a central issue in the provision of current and the development of new treatments. Musculoskeletal disease, including ACL injuries, is under particular scrutiny since, problems of the skeletal apparatus can turn into a lifelong hindrance and diminish quality of life and ability to work.

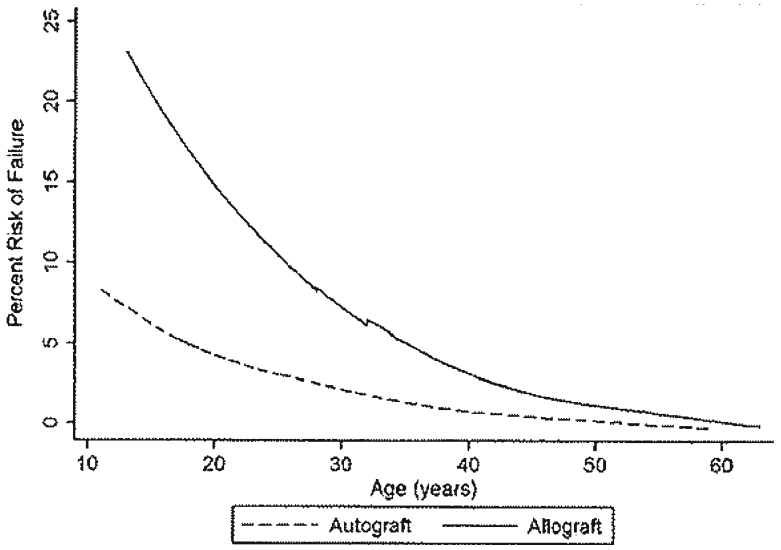

Fig. 1.7 Age-specific ACL reinjury rates. This figure shows the chance of re-tearing an ACL, after ACL reconstruction with one's own tendons (autograft) or with donor tendons (allograft) at different ages. The risk of re-tearing is higher with a donor tendon reconstruction, but this difference reduces with age, as does the overall risk of reinjury (From Kaeding et al. [30], copyright © 2010 by (Sage Publications), reprinted by Permission of SAGE Publications)

The cost of ACL surgery depends on a number of factors. The type of graft that is used is associated with different costs for procurement and different procedure lengths, which in turn affects costs. While there is considerable geographical variation in these costs, an autograft ACL reconstruction (where the patient's own tendons are used) can be estimated to cost about $5,000–$6,000; an allograft ACL reconstruction (where a cadaver or donated graft is used) is about $1,000 more expensive [31, 32]. Among the autografts, hamstring grafts have been shown to be less expensive than patella ligament grafts, because of less operating room time and slightly better functional outcomes [32]. However, for the assessment of medical treatments, cost analyses are not as useful as assessing cost-effectiveness. In addition to the differences in associated costs among the different ACL graft types, there are also differences in their effectiveness. These differences in effectiveness can be expressed in various forms, such as the risk of re-tear or subjective satisfaction of the patient, but the most appropriate way is a formal test using a quality-of-life (QoL) assessment instrument and multiplying this QoL with the number of years the graft is likely to last. This results in the so-called quality-adjusted life years (QALY). Based on earlier studies, hamstring grafts cost about $5,300, bone-tendon-bone grafts about $5,600, and an allograft about $7,000. Thus, hamstring grafts are the least expensive graft type and are the most effective in terms of QALY.

The costs associated with problems that occur during or after ACL surgery are less well documented. An infection in the knee joint has been estimated to produce

additional cost of $9,800 (range $5,000–$30,000). Fortunately, this complication is relatively rare after ACL surgery (less than 1 % of the time). Joint stiffness after an ACL reconstruction will require up to $3,000 in an effort to treat (range $0–$9,000). Revision procedures are even more complicated, riskier than primary surgery because of the tissue changes, scarring, changes in anatomy, the need to remove old implants and fixation devices, and the, now, limited availability of autologous grafts and healthy bone to place them into. Thus, revision surgery is more expensive than primary surgery. If a revision is needed, additional costs of about $20,000 ($14,000–$51,000) should be expected [32]. Naturally, the costs for complications have large variability, depending on how serious these complications are. The rates of full-blown revision for ACL surgery at this time are between 0.7 and 9 %, including the factors outlined above, such as re-rupture or inadequate graft placement or fixation.

Conclusion

ACL tears are a frequent injury with increasing incidence. A number of risk factors, such as female gender or specific types of sports played, predispose some individuals to ACL injury; however, there are modifiable risk factors, which can help to mitigate this risk. Interestingly, the rate of ACL tears seems very stable across a number of countries, as are the high rates of additional injuries that occur in conjunction with ACL tears.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 2
History of ACL Treatment and Current Gold Standard of Care

Martha M. Murray

ACL injuries are increasingly common, with estimates as high as of 400,000 patients each year in the United States sustaining this injury (in comparison with 120,000 patients undergoing hip replacement surgery) [1]. The ACL injury is important, not only due to the number of people affected by the injury but also because of the sequelae of the injury. The ACL does not heal on its own, and as a result, many methods have been designed to treat the ACL-injured knee. However, even our gold standard of treatment, ACL reconstruction, cannot prevent the premature onset of arthritis for patients with ACL injuries. This is worrisome for those of us who care for patients with ACL injuries, and as such, we are extremely interested in finding improved solutions for people with ACL injuries.

There has been a great deal of work done over the past two centuries regarding the diagnosis and treatment of the ACL. We will briefly review some of the highlights that seem most relevant to these injuries and those that give us hints of what might work better. Certainly, any work in this field "stands upon the shoulders" of these wonderful physicians and scientists who have laid a great foundation for future studies.

The earliest reports of ACL injuries largely relied on the history and physical examination of the patient, two areas which remain critical to the accurate diagnosis of this injury today. The first identification of the ACL was attributed to Claudius Galen (150 A.D., Fig. 2.1) who cared for the gladiators and likely had the opportunity to visualize the ACL through gashes in the knee. In 1845, Amedee Bonnet of Lyon reported that patients who heard a snap and developed swelling and loss of function in the knee would most likely have a ligamentous injury, including ACL injury. Thirty years later, Georgios Noulis from Greece performed a series of cadaver studies where he found that forced anterior subluxation of the tibia could

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_2,

Fig. 2.1 Claudius Galen, b. 129 A.D., d circa 200 A.D. Galen was a physician for the gladiators in Rome and is credited with the initial observation of the anterior cruciate ligament and its traumatic injury (From The Wellcome Library, London)

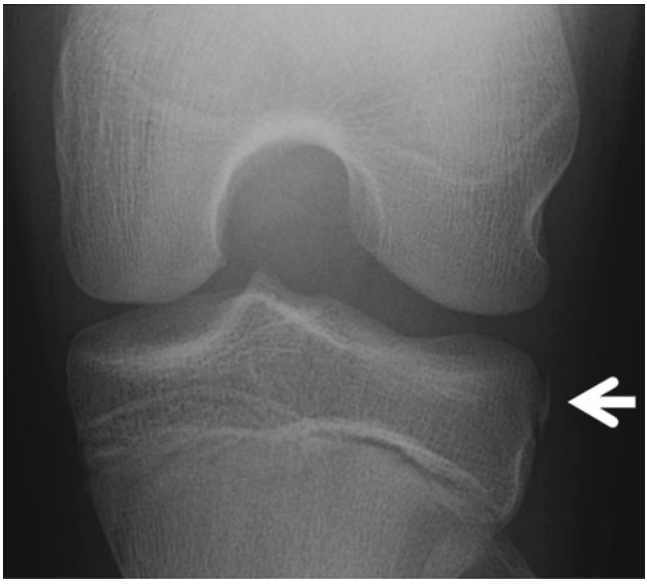

Fig. 2.2 X-ray appearance of a Segond fracture. In 1879, Paul Segond recognized that patients who had a small avulsion fracture off the anterolateral tibial plateau (*arrow*) typically had an ACL tear

cause an ACL rupture, and he described a test very similar to the Lachman test used today for diagnosing a tear of the ACL. After radiography started to become more available in 1879, Paul Segond recognized that patients with a small avulsion fracture off the anterolateral tibial plateau (now called a Segond fracture, Fig. 2.2) typically had an ACL tear. The history, physical exam, and x-ray findings were all critical to the diagnosis of patients with ACL tears.

History of Primary Repair

With the recognition of ACL injury came the realization that untreated ACL tears often caused progressive deterioration of the knee with instability and damage to the menisci and cartilage [2, 3]. For this reason, surgical treatments emerged. The first repair of the ACL is attributed to A.W. Mayo Robson in 1895, who reported on the suture repair of both the ACL and PCL in a miner who had been injured 3 years prior. Six years later, the patient still described his knee as "perfectly strong" and he was able to walk without a limp. Since his discharge from the hospital, he did not miss a day of work due to his knee injury – certainly an outcome of "good patient satisfaction." With the advent of aseptic techniques and general anesthesia, surgery for knee injuries became even more prevalent. In 1938, Ivar Palmer discussed the failure of spontaneous healing of a complete ACL tear and subsequent importance of repair [4]. Palmer thought that early repair was critical to its success. In 1950, D. H. O'Donoghue, from the University of Oklahoma, reported his technique of ACL repair, which consisted of a suture weave through the tibial stump and passing it up through a tunnel in the femur and using postoperative immobilization for 4 weeks with the knee held at 30° [5]. He, like Palmer, thought early repair was critical to successful healing.

O'Donoghue was also very active in the basic science of ACL healing and its repair. In the 1960s, he published a study of repair of the ACL in dogs and found that even with a suture repair of the ligament, the repaired ACL only achieved 10 % of its normal strength at 4 weeks [6]. In 1979, Cabaud et al. evaluated the results of ACL repair in both rhesus monkeys and dogs [7] and found that while repairs reached 45 % of the intact ACL strength at a year in monkeys, the repairs in dogs were less favorable, only achieving 10 % of the ACL strength. The reasons for the failures were unknown, but hypotheses about inadequate immobilization and early stress on the repair were discussed. Interestingly enough, animal studies performed since using ACL reconstruction (the current gold standard of ACL treatment) have also reported high rates of failure and abnormal knee laxity in these large animal models, making one wonder if ACL procedures are less effective in animals who cannot undergo skilled rehabilitation [8–11].

John Marshall expanded the description of primary ACL repair, with placement of multiple loop, varying-depth sutures in both cruciate stumps, and passing the sutures through drill holes in the opposite bone [12, 13]. John Feagin and Walter Curl reported on using a modified Marshall technique with catgut sutures to repair the ACL in West Point cadets (Fig. 2.3). While 25 of 30 patients were doing well at 2 years out from surgery [15], the 5-year follow-up results were less encouraging [16], with 71 % of patients having pain and 94 % presenting with knee instability. One should note, however, that these surgeries were performed in military personnel, the majority of which were commissioned to full duty and many went to fight in the Vietnam War. However, these results were less than heartening to those caring for active patients with ACL tears. Later articles appeared more promising, with Marshall, Warren, and Wickewicz publishing a 2.5-year follow-up

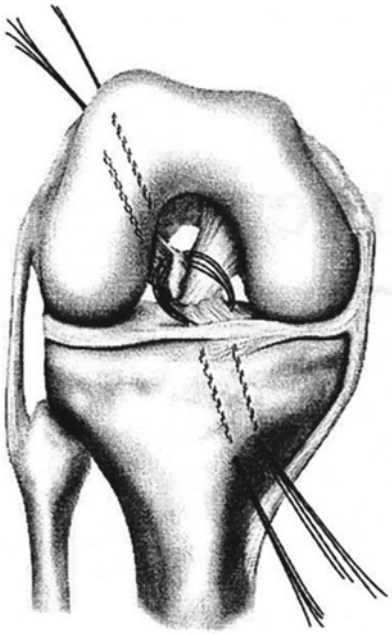

Fig. 2.3 The Marshall technique of primary repair of the ACL. Sutures were placed in a variable depth fashion in the tibial stump and femoral stumps and tied over a bone bridge. In the Feagin and Curl studies, these sutures were made of catgut (Used with permission from McCulloch et al. [14])

study where no patients with repair had giving way symptoms or had needed subsequent meniscal surgery (even though 93 % of them were active in sports) [12]. In 1985, Odensten et al. published the first prospective, randomized trial of suture repair vs iliotibial band reconstruction vs nonoperative therapy for patients with ACL injuries [17]. The suture repair in that paper was described as "the distal fragment of the ACL was sutured with seven or eight non-absorbable sutures to the anatomic insertion point on the lateral femoral condyle. The sutures were pulled out through two drill channels in the lateral femoral condyle and were tied on the outside." The limbs were immobilized for 6 weeks at 30 degrees of flexion. At 18 months out from surgery, there was no significant difference between operated and nonoperated knees in terms of overall outcome scores; however, the authors did note that while 95 % of patients in the repaired group had a stable knee at follow-up (with a negative pivot shift or Slocum test), only 11 % in the nonoperated group did. In addition, twice as many patients in the nonoperative group required further meniscal surgery in the 18 months after injury [17].

In 1987, Sandberg et al. published a second prospective study of primary repair versus nonoperative treatment [18]. As in the Odensten paper, the printed conclusion was that primary repair was no better than non-operative treatment. However, it is interesting to note that the rate of a positive pivot shift test also decreased with surgery (from 62 to 28 %) as did the risk of presenting with a subsequent meniscal tear (24 % vs. 8 %). Thus, while both of these studies concluded that functional

performance was comparable between the two groups, other important characteristics (stable knee, preservation of the menisci) appeared to be better in the group undergoing primary ACL repair.

During the 1980s, a debate raged as to the best surgical treatment for the ACL. Primary repair continued to have its advocates [19], but repair augmented with other tissue (the iliotibial band or a bone-patellar tendon-bone graft) was also coming into favor [20]. While both ACL reconstruction and primary repair had similar results in terms of rate of return to full sports (60–75 %), reinjury (1.5 %), and a normal Lachman exam after surgery (50 %), the repairs needed to be done relatively quickly, while the repairs augmented with a reconstruction could be delayed for months. This convenience factor for both patients and surgeons alike must have been irresistible, as much of the repair literature drops away after these series of reports.

History of ACL Reconstruction

In 1917, Ernest W. Hey Groves reported on the first ACL reconstruction using an iliotibial band transplant, and in 1920, he reported on the results of this procedure in his first 14 patients [21]. None of the patients were made worse by the operation. Four were reported to have no benefit, four had "some benefit," and four were cured (although two were still in rehabilitation at the time the paper was written). In 1935, Willis Campbell of Memphis, Tennessee, reported the first use of a patellar tendon graft and fixation through bony tunnels in the femur and tibia [22]. Additional advances in ACL reconstructive surgery were subsequently made by Kenneth G. Jones of Arkansas, Helmut Bruckner of Germany, and Kurt Franke of Germany.

In the 1970s, nonanatomic ACL reconstruction techniques became more in favor, even though primary ACL repair was commonly performed. The MacIntosh procedure (pioneered by D. L. MacIntosh of Toronto) was a primarily extra-articular procedure that utilized a fascia lata graft left attached to the tibia, passed under the lateral collateral ligament, and attached to the intermuscular septum (Fig. 2.4) [23]. The MacIntosh II procedure involved using a longer graft which could be passed under the lateral collateral ligament, through the intermuscular septum, then over the back of the lateral femoral condyle, and through the joint to a tibial tunnel. This procedure was the predecessor of one of the techniques currently used to reconstruct the ACL in skeletally immature patients (Fig. 2.5) [24, 25].

In the 1980s, intra-articular ACL reconstruction began to come to the forefront. John Insall is often given credit for the early intra-articular reconstructions – he reported a technique where a band of fascia lata was passed through the knee and sutured to the front of the tibia [26]. Another procedure, described by MacIntosh, involved taking a central slip of the patellar tendon with tissue from the top of the patella, leaving it attached distally at the tibia and passing the tendon through the notch to reapproximate the course of the ACL (Fig. 2.6).

A free patellar tendon graft came shortly thereafter. Clancy is typically credited with popularization of this technique [27], although he advocated simultaneous

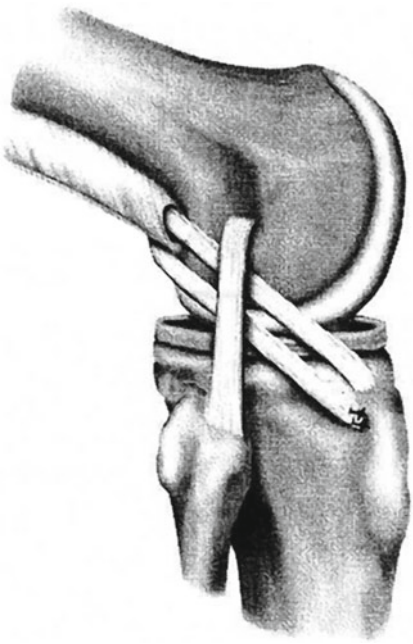

Fig. 2.4 MacIntosh 1. Lateral extra-articular reconstruction with a strip of IT band passed through the intermuscular septum and under the lateral collateral ligament (Used with permission from McCulloch et al. [14])

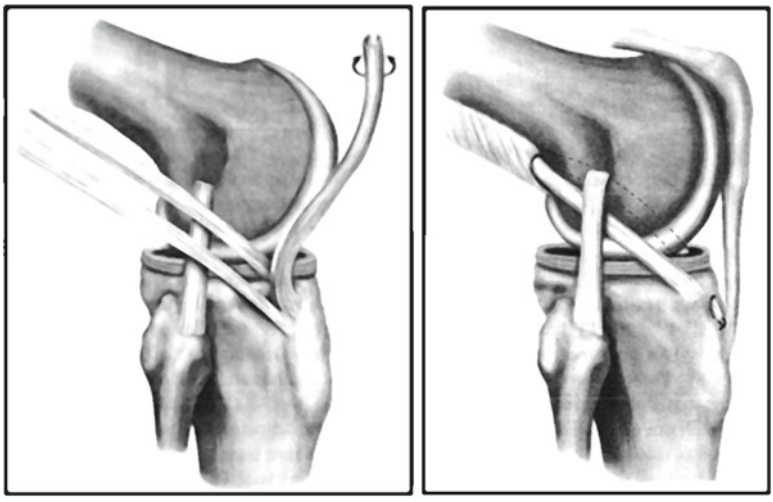

Fig. 2.5 The MacIntosh II. The MacIntosh II procedure involved using a longer graft which could be passed under the lateral collateral ligament, through the intermuscular septum, then over the back of the lateral femoral condyle, and through the joint to a tibial tunnel (Used with permission from McCulloch et al. [14])

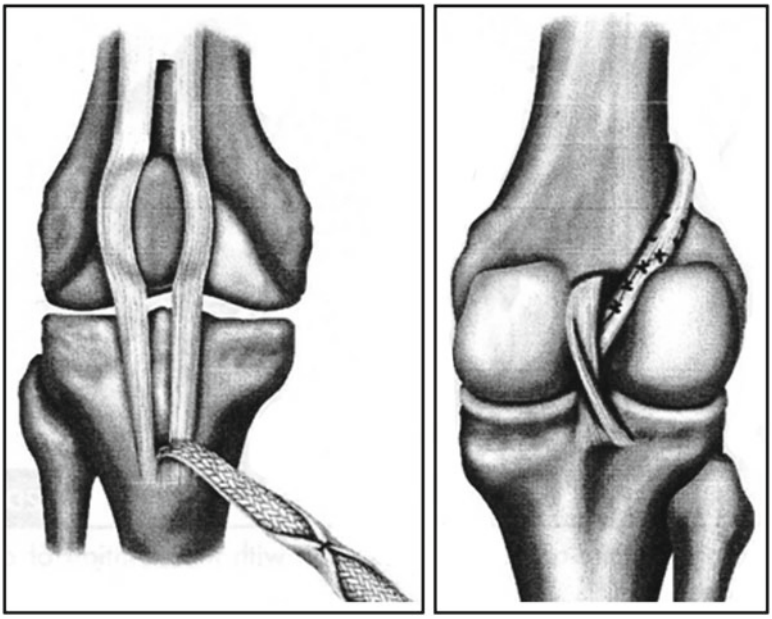

Fig. 2.6 Intra-articular ACL replacement using the central slip of the patellar tendon. Another procedure was described by MacIntosh involved taking a central slip of the patellar tendon with tissue from the top of the patella, leaving it attached distally at the tibia and passing the tendon through the notch to reapproximate the course of the ACL (Used with permission from McCulloch et al. [14])

medial and lateral capsular repairs – techniques later shown to not be necessary for a good outcome by O'Brien [28]. Other tissues were also explored as grafts, including the medial meniscus [29] (no longer in practice) and hamstring grafts [30, 31]. Current standard of practice is to use a free tendon graft (bone-patellar tendon-bone, hamstring, or allograft) through tunnels in the tibia and femur along the course of the prior ACL.

History of Synthetic Replacement of the ACL

The first ACL replacement with synthetic material was reported by F. Lange of Munich, who used a braided silk construct to replace the ACL. The procedure did not work but perhaps set the stage for the next few decades. Since that time, many synthetic materials have been trialed, including Gore-Tex, carbon fiber, and modified silk scaffolds (Fig. 2.7). The details of those materials and their performance will be covered in Chap. 14. In brief, to date there has not been a synthetic ligament which performs well over time. This is likely due to the fact that none of the synthetic materials used have encouraged sufficient cellular and tissue ingrowth.

Fig. 2.7 Photograph of a Gore-Tex graft

Biologic tissues have the amazing ability to engineer their own repair. Small injuries, many of which occur with daily "wear and tear," can be fixed by the cells within the tissues as they occur. Thus, small, repetitive injuries do not typically lead to catastrophic failure for living tissues. However, synthetic materials which do not have biologic incorporation subsequently do not have the ability to heal the small injuries which occur over time. These small injuries thus continue to accrue, until enough damage has occurred to produce catastrophic failure of the ligament. A typical pattern for these synthetics is good stability of the knee for a period of time, even several years, and then a sudden complete failure. In the future, a synthetic ligament replacement which encourages biologic incorporation may be a very useful addition to the armamentarium of an ACL surgeon.

Current Gold Standard of Treatment

The current gold standard of treatment for a skeletally mature patient is an ACL reconstruction with a free tendon graft, placed through tunnels in the distal femur and proximal tibia and anchored at both sides. For young patients, the graft of choice is autograft, with surgeons recommending either hamstring or patellar tendon, based on personal experience and preference. There is currently no data to suggest the superiority of hamstring or patellar tendon grafts as will be detailed in the next chapter. Other surgical decisions, such as choice of fixation method, will also be discussed in the next chapter. The preservation of the ACL remnant, and placing smaller tunnels for the graft through the center of the remaining ACL tissue, is a technique that is gaining much popularity, especially for young active

patients where the preservation of the torn ACL tissue may allow for a source of proprioceptive nerve fibers which may be particularly important on the patient's return to sports.

Conclusions

Our current treatment of ACL injuries has evolved over the past few centuries. Information gained by surgeons and investigators studying and comparing methods of conservative treatment, primary suture repair, and ACL reconstruction has led to the majority of patients being treated with an ACL reconstruction today. This is due to the prior failure of primary suture repair and the superiority of the ACL reconstruction over conservative treatment for the majority of patients. Future improvements in ACL treatment will build on the findings of these prior investigators, and hopefully, the advances in ACL surgery over the next few decades will be as rapid and exciting as that over the past few.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 3
ACL Clinical Outcomes

Carolyn M. Hettrich and Kurt P. Spindler

The practice of medicine has evolved over time. Before the 1900s, medicine was learned through apprenticeships, and what was practiced was entirely based on what one learned from one's mentor and personal experience. In the modern era, we now also use studies in petri dishes and in translational models to try to understand how our bodies work and how to heal them. In addition, particularly over the past 20 years, the quality of clinical research (the study of how patients fare after an intervention) has improved, with an application of the scientific method to clinical studies and a focus on epidemiology, statistics, and patient-reported outcomes. With the improvement in research methodology, there is now an emphasis on practicing "evidence-based medicine." In this chapter, we will review the recent findings for studies with a high level of evidence (LOE) studies (LOE I and II) – these studies involve either randomized control trials (where a patient agrees to participate in a study and then is randomly assigned to one treatment group or another – this is a Level I study) or well-designed cohort or case–control studies where patients are followed systematically over time (this is a Level II study). In addition to these original studies, we will also include systematic reviews and meta-analyses (where multiple studies with similar design are looked at as a whole) focusing on the clinically relevant outcomes in ACL tears with emphasis on anterior cruciate ligament reconstruction (ACLR).

Through this evidence-based medicine approach, we will first focus on understanding the short-term and long-term functions of ACL, meniscus, and articular cartilage. The term "isolated ACL tear" is a misnomer, as some reports state this occurs

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_3,

less than 10 % of the time. Concurrent with an ACL tear, patients have meniscal injuries (60–75 %), "bone bruises" on MRI (80 %), medial and/or lateral collateral ligament tears (5–24 %), and articular cartilage injuries (up to 46 %). Thus, to understand clinically relevant outcomes of ACL injuries and treatment, we must include the damage and treatment to meniscus, articular cartilage, and collateral ligaments.

Indications for ACL Reconstruction

Four Level I studies have determined the indications for ACL reconstruction (ACLR) [2, 6, 10, 19]. First, a randomized control trial by Sandberg et al. [19] found primary repair of the ACL to be no better than nonoperative treatment. Second, in the 1990s, patellar tendon ACL reconstruction was found to be better than ACL suture repair and ACL augmentation [6, 10]. Third, ACL surgery was also determined to result in a significant decrease in the rate of meniscal tears from 32 % in a nonoperative group to 3 % in a surgical group [2]. Fourth, the recently published *k*nee, *a*nterior cruciate ligament, *non*surgical versus surgical treatment (KANON) randomized control trial looked at early ACL reconstruction versus rehabilitation with possible late ACL reconstruction [7]. This study was with patients 18–35 years old from Lund, Sweden. They included 44 % of the ACL tears that were presented to physicians during that period of time; 56 % were excluded due to age, activity, chronic injury, prior surgery, or collateral ligament injury. Their primary outcome measure was the Knee Osteoarthritis Outcome Score (KOOS) at 2 years after the injury. During the first 2 years of the study, 37 % (23/59) of the patients that were initially assigned to the nonoperative group opted for delayed ACL reconstruction. Thirty-six percent (13/36) of the rehab patients also exhibited signs/symptoms of a meniscal tear. Intention-to-treat analysis (where the results are reported for each group of patients as they were assigned at the beginning of the study – the patients who later opted for ACL reconstruction were still included in the results for the nonoperative group) showed the same patient-reported outcomes at 2 years; however, 37 % of the rehabilitation patients had opted for ACL reconstruction by this time. These patients are still being followed by the same research group, and whether the high rate of meniscal loss in the nonoperative group will significantly affect the longer-term outcomes remains to be seen.

Endoscopic or Two Incisions?

A Level I systematic review by George et al. looked at two incisions ("rear entry") versus single incision or endoscopic ACL reconstruction [9]. The data from this study can be seen in Table 3.1. No significant differences were found between the approaches in pain medication postoperatively, postoperative rehabilitation, range of motion, strength, anterior knee pain, Tegner or Lysholm scores, or International Knee Documentation Committee (IKDC) scores. The authors concluded that there were no differences in results between the techniques (statistically significant or clinically relevant) and that surgeons should choose the technique that yields the most reproducible results in their hands.

Table 3.1 Prospective randomized studies of endoscopic versus rear-entry ACL reconstruction

Category	Brandsson et al[2]	Gerich et al[4]	Reat and Lintner[10]	O'Neill[9]
Journal	Br JSM 1999	KSSTA 1997	Am J Knee Surg 1997	JBJS Am 1996
Groups	2	2	2	3*
Patients	59	40	30	80
Follow-up time (testing intervals or range)	24 months† (3, 12, 24 months)	12 months† (6, 12 months)	17 months (endo) 15 months (rear-entry)	24 months (24–60 months)
Percent (%) followup	100 %	100 %	100 % (endo) 80 % (rear-entry)	98 %
Number of surgeons	3	2 (1 each technique)	1	1
Operative time (minutes)	Endo 8 min faster ($p = 0.03$ [86 vs. 94 min])	Endo 22 min faster ($p < 0.05$)	NS	NR
Pain medication	–	NS	–	–
Progression of rehabilitation	–	NS	NS	–
Range of motion	NS	NS	NS	NS
Quadriceps or hamstrings deficit	–	NS	NS	NS
Patellofemoral pain	NS	–	NS	–
Return of activity	–	–	NS	6 % more rear-entry ($p < 0.02$)
Instrumented laxity ≤ 3 mm	NS	NS	NS	8 % more rear-entry ($p < 0.08$)
One-leg hop test	NS	Endo better ($p = 0.046$)	NS	NS
Lysholm/Tegner	NS	NS	–	NS
IKDC	NS	NS	NS	NS

Br JSM = British Journal of Sports Medicine; KSSTA = Knee Surgery, Sports Traumatology, Arthroscopy; Am J knee Surg = American Journal of Knee Surgery; JBJS Am = Journal of Bone and Joint Surgery American edition; Endo = endoscopic; NS = no significant difference; NR = not reported; IKDC = ????

*Reported endoscopic versus rear entry bone-tendon-bone; thus all three used autograft bone-tendon-bone; †studies had in independent examiners

Which Autograft, Hamstring or Patellar Tendon, Should Be Used?

An earlier systematic review by Spindler et al. evaluated only Level I studies looking at the difference between hamstring and patellar tendon autografts [23]. The studies included in this review did not include sport-specific patient-oriented outcome measures (this would include questionnaires answered by patients such as the

Table 3.2 Summary of instrumented laxity data from patellar tendon and hamstring autograft studies. The KT1000, KT2000, Stryker and CA4000 are devices that quantify how loose the knee is following ACL injury and treatment

#[a]	Author	Instrument	Force	PT[b] [mm[c] (variation)[d]]	HG[e] [mm (variation)]	p[f]
1	Andersson	KT1000	maxman[g]	2.1(2.0)	3.1(2.3)	0.05*
			<3 mm	71 %	50 %	
2	Aune	KT1000	maxman	2.7(2.2)	2.7(2.1)	ns[h]
			<3 mm	nr	nr	
3	Beynnon	KT1000	133N[i]	1.1(0.9)	4.4(1.0)	0.004*
			<3 mm	77 %	45 %	
4	Ejerhed	KT1000	89 N	2.0	2.25	ns
			<3 mm	nr[k]	nr	
5	Eriksson	Stryker	18.2kg[j]	nr	nr	ns
			<3 mm	49 %	43 %	
6	Feller	KT1000	134 N	0.5(1.5)	1.6(1.3)	0.05*
			<3 mm	95 %	85 %	
7	Jansson	CA4000	nr	1.7	1.2	ns
			<3 mm	nr	nr	
8	O'Neill	KT2000	maxman	nr	nr	ns
			<3 mm	87 %	83 %	
9	Shaieb	KT1000	134 N	1.5	2.5	0.13
			<3 mm	79 %	45 %	

From Spindler et al. [23]
[a]Study number
[b]PT: Patellar tendon graft
[c]mm: millimeters
[d]variation: Individual study variation
[e]HG: Hamstring graft
[f]p: p-value
[g]maxman: Maximum manual force
[h]ns: Not significant
[i]N: Newtons
[j]kg: kilograms
[k]nr: Not reported
*Statistically significant
ns = not statistically significant

IKDC, KOOS, Marx activity score), as these clinical trials were performed prior to the development of these instruments 1998–2001. These studies instead looked at instrumented laxity, range of motion, and isokinetic testing.

This systematic review data presented in Table 3.2 found no clinically reproducible or statistically relevant differences in instrumented laxity and quadriceps or hamstring strength. It should be noted that two of three studies that reported an instrumented laxity difference were older studies where a 2-stranded hamstring construct was used (as opposed to the stronger 4-stranded construct that is utilized today). When evaluating the incidence of anterior knee pain, one study had significantly more anterior knee pain in the patellar tendon group. Since this was only one study of the nine included in the systematic review, the conclusion of the meta-analysis was that the autograft choice was not a primary determinant of outcome

Table 3.3 Summary of motion and isokinetic data from patellar tendon and hamstring autograft studies

				Extension (Quad)[a]			Flexion (Ham)[b]		
		Instrument	Speed deg/sec[c]	PT[d]	HG[e]	*p*[f]	PT	HG	*p*
1	Anderson	CybexII	60	86	96	ns[g]	96	96	ns
			180	91	99	ns	100	96	ns
2	Aune	Cybex6000	60	90	90	ns	94	90	ns
			240	90	92	ns	100	85*	0.01*
3	Beynnon	Cybex	60	95	88	ns	99	96	
			180	96	92	ns	96	91	
			240	97	93	ns	100	89*	0.04*
4	Ejerhed	Cybex	60	210	215	ns	100	190	Inj vs Uninj ns
5	Eriksson	–	–	–	–	–	–	–	–
6	Feller	CybexII	60	77	89	ns	98	91*	0.05*
			240	85	91	ns	106	99	
7	Jansson	Dynometer	60/180	nr[h]	nr	ns	nr	nr	ns
8	O'Neill	Biodex	60/180/240	nr	nr	ns	nr	nr	ns
9	Shaieb	–	–	–	–	–	–	–	–

From Spindler et al. [23]
[a]Quad: Quadriceps extension
[b]Ham: Hamstrings flexion
[c]deg/sec: Degrees per second
[d]PT: Patellar tendon graft
[e]HG: Hamstring tendon graft
[f]p: p-value
[g]ns: Not significant
[h]nr: Not reported
*Statistically significant

after ACL reconstruction. The authors hypothesized that injuries and treatment to meniscus and articular cartilage are the most important predictors with current ACL reconstruction techniques and that surgeons should focus on the details of a specific technique and perfect that technique.

A summary of recent meta-analyses of autograft ACL reconstruction with either hamstring or patellar tendon had similar findings, with the only significant difference between the two graft types being increased anterior knee pain in the patellar tendon group, with a 9 % higher incidence of knee pain in that group [3, 4]. There were small differences in the rate of positive pivot shift exams, anterior knee pain, extension deficit, percent normal IKDC score, and return to preinjury activity level between the groups with some favoring hamstring and others favoring patellar tendon. The statistical evaluation and complete data from the 19 RCTs are shown in Table 3.3.

Finally, a Cochrane meta-analysis (2011) on autograft choice between hamstring and patellar tendon found no difference in patient-reported outcomes (KOOS, IKDC). They concluded that they were unable to recommend one graft over the other, as some of the specific functional testing had slightly better results for patellar tendons and other tests showed improved performance for hamstring grafts. This data can be seen in Table 3.4.

Table 3.4 Data from meta-analysis on differences between hamstring and patellar tendon autografts

	Pivot shift[a]	Ant knee pain[a]	Extension deficit[a]	% IKDCA[b]	Return[c] to preinjury activity
HG (%)	24	13	6	33	67
BTB (%)	19	22	9	41	76
Absolute difference (%)	5	9	3	8	9
Method	PE	Undefined	PE	Composite scale	Single?
Significant	No	Yes	No	No	No
HG/BTB relative risk				0.90 (0.79–1.03)	0.94 (0.85–1.05)

HG hamstring graft, *BTB* bone-patellar tendon-bone graft

What About Other Autografts?

Other autograft choices have not been studied as intensely. To our knowledge, there has not been a randomized control trial (LOE I) or prospective longitudinal cohort with concurrent controls (LOE II) performed on quadriceps or contralateral patellar tendon versus either hamstring or patellar tendon. Therefore, there are only lower levels of evidence studies supporting the use of quadriceps tendon (LOE IV) [8, 15] and contralateral patellar tendon and hamstring [21].

Single or Double Bundle?

Standard ACL reconstruction is performed with one bundle of graft going through a single tunnel in the tibia and then through another single tunnel in the femur. "Double-bundle" ACL reconstruction involves placing two smaller grafts across the knee, each with its own set of tunnels (thus two tunnels in the tibia and two in the femur). The justification for this increase in complexity of the surgery is that the ACL has two major functional bundles, and a procedure which replaces both bundles individually (double bundle) would be better than one which replaces the two bundles with one larger bundle (single bundle). However, it is not yet known whether this plausible hypothesis is borne out in clinical outcomes.

Meredick et al. [16] did a meta-analysis of the outcome of single- versus double-bundle grafts in randomized controlled trials in the literature. They concluded, "Double-bundle reconstruction does not result in clinically significant differences in KT-1000 arthrometer or pivot shift testing." When they also included prospective cohorts and retrospective comparative studies, they still did not find any difference between single- and double-bundle graft outcomes.

Aglietti et al. [1] (LOE I) randomized 70 subjects to either single ($n=35$) or double bundle ($n=35$). Minimum follow-up was 2 years. There were no clinically relevant or statistically significant differences in clinical outcome (KOOS, IKDC) or in the return to previous level of activity. The KT side-to-side difference was 2.3 mm for single-bundle and 1.3 mm for double-bundle grafts. While this was statistically significant, it was not clinically relevant. Similar findings were reported in other Level I studies [12, 20].

A randomized control trial assessing cost-effectiveness of single versus double bundle by Nunez et al. found similar health-related quality-of-life and medical outcomes between the two groups at 2-year follow-up; however, the single-bundle technique was more cost-effective [17].

Should I Use Allograft?

Allografts are tissues that are obtained from a donor after their death. Tendons, including the tibialis anterior, Achilles, and patellar tendon, can be stored frozen and then used in ACL reconstruction. The use of an allograft means the patient does not have to have their own tissue harvested for the graft. However, with allografts, there are differences in the processing of the grafts that may not only affect tissue integrity biomechanically but potentially have clinically relevant effects on the biologic incorporation of the graft in the knee. Review of the literature indicates one has to be aware of the variation in processing when choosing allograft.

A randomized control trial of 102 patients allocated to bone-patellar tendon-bone (BTB) autograft ($n=33$), irradiated BTB allograft ($n=32$), or nonirradiated BTB allograft ($n=34$) found no difference in failure rate between nonirradiated allograft and autograft, but increased failure rate with the irradiated grafts [24]. The irradiated allografts in this study were sterilized with 2.5 Mrad of irradiation.

A meta-analysis by Kyrch et al. compared the results of BTB autograft and BTB allograft in ACL reconstruction [14]. Six prospective studies, with minimum 2-year follow-up, were included. Allograft patients were more likely to rupture their graft than autograft patients (odds ratio [OR] 5.03) and more likely to have a hop test less than 90 % of the nonoperative side (OR 5.66). When irradiated and chemically processed grafts were excluded from analysis, no significant differences were found in graft rupture rate, reoperation rate, IKDC scores, Lachman and pivot shift testing, patellar crepitus, hop test, or return to sport.

Is Allograft Versus Autograft a Cause of Primary ACL Reconstruction Failure?

A Level I study by Kaeding et al. using patients enrolled in 2002 and 2003 in the Multicenter Orthopedic Outcomes Network ACLR study included ~1,000 patients, with 94 % follow-up via questionnaire or phone contact [13]. ACL failure was defined as the patient having revision ACL reconstruction. In 18-year-old patients, 20 % of the patients who had allograft had failed versus 6 % of the patients who had used their own tissue (autograft). In 40-year-old patients, there was 3 % failure in the allograft patients versus 1 % failure in the autograft patients. The number needed to harm (NNH) from using allograft was thus 7 for high school age patients, meaning that for every seven high school patients who have an allograft ACL reconstruction, one extra graft failure will occur than if all seven had treatment with autograft. Graph of the data for risk of failure by age, for both autograft and allograft, can be seen in Fig. 1.7.

A case–control study consisting of 21 ACL revisions due to graft failure included 5 patients who had an autograft reconstruction (quadrupled hamstrings) and 16 patients who had had an allograft reconstruction (tibialis). These patients were compared to age- and sex-matched controls ($n=42$) with no graft failure at minimum 2-year follow-up [5]. They found that patients with higher activity level had 5.53 greater odds of ACL graft failure (5.53 OR, 95%CI, 1.18–28.61; $p=0.03$) and the patient who had an allograft reconstruction had 5.56 greater odds of graft failure than autograft (5.56 OR, 95%CI, 1.55–19.98, $p=0.009$). They concluded that the "odds ratios suggest a multiplicative interaction between higher activity level at time of graft failure and allograft for ACL graft failure after ACLR." This means that a young active patient with an allograft reconstruction has a much higher risk of graft failure than an older patient with an autograft reconstruction. Thus, the optimal graft choice may depend on age and activity level.

Metal or Bioabsorbable Screws?

In a meta-analysis looking at metal versus bioabsorbable screws, Shen et al. included 10 Level I articles [22]. They looked at functional outcomes (IKDC and Lysholm) and laxity measures (pivot shift and KT). They concluded that there were no clinically significant differences between grafts fixed with either metal or absorbable interference screws. They did find a statistically significant lower risk of knee effusion (knee swelling) after reconstruction with metal screws but noted that the effusion is of unknown etiology.

Rehabilitation ACL Reconstruction (Level I)

Wright et al. [25, 26] performed a systematic review of 54 randomized controlled trials and found that postoperative rehabilitation programs with immediate postoperative weight bearing were safe, continuous passive movement and postoperative functional bracing were of no benefit, self-directed home therapy after initial education and with periodic monitoring had the same results as with a therapist, closed-chain exercises were safer than open-chain, and accelerated rehabilitation with goal of 6-month return to play is safe.

Complications After ACL Reconstruction (Level I)

Spindler et al. [23] in a Level I systematic review found that the rate of graft failure at 2 years is 3.6 % (24/664), with a 95 % confidence interval of 2.3–5.3 %. There was no difference in graft failure between hamstring and patellar tendon grafts (3.1 % PT (10/325); 95 % CI 1.2–5.0 %; 4.1 % HG (14/339); 95 % CI 2.0–6.2 %). The average infection rate was 0.8 % (5/608). The risk of additional arthroscopic

surgery at 2 years was 14.7 % (72/491). There were no reported deep venous thromboses and one nerve injury recorded.

A systematic review looking at knee osteoarthritis after ACL injury looked at 7 prospective and 24 retrospective studies [18]. They found that meniscal injury was consistently related to radiographic osteoarthritis. At 10-year follow-up, the prevalence of radiographic knee arthritis with an "isolated" ACL injury (no meniscus tear) prevalence was 0–13 %, and if there was combined ACL and meniscal injury, the prevalence was 21–48 %. This study was limited by heterogeneous classification systems for osteoarthritis, lack of inter-rater studies, and lack of multivariable analysis. The authors concluded that future studies should (1) include prospectively defined aims and end points; (2) report percent follow-up; (3) use a single common radiologic classification system with reliability data and an independent, blinded examiner to evaluate these; (4) report rehabilitation programs; and (5) perform regression analysis to evaluate risk factors for knee OA.

Can ACL Ruptures Be Prevented?

The Level I prevention studies to date have primarily been aimed at female athletes. Primary prevention focused on neuromuscular training, balance, and strengthening exercises a minimum once per week for at least 6 weeks. The results of these studies are very encouraging as half of them showed a decreased risk of ACL tears with training, with an average 60 % decrease in injury rate. Three of six trials in a meta-analysis showed reduced risk of ACL tears with overall odds ratio 0.40 (95 % CI 0.26–0.61) [11].

Areas of Remaining Uncertainty

The major questions after ACL reconstruction that still need to be answered are:

1. What are the risk factors and predictors for future premature osteoarthritis?
2. Is there a secondary prevention for ACL graft and contralateral ACL tears?

Other areas needing further study are timing and performance with return to play, efficacy and optimal method of meniscus repair with ACL reconstruction, and how to treat partial ACL tears.

Summary

Based on the best literature, what outcomes are expected after ACL reconstruction? With either a hamstring or patellar tendon graft, most patients should expect:

- A knee with less than 2 mm of increased laxity compared to the other, uninjured side (KT-1000 difference of <2 mm).

- Isokinetic strength greater than >90 % of the opposite, uninjured side.
- Normal range of motion.
- A mild decrease in the total activity level (Marx activity level down by 4).
- A mild decreased in the overall function of the knee (IKDC composite score – <40 % normal).
- A 40 % chance that they will develop osteoarthritis on x-ray within 10 years.
- Their graft will fail 1–20 % of the time, with higher rates of graft failure seen in young, active patients.
- They are more likely to tear their opposite (contralateral) ACL than their graft.
- The rate of postoperative infection is low –0.9 %.
- If they need a meniscus repair at the time of their ACL reconstruction, the meniscal tear should heal 87–96 % of the time.

Patient Education by Level of Evidence (see Table 3.5)

Table 3.5 Summary of data from George et al. [9], endoscopic versus rear entry (2 incision)

What is the risk of ACLR graft failure at 2 years?	1–20 % (LOE I)
What is the risk of ACL tear in the normal contralateral knee at 2 years?	3–6 % (LOE II)
What will my future activity level be after ACLR?	Decreased (↓ 4 Marx levels) (LOE I)
What is the risk of future OA (radiographic) after ACL tear/ACLR?	Isolated ACL tear: 0–13 % ACL tear with meniscus tear: 21–48 % (LOE II)
What is the success of meniscus repair during ACLR?	94 % (LOE I)
What causes knee pain at ACLR?	BMI, female gender, older age, and LCL injury (not bone bruise) (LOE I)
What is my risk of infection after ACLR?	0.8 % (LOE I)
Will my knee feel stable after ACLR?	Expect average 1–2 mm instrumented laxity (KT): not clinically significant (LOE I)
What will my ROM be after ACLR?	Normal (LOE I)
Will I be able to return to sports?	Unknown, except football where high school and college athletes ~70 % RTP
How will my knee feel?	Improved from preoperative, but not normal
What is the best graft source (autograft or allograft)?	Lower failure rate in autograft, especially in younger patients (LOE I)
What is the best autograft choice (patellar tendon or hamstrings)?	No difference (LOE I)
Should I use a brace after ACLR?	No evidence to support routine use of brace with isolated ACLR (LOE I)
What decreases long-term outcome after ACLR?	Worse outcome allograft, higher BMI, and smoking
Will my results be same over time?	2- and 6-year outcomes are similar

Conclusion

To continue to improve outcomes after ACL reconstruction, we need to identify modifiable predictors of failure and other poor outcomes. This can be best done through prospective multicenter longitudinal studies. These studies should be used to determine how an individual responds in clinically meaningful ways rather than focusing only on population "average." Randomized control trials may have a role in determining the efficacy of a new treatment as compared to standard ones, however, prospective longitudinal cohorts can also be very helpful in understanding how to incorporate new treatments into clinical practice.

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Chapter 4
ACL Treatment in the Skeletally Immature Patient

Patrick Vavken and Martha M. Murray

Pediatric ACL Tears

Recent reports from registries of ACL patients suggest that one-third to one-half of all surgical ACL procedures are performed in children or adolescents [1]. While it had previously been thought that skeletally immature athletes pull the ACL off the bone where it attaches to the tibia (shinbone) with a tibial spine fracture, it has recently come to light that many tears sustained by children are within the ligament midsubstance, similar to those seen in adults.

Treatment of ACL tears in skeletally immature patients has the same goals as treatment in the adult population, namely, recreating a stable knee and avoiding secondary joint damage. In young patients, however, the consequence of secondary damage can be far more severe due to the length of time the knee will need to function after injury. Furthermore, due to the prolonged exposure to high activity of skeletally immature patients when compared to adults, the risk of significant secondary injuries to the cartilage or menisci is higher for these patients. For example, for most skeletally immature patients, the onset of osteoarthritis 10–14 years after ACL injury would occur in their mid to late-twenties. In addition, in the growing skeleton, ACL reconstruction surgeries, where tunnels are drilled in the top of the shinbone and bottom of the thighbone, can potentially damage the growth plates in each of these locations and lead to growth disturbances. These factors must all be

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_4,

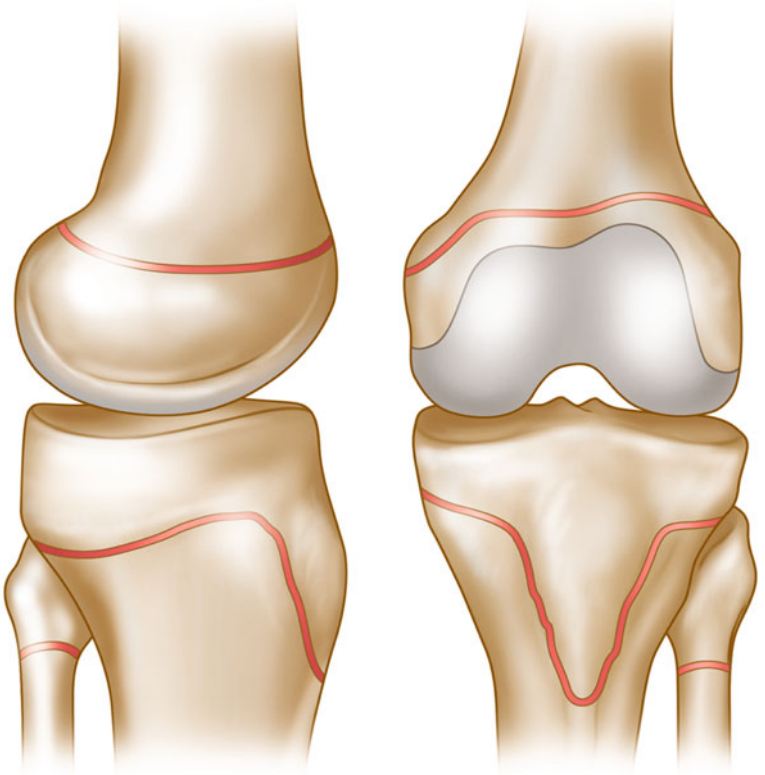

Fig. 4.1 Schematic of knee with growth plates. Illustrates the growth plates around the knee. Sixty-five percent of lower leg length derives from these two growth plates. Both growth plates, although often described as disc, have a complex three-dimensional structure. Injury to the growth plate can lead to focal or global growth disturbances

considered when choosing a treatment for a skeletally immature patient with an ACL tear.

Children and young patients with a significant amount of remaining growth are at risk of growth deformities if the growth plates (or "physes") are injured (Fig. 4.1). The growth plates close and height changes stop around 13–15 years of age in girls and around 16–18 years of age for boys. The progression of growth can be assessed and documented using the method described by Tanner and Davis [2], using physiologic signs of outer sexual development or radiographically using X-ray images of the hand and wrist, the pelvis, or the knee. During skeletal growth, 65 % of the lower leg growth derives from the growth plates at the end of the thighbone (distal femur) and the top of the shinbone (proximal tibia). To perform a standard "adult" ACL reconstruction, tunnels are drilled both in the distal femur and proximal tibia and these tunnels cross the region of the "closed" growth plates (Fig. 4.2).

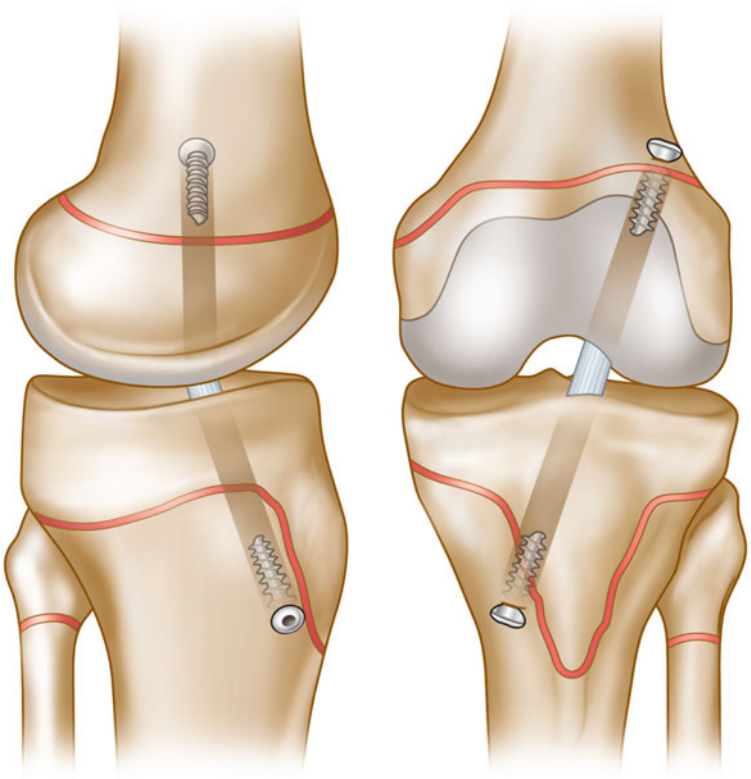

Fig. 4.2 The relationship of the physes (growth plates) to standard ACL reconstruction techniques using interference screw fixation. Both the peripheral tunnel placement and fixation techniques can injure the growth plate if care is not taken to appropriately modify the technique for a skeletally immature patient

Tunnel placement in ACL reconstruction thus can potentially jeopardize both growth plates if it is performed in a patient who is still growing. Damage to the growth plates can lead to the leg being shorter on one side or the leg growing in a crooked fashion rather than straight (Fig. 4.3). This potential risk to the physis has discouraged orthopedic surgeons from performing surgical ACL reconstruction in skeletally immature patients for a long time.

The risk of damage to the growth plates after drilling a tunnel across them has been studied in a number of large animal studies. These studies assessed the risk of length discrepancy and angular deformities after placement of tunnels as they would be performed in ACL reconstruction. These studies suggest that the risks of growth disturbance can be minimized by adherence to several basic principles. Placement of the distal femoral tunnel in a posterior position can lead to the tunnel disrupting the periosteal ring of Ranvier, which can result in tethering of the growth plate at the

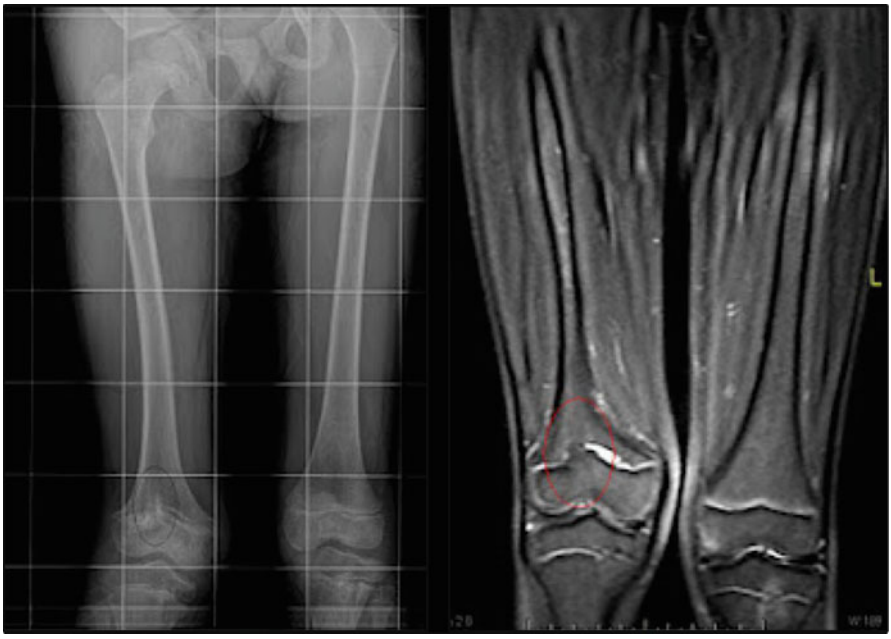

Fig. 4.3 Closure of physis leading to shorter leg, off-center closure leading to angular deformity. If the growth plate is injured, growth disturbances can ensue. If the damage is large or central, growth across the whole physis is disrupted and the leg will be shorter. The extent of leg-length discrepancy (LLD) will dictate the clinical ramifications (70 % of all people have up to 7 mm LLD at baseline). If the defect is off-center, a growth disturbance will occur at the site of defect, while the rest of the physis will grow normally. This will lead to an angular deformity with physeal opening away from the defect. A defect of the inner side of the knee will lead to bowleggedness, a defect on the outer side to knock-knees. Defects in the front of back will need to a forward or backward bend of the knee, called antecurvatum and recurvatum. Again, the extent of this defect dictates the problem it causes. The figure shows a physeal defect with a bone bridge across the growth plate of the lateral femoral growth plate that has led to a shorter leg (look at the hip in the top area of the X-ray) and a valgus (knock-knee) deformity (Image courtesy of Sumer Sethi, MD., Sumer's Radiology Site; URL, http://sumerdoc.blogspot.com)

back so the front of the bone grows more than the back, leading to an angular deformity in the bone [3, 4]. In addition, placing large tunnels in a small bone can also lead to a disturbance of growth plate function [5–7]. In addition, making the graft too tight [8] or failure to completely fill the tunnel with graft [9, 10] can also lead to problems. Lastly, placing fixation devices across the physis can also prevent it from functioning effectively [11]. By using a slightly more anterior femoral tunnel, small tunnels that are completely filled with a soft tissue graft, only moderate graft tension and avoiding putting fixation across the physis, the risk of growth disturbances after a transphyseal ACL reconstruction can be reduced to below 1 %, as reported in two recent meta-analyses [1, 12] (Fig. 4.4).

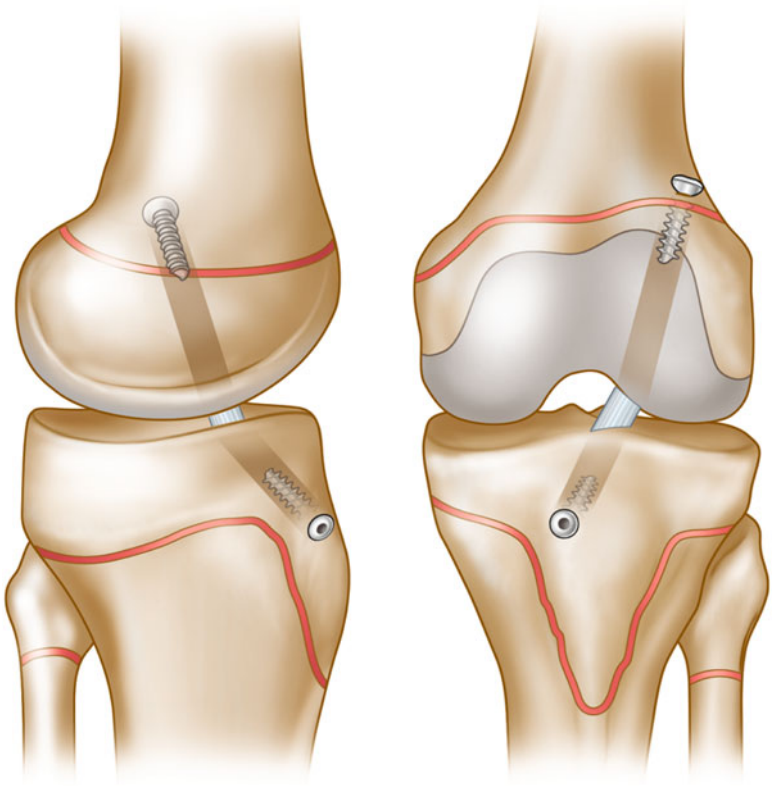

Fig. 4.4 Schematic of femoral transphyseal ACL reconstruction with central small tunnels filled with graft and fixation away from physes. From clinical experience, as well as from animal trials, it is known that small bone tunnel in the center of the growth plate poses only a marginal risk for growth disturbances. Other important factors are that soft graft and not bone plug is placed in tunnel and that the tunnel is completely filled

History of Nonsurgical Treatment for Pediatric ACL Injuries

With the concern of harm to the growth plate during ACL reconstruction, many surgeons have elected to pursue nonoperative treatment of these injuries until the child reaches skeletal maturity. This course typically consists of limited weight bearing with or without a brace for up to 8 weeks combined with physical therapy to regain and maintain muscle strength. The training often starts with isometric exercises first, gradually progressing to closed kinetic chain exercises over approximately 2 months. Thereafter, moderate sports participation, particularly sports that involve "straight-ahead" activities such as indoor cycling or swimming, are encouraged. Pivoting sports should be avoided until definitive treatment, which is usually ACL reconstruction after skeletal maturity [1, 13–15]. Some investigators advocate return to pivoting sports with a brace after 1 year of physical therapy [14].

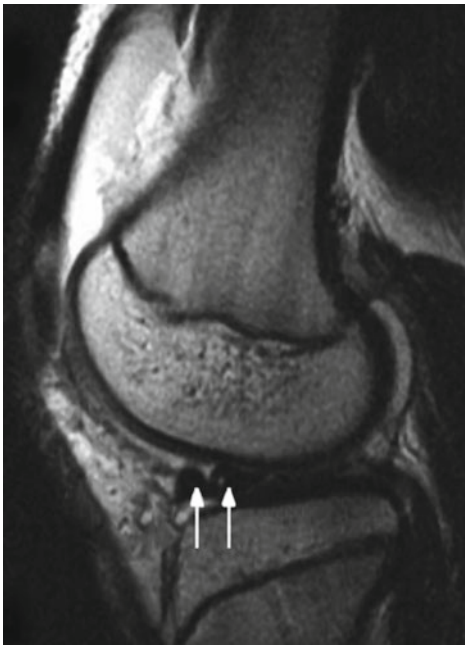

Fig. 4.5 MRI picture of skeletally immature patient with meniscal tear. This figure shows the MRI of a skeletally immature patient with a meniscus tear. The picture is T2-weighted; thus, bright white means water, as it is seen in the middle of the black triangle that is the meniscus. The wedge-shaped menisci acts a "doorstop" for the knee and, together with the ACL, holds the thighbone in place. If the ACL is torn, the stress on the meniscus rises and eventually it may tear. Just like the ACL, meniscus tear very rarely heals but usually extends. Thus, a patient with an insufficiently treated ACL tear may acquire meniscus symptoms and, if the meniscus fails completely, this may accelerate the development of osteoarthritis (with permission)

Nonoperative treatment until skeletal maturity has been considered the first-line treatment for immature patients with ACL tears for a long time and is still favored by many [1, 12]. It has been favored because it was assumed that surgical treatment, i.e., transphyseal ACL reconstruction, would expose immature patients to an undue risk of growth plate damage, resulting in limb-length discrepancy and/or angular deformities (see Fig. 4.3).

However, careful follow-up of pediatric patients undergoing nonoperative treatment has revealed a troubling effect: these patients remain at high risk for additional damage within the ACL-deficient knee, even with bracing and sports avoidance (Fig. 4.5). Long-term studies have reported problems such as subsequent meniscal damage and early osteoarthritis [1, 12–25]. Many of the changes are not reversible or repairable.

A recent meta-analysis of 476 young patients followed for over 4 years of nonoperative treatment as outlined above reported a high proportion of unstable, symptomatic knees with chronic degenerative changes in the ACL, meniscus, and cartilage – leading to half of the patients electing to proceed to surgical

reconstruction during the 4-year follow-up period [1]. While it is not clear whether the meniscal and cartilage damages were caused by a treatment failure (recurrent instability of the knee leading to meniscal damage) or are the cause of the treatment failure (meniscal injury causing locking and feelings of instability), it would seem as if the outcomes of nonoperative treatment could be improved upon.

Surgical Treatment

If nonoperative treatment has problems of high rates of additional knee damage, one might then consider surgical stabilization of the knee as an alternative to improve these outcomes. There are two major categories of ACL reconstruction performed in the pediatric population. "Transphyseal" ACL reconstruction, where tunnels are drilled through the distal femur and proximal tibia for graft placement, is similar to that performed in adults (see Fig. 4.2). "Physeal-sparing" ACL reconstruction involves techniques that avoid drilling a tunnel across the physis. Physeal-sparing techniques achieve this either by fixing the graft on the outside of the bone, away from the physis, or by drilling tunnels within the end of the bone (and not crossing the growth plate).

Transphyseal ACL Reconstruction

Standard, "transphyseal" reconstruction has been largely reported in adolescents who are within a few years of their growth plates closing. To date, 31 studies present findings for ACL reconstruction with at least one transphyseal tunnel in total of 479 patients with an average age of 14 years [14, 16, 17, 19–21, 23, 26–48]. In these studies patients were typically followed for 42 months (± 19 months) after surgery.

The grafts used in these studies were largely similar to those used in adults – hamstring graft, quadriceps tendon graft, and patellar tendon graft. Gebhard et al. in 2006 published a direct comparison of four different graft types (hamstrings $n=28$, patellar tendon $n=16$, fascia lata $n=12$, and quadriceps tendon $n=12$) in a multicenter study including 68 patients at Tanner stage 1–3 and 28 patients at Tanner stage 4–5 [34]. After an average follow-up of 33 months, Tegner activity, Lysholm, and International Knee Documentation Committee (IKDC) scores and joint stability (i.e., KT-1000) were assessed. While all four groups showed a significant improvement over the nonoperative treatment group, there were no significant differences between the surgical groups for Tegner activity, Lysholm, and IKDC scores, or knee stability.

Looking at the combined results of recent studies of transphyseal ACL reconstruction in immature patients (including almost 500 individuals), only three angular deformities and two limb-length differences of more than 10 mm were observed, a risk of roughly 1 % [1]. Ten patients had MRI results consistent with physeal

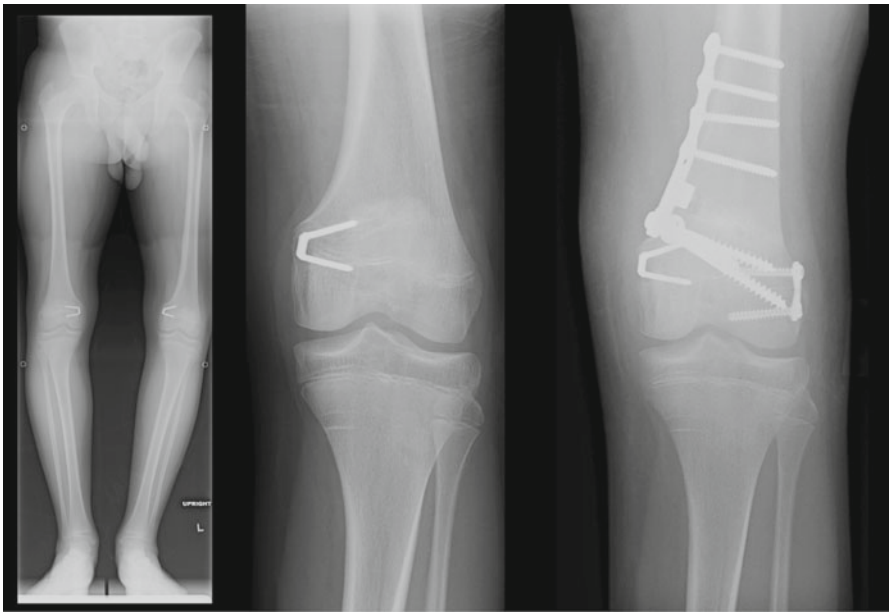

Fig. 4.6 X-ray of staple fixation across the physis. The bone tunnels needed for ACL reconstruction are not the only threat to the physis. Even with perfect tunnel placement, the graft has to be fixed to the bone, using screws, staples, or other devices. If such a fixation device is placed over or too close to the physis, it can disturb growth. As a matter of fact, in children with angular deformities, a treatment called epiphysiodesis can be used, wherein the physis is artificially closed on the side away of the deformity shortly before the stop of skeletal growth to balance out the angulation. In this case the staples were fixed in such a way that they compress the growth plate, resulting in bowed legs (*left panel* and *middle panel*). Over time this deformity became symptomatic (inner-sided knee pain) and a corrective osteotomy was required (*right panel*)

narrowing, but without angular or limb-length deformities. Another meta-analysis of 55 studies, which included 935 ACL-injured patients 13 years of age on average, showed a slightly higher risk for growth disturbances of 1.8–2 % [49]. However, when interpreting these numbers, it is helpful to keep in mind that 70 % of the non-injured population also have leg-length difference of up to 7 mm, while 7 % of the noninjured population have a leg-length difference of 12.5 mm or more [50]. Other studies have gathered information on the potential causes for leg-length differences after ACL reconstruction. A study of orthopedic surgeons listing the problems they had seen identified graft fixation devices or bone plugs placed across the physis as particularly problematic (Fig. 4.6). Both of these problems can lead to bony bars (that cease the function of the growth plate) forming across the growth plate at the end of the thighbone (54 % of angular deformities) or the top of the shinbone (27 % of angular deformities) [51]. These are the most common causes of growth disturbance after ACL reconstruction, followed by tunnel placement and tunnel diameter [51].

The clinical outcomes after transphyseal ACL reconstruction in immature patients are similar to those reported for adult patients. Patient-oriented outcome scores show that about 85 % of patients feel their knee is nearly normal after surgery [49], and Lysholm scores and OAK scores, two measures of knee function, of 95–98 (of 100 as best result on both scores) can be expected [1, 12]. Over 90 % of young patients return to their pre-injury activities, and over 90 % have a knee that is similar in terms of stability to the opposite, uninjured knee [1, 12, 34]. However, re-rupture rates of 4–10 % have been reported for ACL reconstruction in young patients [49], and graft failure rates in the long term can vary between 25 and 41 % [52, 53]. Furthermore, long-term follow-up studies have shown the rate of posttraumatic osteoarthritis despite ACL reconstruction to be as high as 41–75 % within 10–14 years of surgery [52, 53]. This may be the biggest problem facing a 14-year-old with an ACL tear, as osteoarthritis in a young, active 30-year-old is a debilitating problem currently without a good solution.

Physeal-Sparing ACL Reconstruction

Physeal-sparing ACL reconstruction aims at surgical stabilization of the knee without damaging the growth plate. This is usually accomplished by placing the graft tunnels only within the very ends of the bone, staying away from the growth plate, or by using extraosseous stabilization techniques.

Physeal-Sparing, Transosseous ACL Reconstruction

Physeal-sparing procedures can be performed with a soft tissue graft [5, 6, 26] or a patellar tendon graft [54, 55]. Using X-ray to help keep placement of the tunnel away from the growth plate, a guidewire is drilled from the outside of the knee to the starting point of the ACL (Fig. 4.7). Once the guide pin is in satisfactory position, a cannulated drill (6–8 mm) is used to make the tunnel large enough for the graft [5, 6, 26]. The graft can be fixed in place with staples, interference screws, sutures, or a combination of these devices without violating the growth plates (Fig. 4.8).

There are data for 56 patients from five scientific papers undergoing physeal-sparing, intra-articular, transosseous stabilization [5, 6, 44, 54, 56, 57]. The average age of the patients in these studies was 13 ± 2 years, and all patients were followed for an average of 4 years.

While such procedures have been reported to have both a good clinical outcome and a low risk of growth disturbance, there are a few potential risk factors for physeal disturbance with these procedures. The following risk factors are mentioned without ranking the order of importance. Tunnel placement is usually not in the center of the tibia, which is a known risk factor for physeal damage in ACL

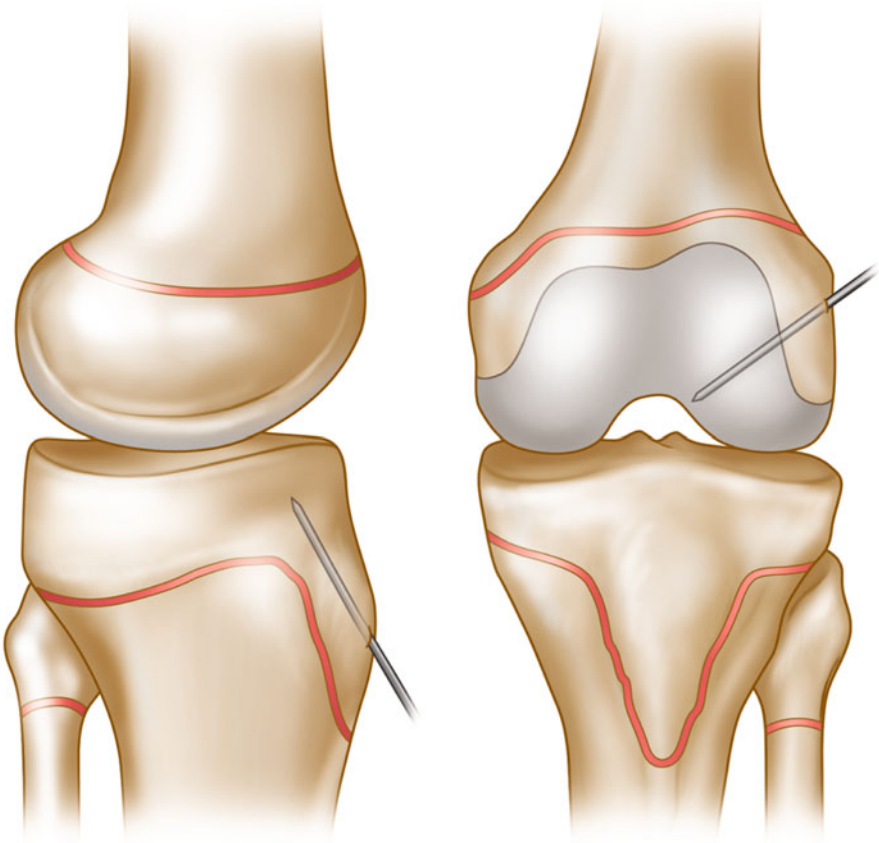

Fig. 4.7 All epiphyseal ACL reconstruction – guide pin with X-ray. Alternatively to transphyseal ACL reconstruction, the graft can be placed within the limits of the growth plates. As the first step, guide pins are placed away from the physis with the use of intraoperative X-rays and tunnels are drilled over these guides

procedures [1]. The distal fixation with staples can potentially tether the growth plate, and the graft lying parallel to and on top of the growth plate can cause a similar effect [1]. Furthermore, drilling though the epiphysis parallel to the growth plate must be done carefully to avoid thermal damage to the nearby growth plate. In an earlier analysis of these techniques, Frosch et al. calculated the risk ratio for growth deformities to be three times as high for physeal-sparing versus transphyseal ACL reconstruction [49]. If the above-mentioned facts are to be considered, this risk ratio can be substantially reduced, but if not, the growth plate might suffer more

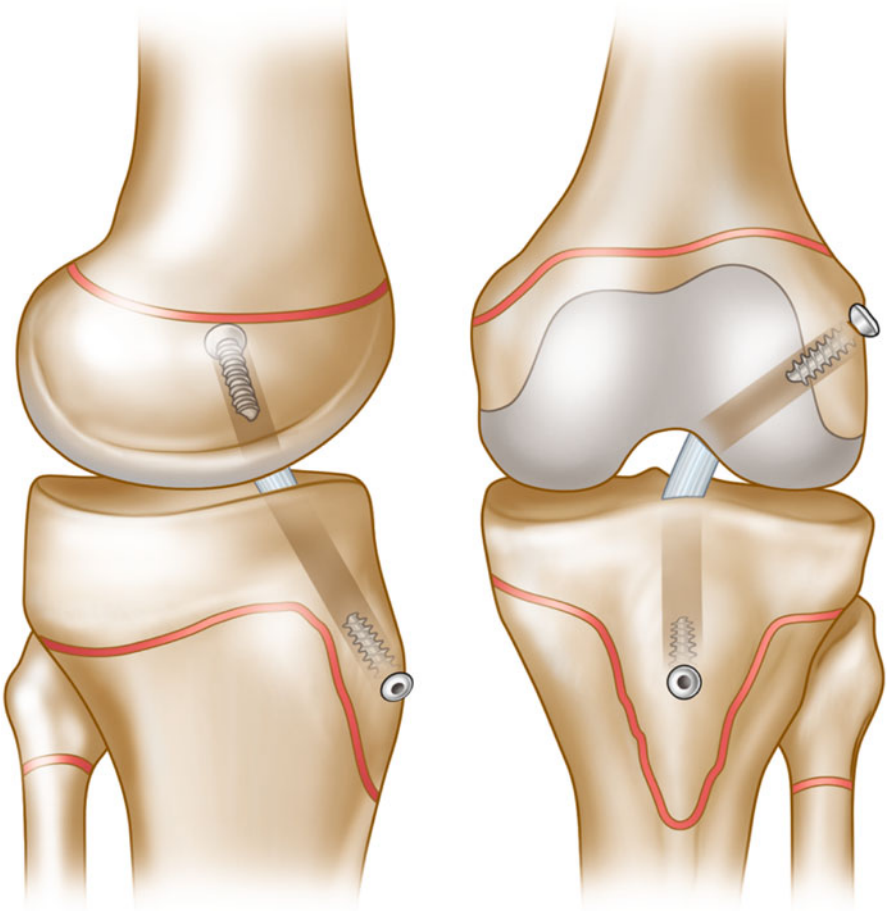

Fig. 4.8 All epiphyseal ACL reconstruction – views from the front (AP) and side (lateral) of the knee. In epiphyseal ACL reconstruction, the graft is placed within the limits of the femoral and tibial growth plates. Thus, in theory, the risk of growth plate injury is avoided. However, the geometry of the growth plate is complex and there is a risk to affect the physis at least partially. If this happens, it typically happens tangentially and off-center – thus, the risk of growth disturbance is actually higher than in transphyseal ACL reconstruction. Even if the tunnels are perfectly parallel, drilling releases a lot of heat and debris which, again, might injure the physis

damage from such "physeal-sparing" procedures than it would from transphyseal placement.

Apart from the potential growth disturbances, the clinical outcome of physeal-sparing intraosseous ACL reconstruction is excellent, with over 90 % of patients reporting excellent function of the involved knee. The final follow-up scores for the population described above were 98 for the OAK score and 96 for the International

Knee Documentation Committee score, on average. The average side-to-side difference in anterior-posterior laxity compared to normal knees was 1.5 mm.

Extraosseous Stabilization Techniques

Rather than keeping the drill holes close to the joint and to avoid drilling through the physis, alternative physeal-sparing approaches exist that involve no tunnel drilling at all. The best-known technique is a "combined intra- and extra-articular, physeal-sparing, extraosseous reconstruction," i.e., a modification of the technique originally developed by Micheli [58]. In this procedure (Fig. 4.9), the iliotibial band (ITB) is incised, tubularized, and brought to the over-the-top position at the back of the intercondylar notch by wrapping it around the lateral femoral condyle. At this position it is sutured to the condyle for additional fixation and inserted into the knee through the posterior capsule. From there, the ITB is brought to the front of the tibial ACL footprint, led through a groove placed underneath the intermeniscal ligament, and sutured to the periosteum or attached to the tibial cortex with staples. This configuration creates an extra-articular, anterior-posterior stabilization between Gerdy's tubercle and the lateral femoral condyle as well as an intra-articular stabilizer against anterior-posterior translation and rotation.

The results from 106 patients with an average age of 12 ± 1 years treated in such a fashion are available [16, 19, 34, 59–61]. After an average observation period of 47 ± 21 months, no growth disturbances such as leg-length differences or angular deformities were seen [1]. Lysholm scores at final follow-up ranged from 94.3 to 97.4 (where 100 is the best possible score), with no knee instability reported in any of the included patients. Direct comparison of such extraosseous stabilization with standard, transphyseal ACL reconstruction reported no difference in functional outcomes at 32 months postoperatively [34]. Although this treatment is usually considered a temporizing procedure, results are so good that it has functioned as a definitive reconstruction for a number of patients [59, 62].

An alternative procedure uses the semitendinosus and gracilis (hamstring) tendons, which insert at the inner side of the tibia just below the knee. The tibial insertions of these tendons are left intact, while the proximal (high-end) portion of the tendons are cut free underneath the skin at a level above the knee. These high ends are then folded backward and wrapped around the knee in a similar fashion as in the procedure described above but starting at the other end [61]. The important difference is that in this procedure the end of the stabilizing loop is at the medial side of the tibia, while in the procedure above, it is in the center of the tibia, resulting in a more natural construct. The semitendinosus-gracilis loop has been assessed in one clinical study including nine patients and produced no growth deformities and good clinical scores. However, none of the nine patients of the original study population returned to sports without bracing.

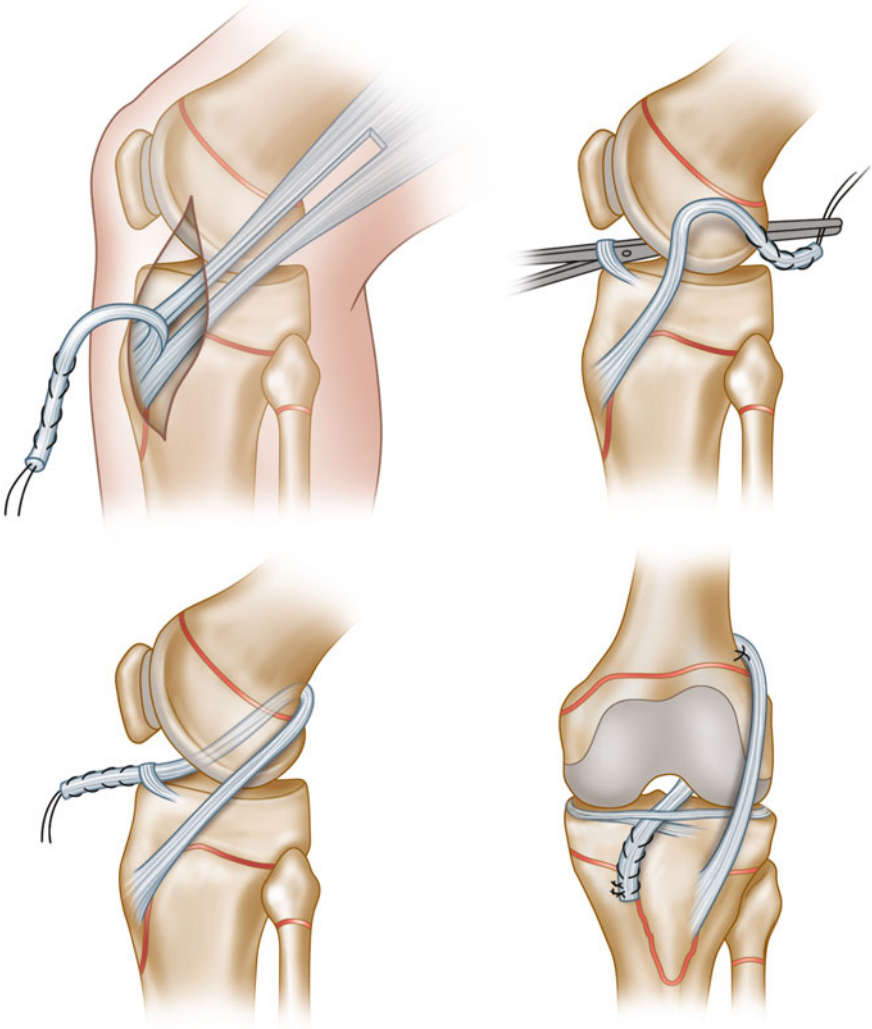

Fig. 4.9 ITB schematic. In this technique, the iliotibial band (ITB) is wrapped around the lateral femoral condyle, inserted into the knee through the posterior capsule, and brought to the front of the tibial ACL footprint where it is sutured to the periosteum or attached to the tibial cortex with staples. This configuration creates an extra-articular, anterior-posterior stabilization as well as an intra-articular stabilizer against AP translation and rotation

Surgery for the Very Young Patient: Tanner I and II

Most of the patients collected in scientific papers on ACL reconstruction in skeletally immature individuals are 13 years old on average and as such are usually in the later stages of skeletal development. Of particular interest is the management of

ACL tears in the very young patients, such as Tanner stage I and II which typically corresponds to a chronological age of 11 years or younger.

Liddle et al. followed 17 prepubescent patients for 44 months after standard, transphyseal ACL reconstruction and found 15 excellent, 1 good, and 1 poor result [39]. There were two complications reported (1 re-rupture of the ACL and 1 superficial wound infection right after the procedure that cleared quickly and without causing further problems). One patient in this group also developed a 5° valgus (knock-knee) deformity, but without functional disturbance. Bollen et al. observed five adolescent males treated with standard, transphyseal ACL reconstruction for 35 months and reported no growth disturbances. All children returned to their pre-injury level of activity. Streich et al. directly compared 12 patients treated nonoperatively with 16 patients treated surgically with standard, transphyseal ACL reconstruction 70 months after the procedure and found no angular deformities or leg-length discrepancies (≥15 mm side-to-side difference) [14]. Unsurprisingly, the surgical group had significantly better clinical outcomes. Within 2 years after the initial injury, 7 out of the 12 children in the nonoperative group (58 %) opted to receive surgical stabilization by transphyseal ACL reconstruction.

Micheli et al. used the ITB stabilization procedure described above for 17 prepubescent patients with ACL tears [63]. Eight patients were assessed after reaching skeletal maturity, on average 67 months after surgery. All patients reported subjectively stable knees, which were confirmed objectively by KT-1000 knee stability testing. No leg-length discrepancies or angular deformities were reported. The average Lysholm score for all patients at final follow-up was 97.4 of 100. Kocher et al. extended this treatment group to 44 patients, followed to 5 years postoperatively, on average [59]. Again, no leg-length discrepancies or angular deformities were seen. However, two patients underwent a second surgery because of graft failure at 5 and 8 years postoperatively. For the remaining patients, the mean IKDC score was 97 of 100, and the mean Lysholm knee score was 96 of 100.

No Treatment: ACL Tear Prevention

Earlier in this chapter, we outlined the treatment options for ACL tears in skeletally immature patients. While the described surgical treatments result in good and excellent clinical results in the midterm, the long-term outcomes are characterized by a considerable risk of graft failure and early osteoarthritis. New treatments might mitigate this situation, but are not clinically available yet. Hence, the current best option for managing ACL tears is their prevention.

Earlier studies have shown that approximately 80 % of all ACL tears in adolescent patients are noncontact injuries that involve quadriceps-active, valgus stress incidents. Typical reasons for such incidents are too narrow a stance during landing after a jump or direction changes with knees and hips close to full extension (particularly in girls). Based on such knowledge, various ACL tear prevention programs have been developed that aim at improving motion patterns, proprioception, and neuromuscular response (see Fig. 1.6).

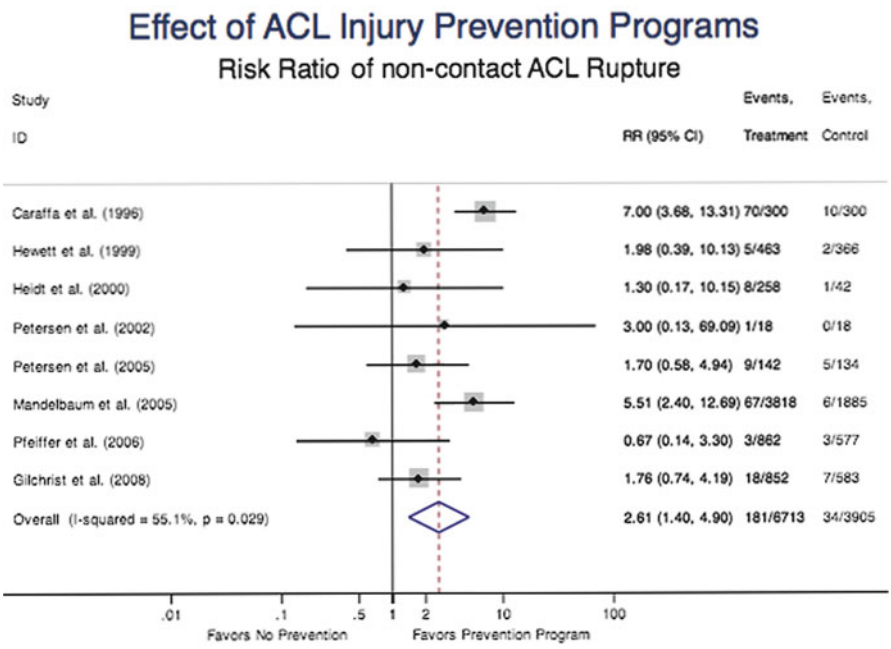

Fig. 4.10 Risk analysis for ACL injury prevention programs. While ACL injuries in skeletally immature patients can be successfully treated, avoiding them all together is a clearly favorable approach. Roughly 80 % of ACL tears are noncontact injuries in situations of excessive biomechanical stress due to poor knee alignment. Very frequently, alignment can be improved with very simple exercises (compare Chap. 1). A recent meta-analysis showed that the risk of noncontact ACL injury can be reduced by more than half by such exercises

Two recent meta-analyses have assessed the effectiveness of such programs in reducing the injury rate for noncontact ACL tears. Abernathy et al. assessed the effectiveness of strategies to prevent adolescent injury in sports in general, including a subgroup of knee and ACL injury, and found evidence for effectiveness of preseason conditioning, functional training, balance training, sport-specific skills, and education, but no evidence supporting the effectiveness of protective equipment such as braces [64]. Sadoghi et al. focused on programs for noncontact ACL injury prevention specifically. In their meta-analysis of nine controlled trials, they calculated a pooled risk ratio (RR) of 0.38 in favor of intervention programs versus untreated controls. This means there was a 62 % reduction in the risk of noncontact ACL ruptures in the prevention group. They also report a substantial difference in this effect across genders, with females showing a reduced risk of injury of approximately 50 % and males a reduced risk of injury of 15 % (Fig. 4.10). According to their statistical evaluation, the number needed to treat, i.e., the number of adolescents that need to receive training in order to avoid one ACL tear, was 38, which is fairly small considering that such prevention programs could be used for whole teams or high-school classes.

Summary

ACL tears in skeletally immature patients are an important clinical problem because they occur frequently, and the long-term effects can be significant. Because these patients are still growing, there is an increased likelihood of chronic secondary damage such as early onset osteoarthritis, but there is also the risk of growth disturbances with surgery. Management of ACL tears in such a population should consider all of these risks. Conservative treatment, which has been considered the first-line treatment to avoid growth disturbances, often results in continuing knee instability and the destruction of the menisci and cartilage. However, surgical treatment carries a small, but not zero, risk of growth disturbance.

Standard, transphyseal ACL reconstruction can be done even in the youngest patients. If a few simple principles are considered, the risk of growth disturbance remains well below 1 %. Physeal-sparing placement of ACL grafts is possible but could potentially lead to even more growth plate damage than transphyseal placement. Extraosseous stabilization, such as the ITB technique, has shown excellent results, but follow-up data beyond 5–8 years is scarce. Despite the impressive clinical results and a low risk of growth disturbances, surgical ACL reconstruction has less impressive long-term results, with roughly every other patient suffering from a failed graft or onset of osteoarthritis one to two decades after the initial injury. This is particularly troublesome in adolescents, who are in their twenties at this time.

New treatment options aiming at biological regeneration of the ACL are being developed and may help us improve the treatment of ACL injuries in this vulnerable population. Such treatments have shown promising results in large animal studies and are of special interest for immature patients because of their higher healing potential. Others have suggested to support ACL injury prevention programs, which have been shown to be effective – 38 adolescents have to be treated to avoid one extra, noncontact ACL tear – and easy and cost-effective to implement on a large scale.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR052772 and RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Part II
ACL Injury: The Biologic Problem

Chapter 5
The Biology of the Normal ACL

Martha M. Murray and Braden C. Fleming

In the previous section, we learned about ACL tears and their current treatment. To set the stage for understanding the development of future treatments, we will now review the known biology of the normal ACL, as restoring this biology is the goal of any future ACL treatment.

Physiologic Function of the ACL

The anterior cruciate ligament is a fibrous connective tissue that connects the end of the thigh bone, or femur, with the top of the shin bone, or tibia. It goes from the back, or posterior aspect, of the distal femur to the front, or anterior aspect of the tibia (Fig. 5.1), and serves to mechanically stabilize the knee joint, particularly for twisting and pivoting motions, like the motions a soccer player might make when they plant their foot and change the direction they are running ("plant and pivot," Fig. 5.2). The anterior cruciate ligament lives within the knee joint and is surrounded by fluid within the joint, called synovial fluid. The ACL is considered to be an "intra-articular" ligament because it passes through the middle of the knee joint, which represents the articular space. Other ligaments of the knee, including the medial and lateral collateral ligaments, are called "extra-articular" because they exist on the outer surface of the joint (outside the articular space of the knee).

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_5,

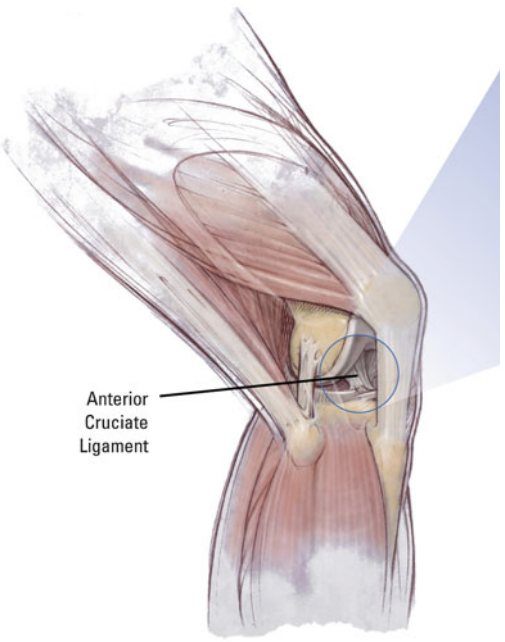

Fig. 5.1 Schematic of the anatomy of the anterior cruciate ligament (ACL) in a view of the knee from the side. The ACL courses from the back, or posterior aspect, of the distal femur to the front, or anterior aspect of the tibia

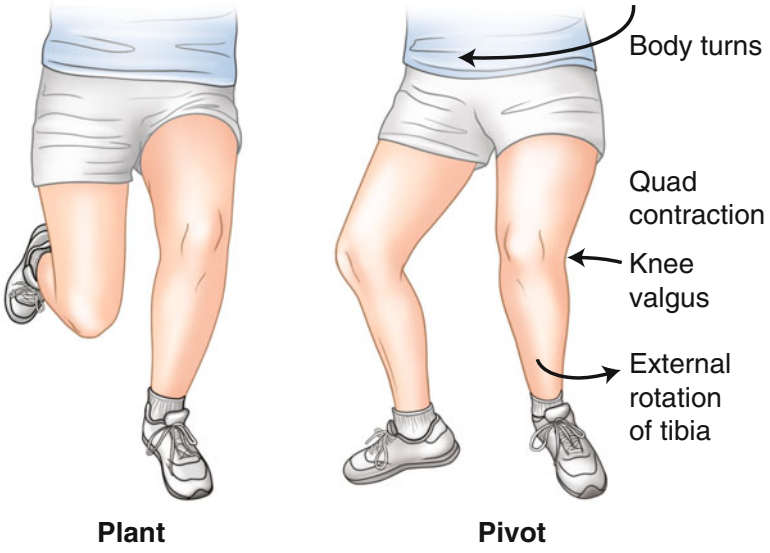

Fig. 5.2 "Plant and pivot" motion. In this movement, the player is planting her left leg in a flexed position. As she goes to kick the ball, she will pivot around this planted leg. A normal ACL is critical for stability during this maneuver. This can also happen during simple changes of direction with running, where the player plants one foot and then pushes off that foot in another direction (pivoting around the planted foot)

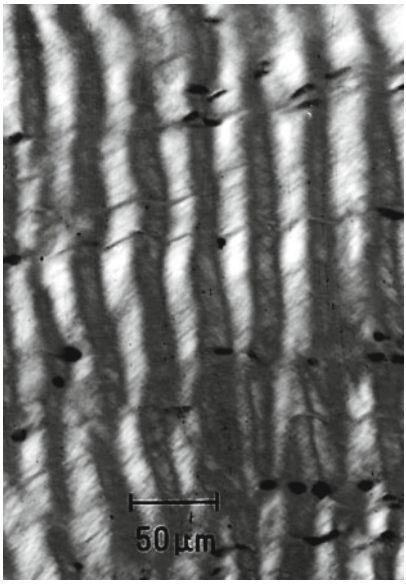

Fig. 5.3 Photomicrograph of the "crimp" or waviness seen in a normal ACL

These "extra-articular" ligaments are not surrounded by joint fluid; rather, they are typically surrounded by solid tissues that have a good blood supply.

Ligaments typically have a waviness to their fiber structure, which is important in their ability to function correctly (Fig. 5.3). The waviness, or "crimp" of the fibers, allows the ligament to stretch easily to a certain extent (while the waviness straightens out), but once straightened, the fibers can support high loads without stretching much (Fig. 5.4). This makes it so the bones of the knee can move relative to each other through a normal range of motion that the ligament is designed to allow. However, if the bones try to move any further away from each other, the ligament restrains the motion and prevents the abnormal motion of the joint from occurring. It should be noted that if the load becomes too excessive (i.e., exceeds the failure load of the ligament), it can cause the ligament to tear.

The anterior cruciate ligament also functions in this way. When the knee moves from a straight position (extension) into a bent position (flexion), the ACL is able to change in length like a spring as the joint moves. In a normal range of flexion/extension, the ACL provides minimal resistance to motion. However, if the knee goes into hyperextension (i.e., bending backward), or if the tibia moves too far anterior relative to the femur, or if it tries to bend to far inward (valgus), the ACL will resist these movements. The anterior cruciate ligament also provides proprioceptive feedback to the muscles surrounding the joint, particularly the hamstring musculature. This means that when the joint starts going into an abnormal position, the ACL stretches a bit and this stretch sends a nerve signal back to the large muscles in the back of the thigh (the hamstrings) which go in the same direction as the ACL (from the back of the thigh bone to the front of the shin bone). The stimulation of these large muscles can help the ACL stop the knee from going into an unhealthy

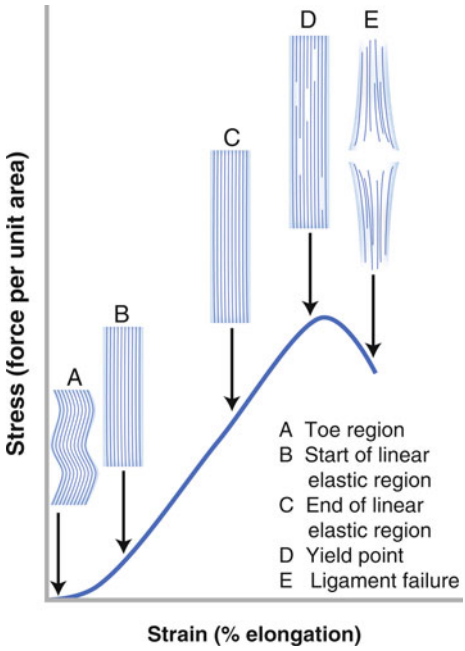

Fig. 5.4 A schematic of a load–displacement curve for the wavy fibers of the ACL. The waviness, or "crimp" of the fibers, allows the ligament to stretch easily to a certain extent (while the waviness straightens out), but once straightened, the fibers can support high loads without stretching much, thus keeping the knee stable

position. However, if a high enough force is applied to the knee or if it were applied so quickly that the muscles cannot respond, abnormal positions of the tibia and femur may occur that causes the disruption of the ACL (see Fig. 1.2). This can happen during a contact injury (i.e., a 300-lb lineman falls on the straight knee of the 120-lb kicker and forces it backward) or as a "noncontact" injury (i.e., subject lands on the ground with his or her center of gravity out of line with the knee). The quadriceps muscle (the muscle in the front of the thigh that goes down to the front of the shin bone) is used to straighten the knee. To do this, it pulls on the front of the tibia which can pull against the ACL. Typically, the hamstring muscles contract at the same time and stabilize the knee. However, athletes who have very strong quadriceps and relatively weak hamstrings can put their ACL at risk if the quadriceps has a sudden, unopposed strong contraction (like during a planting maneuver) as the quadriceps can pull the tibia forward and pop the ACL when the knee is extended or partially flexed.

Structure of the ACL

The anterior cruciate ligament has a complex structure [1–5]. The main molecule that makes up this structure is called collagen, specifically type I collagen. Type I collagen is made up of three intertwined chains of amino acids with links forming between the three chains as well as between the larger collagen fibers (Fig. 5.5). The

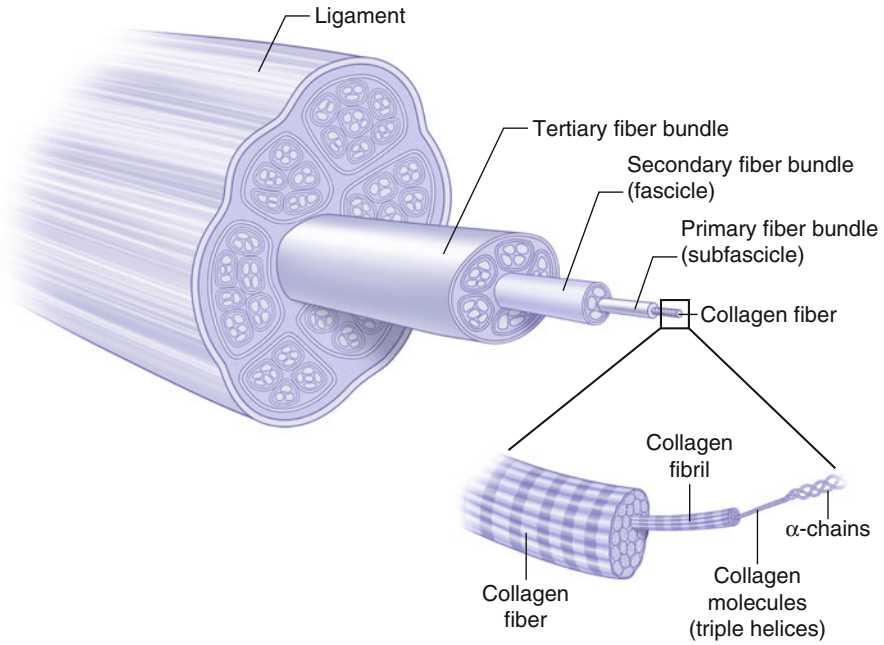

Fig. 5.5 Schematic of the ultrastructure of collagen. Type I collagen is made up of three intertwined chains of amino acids with links forming between the three chains as well as between the larger collagen fibers as seen in this figure. The fibers are typically going in the same direction and are lined up in parallel to provide the most strength of the tissue in this one direction (anisotropic). The bonds between the fibers, or cross-links, are primarily responsible for the strength in the direction perpendicular to the aligned fibers, and this cross-strength is typically orders of magnitude lower than the strength of the ligament along the direction of the fibers

fibers are typically going in the same direction and are lined up in parallel to provide the most strength of the tissue in this one direction (anisotropic). The bonds between the fibers, or cross-links, are primarily responsible for the strength in the direction perpendicular to the aligned fibers, and this cross-strength is typically orders of magnitude lower than the strength of the ligament along the direction of the fibers.

Collagen is an extracellular matrix molecule that comprises 70–80 % of the dry weight of ligaments (what is left when all the water is removed from the ligament). More than 90 % of the collagen is type I, with the remainder being type III collagen. The differences between type I and type III collagen are in the fibrils that make up the fibers, but the function of both types of collagen is to resist load pulling on the ends of the fibers. Both type I and type III collagen are produced inside an ACL cell (intracellularly) but are then excreted by the cell and modified outside the cell "extracellularly." Once the extracellular modifications are made, the collagen molecules then self-assemble into microfibrils. Collagen in ligaments is synthesized and degraded continuously, with a half-life of 300–500 days [6]. The factors regulating collagen turnover have yet to be determined. Other molecules like

proteoglycans, such as chondroitin-4 sulfate and dermatan sulfate, comprise less than 1 % of the dry weight of ligaments, although the exact amounts and composition are considered ligament-specific. Ligaments also contain elastin, fibronectin, and other glycoproteins. As much as 70 % of the wet weight of ligaments is water, which is both structurally bound to collagen and freely associated with the interfibrillar gel [7].

ACL Cells

The cell type most evenly dispersed among the collagen structure is the ACL fibroblast. These ligament cells are typically oriented in line with the collagen fibers of the ligament. There are more cells per unit volume in the ACL than in other joint tissues such as articular cartilage and cortical bone, but the ACL has far fewer cells in it than cell-dense organs such as liver or kidney. Typical methods for looking at cells in the ACL often show the central nucleus of the cell (where the DNA resides), but it has been more difficult to see the body of the cell, or cytoplasm, using standard methods. This is likely because the cytoplasm is thinly spread out and far reaching along the collagen fibers. Recent work with low-power electron microscopy has shown that there are extensive arms and legs for the ACL cells which extend great distances along the collagen fibrils [8]. The cells within the ACL depend predominantly on diffusion of nutrients from the blood vessels coursing between the ACL fiber bundles, and these long arms and legs may be one way the cell is able to feed itself, even when the central nucleus of the cell is relatively far from the nearest blood supply.

Because of the ease with which we can see the nucleus of the ACL with a microscope, the cells of the ACL have been described in terms of the nuclear shape of the cell. The major nuclear shapes are spindle-shaped or fusiform, ovoid, and spheroid (Fig. 5.6). Actin is a major protein within the ACL cell which helps to maintain the cell shape. Actin has six isoforms. The alpha-smooth muscle actin (SMA) isoform is a contractile form of actin and has been considered to be the signature of a fibroblast that is able to contract and tighten collagen fibers, also known as the myofibroblast [9]. Recently, ligament cells which contained this actin isoform were noted in certain stages of healing of the rabbit medial collateral ligament [10] and human ACL [11], and this cell type may also play a role in the normal maintenance of certain levels of structural organization of the anterior cruciate ligament.

The microscopic appearance, or histology, of the human ACL is characterized by how many cells are in a specific area of the ligament, what those cells look like, and the structural organization of the extracellular matrix. In the human ACL, there are three histologically different zones along the anteromedial bundle as one moves from the femoral to the tibial attachment sites (see Fig. 5.6). In the proximal ¼ of the ligament, the cells are spindle-shaped or fusiform, transitioning to a ovoid nuclear shape and then to a spheroid shape in the distal ¾ of the ligament [12]. The fusiform cell zone has a high cell density, with the cells oriented longitudinally, as

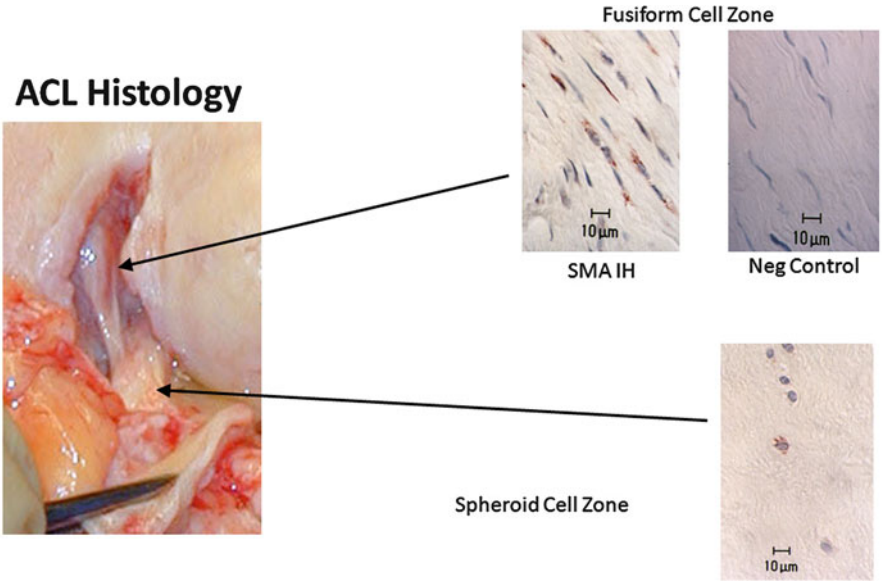

Fig. 5.6 Illustration of the various fibroblast cell shapes seen in the ACL. The cells of the ACL have been described in terms of the nuclear shape of the cell. The major nuclear shapes are spindle-shaped or fusiform, ovoid, and spheroid as seen here. In the human ACL, there are three histologically different zones along the anteromedial bundle as one moves from the femoral to the tibial attachment sites. In the proximal ¼ of the ligament, the cells are spindle-shaped or fusiform, transitioning to an ovoid nuclear shape and then to a spheroid shape in the distal ¾ of the ligament

well as longitudinal blood vessels and a high crimp length (or a long distance between the peaks of the crimp waves). The cells in this region are positive for alpha-smooth muscle actin, particularly in areas of crimp disruption. The ovoid cells live in a region between the fusiform and spheroid cells, and this region also has a high crimp length. The spheroid region has a low density of round-nuclei cells, fewer blood vessels, and a shorter crimp length [12]. How the morphology and distribution of the cells affect their function within the ligament requires further study.

Additional studies have investigated whether this distribution of fibroblasts noted in the human ACL is also present in other large animal knees. In a study comparing human, dog, cow, and sheep ligaments [13], the human ACLs were similar to the canine ACLs in all four measured parameters: cell number density, SMA expression, vascularity, and cell nuclear morphology. The normal human ACL was similar to the ovine ACL with respect to all parameters except cell nuclear morphology. The normal human ACL was significantly different from the bovine ACL in both vascularity and cell nuclear morphology. The human ligaments demonstrated a significant effect of location on cell morphology, which was not seen in the other three species. The variation in cell phenotype among species is important as these different cell types may represent differences in the intrinsic properties of the cells [13].

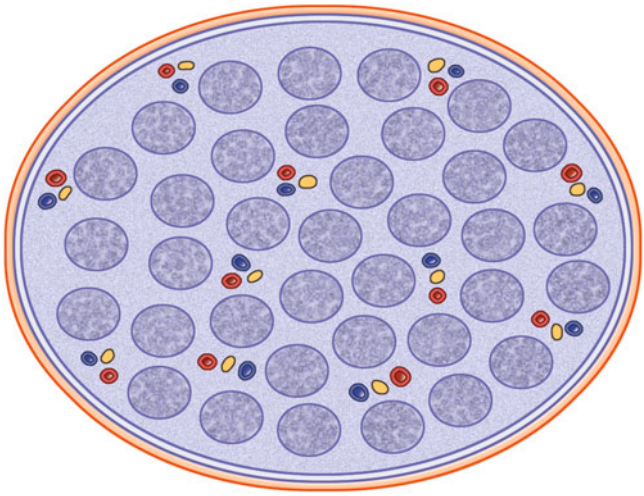

Fig. 5.7 Schematic of the various tissue structures that comprise the ACL in a cross-sectional view. The bundles of collagen fibrils (*dark gray*), or fibers, are surrounded by the endoligament (*lighter gray*) which also contains the blood vessels (*red and blue*) and nerves (*yellow*). The surface of the ligament is covered by an adherent, fibrous, cellular layer called the epiligament (*orange outer ring)*, which is also well vascularized. The vascular supply for most ligaments comes from vessels that cross joints and send branches to the periarticular ligaments. Within the ligament, the vessels typically course with the nerves of the ligaments, as neurovascular bundles

The bundles of collagen fibrils, or fibers, are surrounded by the endoligament (Fig. 5.7). The surface of the ligament is covered by an adherent, fibrous, cellular layer called the epiligament, which is also well vascularized. The vascular supply for most ligaments comes from vessels that cross joints and send branches to the periarticular ligaments [14]. Within the ligament, the vessels typically course with the nerves of the ligaments, as neurovascular bundles [15].

Ligaments also contain mechanoreceptors, which are thought to send out the signals to the hamstring muscles of the thigh when the ligament is stretched. Three types of nerve endings that can sense stretch (mechanoreceptors) have been identified in the ligaments of the knee, including Ruffini receptors, Pacinian receptors, and Golgi receptors [16]. A fourth type of receptor, the free unmyelinated nerve ending, is thought to function as a pain receptor [15]. The neurosensory role of these receptors has been supported by studies demonstrating changes in muscle and nerve function after disruption of the anterior cruciate ligament of the knee.

Growth and Development

When ligaments first form in the embryonic knee joint, they are simply a condensation of cells aligned between two bony attachment sites. As the fetus starts to move, the cells in the area of the future ACL start to make collagen molecules, which move

outside the cell and start to self-assemble. Interestingly, if joint movement is stopped, the cellular condensations disappear and the ligament does not form [17]. The cell body, or cytoplasm, of fetal and young adult rat ligament cells has a great deal of machinery, including abundant rough endoplasmic reticulum and a prominant Golgi apparatus, that produces these collagen fibrils. The presence of this machinery suggests the cells in the young ligaments are very active secretory cells [18]. As the ligament cells deposit more and more collagen, the bundles of matrix separate the cells, thus forming the mature ligament with a lower cell number density and higher collagen content.

Most bones are known to grow from one or more growth centers. In contrast, ligaments grow throughout their length, rather than at discrete sites. The area within the ligament where it attaches to the bone (the insertion site) is an area of rapid cell division. With growth, the collagen of the insertion site is incorporated into the adjacent bone. This active formation of the insertion site allows the insertion to remain metaphyseal rather than gradually becoming diaphyseal with bone growth. Mechanical tension, or load on the ligament, has been found to accelerate the rate of ligamentous growth in immature animals [19].

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR052772, RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 6
The Role of Inflammation and Blood Cells in Wound Healing

Linda H. Chao and Martha M. Murray

So now we know what the ACL looks like before it is injured and that the ACL does not heal once it is ruptured. However, other tissues such as the medial collateral ligament (MCL) heal very well after injury. In order to understand where the ACL healing process goes wrong, we need to understand what a successful wound healing process looks like. This will then give us a template against which to compare the ACL and find the specific deficiencies in the ACL's response to injury.

Wound healing is complex and intricate process, involving multiple cell types, cytokines, and proteins. The goal of functional wound healing for ligaments is to fill the wound defect with a tissue that can support the mechanical loads placed on the ligament and, preferably, that will continue to remodel and repair the structure as small amounts of everyday damage occur. Most wounds which functionally heal, including skin wounds, do so with a structure that is not identical to the original tissue (you can see a scar on the skin for years), but the function of the healed wound is excellent and therefore recapitulation of the exact original structure may not be necessary.

Connective tissues that heal successfully do so in an orderly and predictable pattern. Tissues that heal routinely include skin, the medial collateral ligament, and the Achilles tendon. However, there are other connective tissues, including the ACL, which do not heal after injury, even with suture repair. The reasons for this are unclear. If we focus solely on the ACL for a moment, some of the reasons which

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_6,

have been proposed for the failure of the ACL to heal after injury include a lack of vascular supply, "poisoning" of the ACL cells by synovial fluid, and an intrinsic failure of the cells to make new collagen or perform functions essential to normal wound healing. These factors will be discussed in greater detail in Chaps. 7 and 8.

In this chapter, we will focus on the basic science of wound healing for tissues which are able to functionally heal. The majority of the information we have on wound healing comes from the dermatological literature involving studies of skin (dermis), but there has also been excellent work done on the healing of the medial collateral ligament and other ligaments and tendons which we will also review.

The Principal Phases of Wound Healing

There are three major phases that have been described for functional connective tissue wound healing: the inflammatory phase, the proliferative phase, and the remodeling phase. The inflammatory phase typically lasts a few days, the proliferative phase lasts a few weeks, and the remodeling phase continues for months to years after injury (Fig. 6.1). We will review each phase here and highlight specifics that are critical to each.

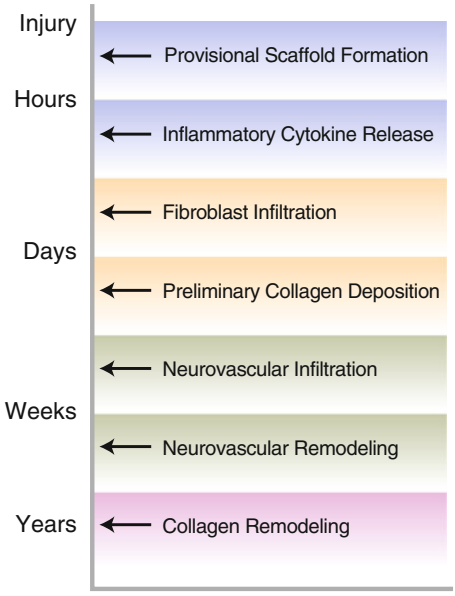

Fig. 6.1 The major phases of wound healing. There are three major phases that have been described for functional connective tissue wound healing. The inflammatory phase (shown here in blue which includes the processes of provisional scaffold formation, cytokine release and early fibroblast infiltration) typically lasts for a few days. The proliferative phase includes continuing fibroblast infiltration and proliferation, as well as collagen deposition and neurovascular infiltration, and typically lasts a few weeks. The remodeling phase can go on for years, and included remodeling of both the collagenous structure of the tissue, as well as the neurovascular structures

The Inflammatory Phase

The first thing that happens after the ACL is wounded is that blood flows from the torn blood vessels to fill the site of injury (Fig. 6.2). When platelets in the blood contact the exposed collagen on the edges of the wound, they become activated and initiate the wound healing process. The platelets become "sticky" and adhere to the damaged tissue, forming a platelet plug (primary hemostasis). The platelets then degranulate (i.e., break apart) or release the growth factors stored inside them, thus initiating the clotting cascade. This cascade results in the formation of a sticky fibrin-platelet clot in the wound site (secondary hemostasis) that stops the blood flow from the damaged blood vessels. The fibrin-platelet clot also serves as a bridge, or provisional scaffold, between the two torn ends of the ligament.

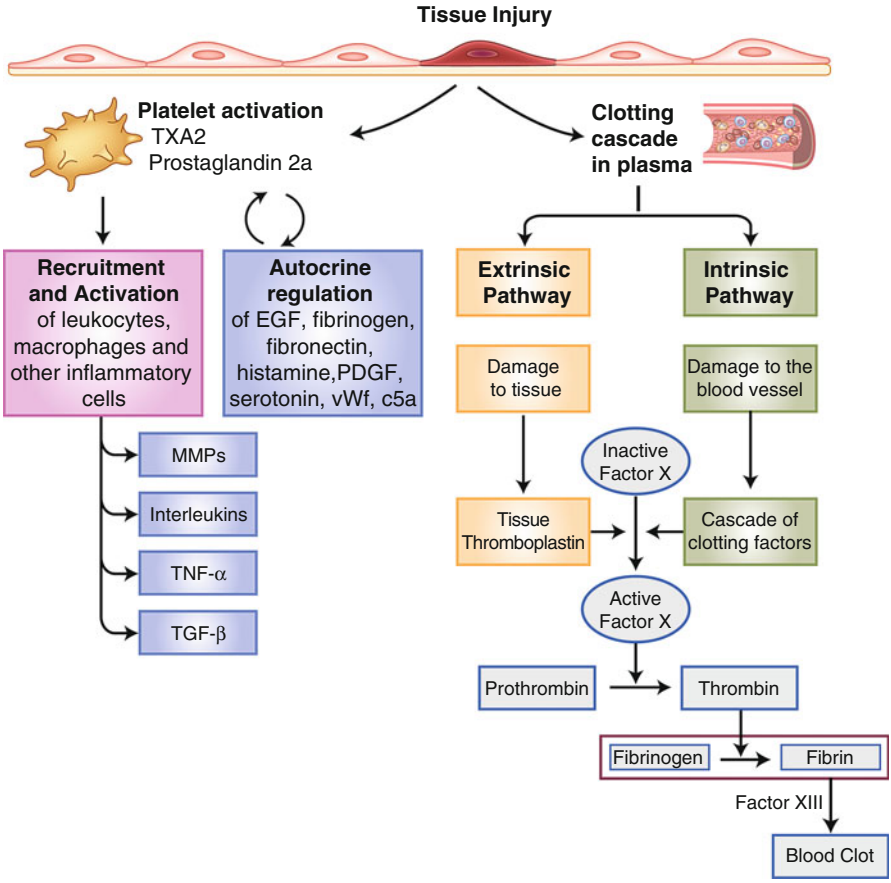

Fig. 6.2 The initial processes of wound healing. Platelets contact the exposed collagen on the edges of the wound, become activated (Pink square), and initiate the wound healing process. The platelets release growth factors which recruit and stimulate other cells into the wound (blue boxes). Simultaneously, the coagulation cascade starts working and a fibrin-platelet plug forms in the wound site (yellow and green boxes). The leukocytes that migrate into the wound site also begin to release various growth factors and cytokines

Within this bridge, or scaffold, multiple important processes begin happening all at once. The activated platelets first release thromboxane A2 (TXA2), which stimulates activation of new platelets and increases platelet aggregation. The platelets also release prostaglandins like PGH2 (the precursor for TXA2) while continuing to produce Factor VII, resulting in continued stimulation of the clotting cascade. The platelets then also begin releasing EGF, fibrinogen, fibronectin, histamine, PDGF, serotonin, and von Willebrand's factor, in addition to other growth factors and proteins. Some of these platelet-derived proteins are growth factors that function as signaling molecules for other cells to come in to the wound site. Platelet-derived factors stimulate neutrophils in the blood circulation to migrate through the blood vessel walls and invade the wound site. The neutrophils are the first type of white blood cell, or leukocyte, to migrate to the site of injury, and these neutrophils secrete additional growth factors to attract additional neutrophils, as well as monocytes, to the wound site. Once monocytes enter the tissues, they transform into macrophages. Macrophages are essential to clearing the wound of tissue debris and pathogens. The macrophages also secrete additional cytokines and growth factors to recruit cells involved in later stages of wound healing, including fibroblasts. We will now review the actions of these cell types in the inflammatory phase of wound healing in more detail.

Platelets

Platelets are essentially growth factor delivery vehicles, specialized for wound repair. They are cell fragments that do not have a nucleus. Platelets form by budding from a larger progenitor cell in the bone marrow called the megakaryocyte (Fig. 6.3). Platelets contain large quantities of growth factors important in wound healing, which include platelet-derived growth factor β-1 (PDGF-AB), transforming growth factor-β1 (TGF-β1), and vascular endothelial growth factor (VEGF). These molecules, released by platelet degranulation, stimulate the cells of the wounded tissue to proliferate and encourage the ingrowth of new vessels into the wound site (angiogenesis). The molecules released by platelets also stimulate cells from the injured tissue to release proinflammatory growth factors, for example, interleukin-1 and interleukin-8 (IL-1 and IL-8). These cytokines have many critical functions, for example, IL-8 works to attract neutrophils to the wound site to remove any contaminating pathogens.

Platelets play an essential role in wound healing. They are the critical "first responders," forming a plug to get the bleeding to stop. Thus, they are an important player in forming the provisional scaffold for the wound. In addition to their structural role, they also release multiple growth factors and chemokines with functions known to be important in wound healing. However, the optimal concentration of platelets within a wound site is not clear. Studies using platelet-rich plasma (PRP), a blood product in which the platelet concentration of a patient's blood can be greatly increased before it is placed in a wound site, have not consistently reported

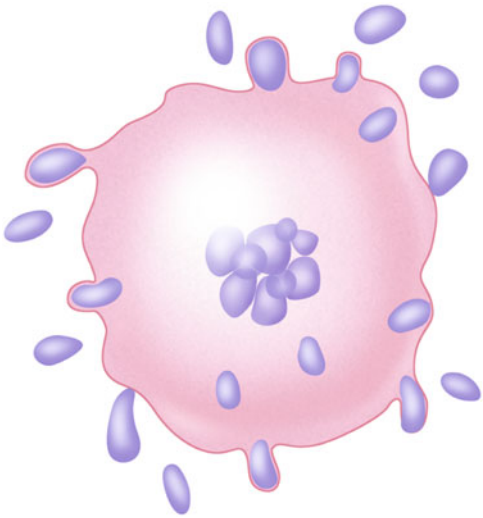

Fig. 6.3 Platelets budding of a megakaryocyte (Reprinted from medcell.med.yale.edu)

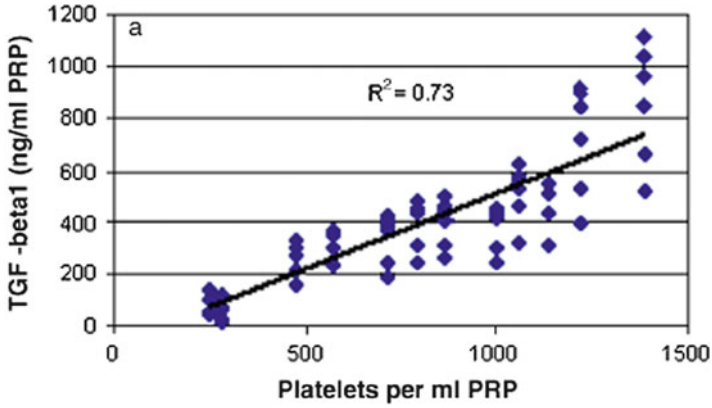

Fig. 6.4 Platelet number versus growth factor release. When more platelets are incorporated into a blood clot or PRP clot, more growth factors are released from that clot. For the case of TGF-b, the relationship is linear as shown here (Used with permission from Jacobson et al. [1])

improved results with the inclusion of more platelets. From work in a petri dish, we do know that including more platelets in PRP results in a greater release of growth factors such as TGF-β1 (Fig. 6.4), but whether the increase in TGF-β1 release is helpful or harmful for functional wound healing in the body remains unknown. Theoretically, overdosing of PRP and its growth factors can be detrimental for two reasons, first, because it could overstimulate cells and lead to a poorly differentiated scar tissue and, second, because some of the released growth factors might have negative effects, such as increased inflammation or excessive tissue destruction. There is evidence available from large animal models to suggest that placing PRP with platelet concentrations three to five times higher than normal in a wound site

can both stimulate ACL healing in an animal knee [2], but further work is needed to justify the expense and use of platelet concentration vehicles to enhance wound healing.

While platelets are the first responders, there are other blood cells that also play a critical role in the inflammatory phase of healing. Two major groups of these cells are red blood cells (erythrocytes) and white blood cells (leukocytes), which we will discuss in detail below.

Red Blood Cells (Erythrocytes)

While platelets and white blood cells have been generally recognized to participate in wound repair, the role of the erythrocyte is less well understood. In fact, many common methods for making platelet-rich plasma (PRP), a substance that is increasingly popular for use in promoting healing in sports medicine, remove the erythrocytes altogether to allow for greater concentration of the platelets. However, it has been recently recognized that erythrocytes also play a key role in wound repair [3]. Erythrocytes change the behavior of cells within the ligament during wound healing. For example, including erythrocytes in a 3D-simulated wound site results in an increase in production of collagen by the other cells in the model wound [4]. This increase in collagen expression may be due to the high concentration of hemoglobin released from the erythrocytes as they die over a 10-day period. Hemoglobin, the major functional protein of the erythrocytes, is a carrier not only for oxygen but also for nitric oxide, and the release of this nitric oxide as the cells die may be responsible for the mechanism by which red blood cells stimulate collagen synthesis by fibroblast in vitro [5, 6], as well. The exact role of these cells within the wound site remains relatively unknown, but it is very likely these cells play a critical role in functional wound healing and perhaps an even more important role where the wound site tends to be hypoxic until it is revascularized, as is the case for intra-articular ligaments like the ACL.

White Blood Cells (Leukocytes)

Multiple types of white blood cells (leukocytes) are active in the wound site, including granulocytes (neutrophils, basophils, and eosinophils), macrophages, and lymphocytes (Fig. 6.5). White blood cells are present in the initial fibrin-platelet clot in the same concentrations as found in the peripheral blood. However, within hours, the concentration of white blood cells in the wound site increases dramatically as white blood cells are stimulated to migrate from the circulation into the wound site. The cells are able to crawl through the blood vessel wall, via a process called diapedesis, to enter the wound. This process is initiated by the growth factors released by the activated platelets in the wound site. Once they are in the wound site, leukocytes

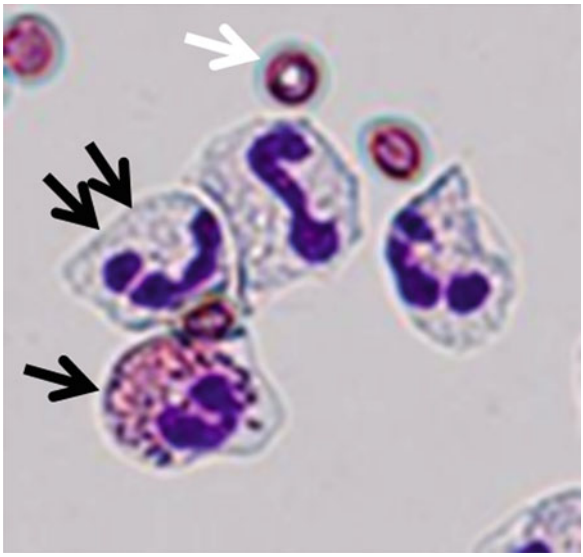

Fig. 6.5 Photomicrograph of erythrocytes (*white arrow*), eosinophils (*black arrow*), and neutrophils (*double black arrow*). Neutrophils are characterized by the multilobed nuclei (*black arrow*) and the granules in the cytoplasm, most of which are neutral staining but about a third of which are azurophilic (purple staining) and contain a wide variety of antimicrobial defensins. Eosinophils are characterized by the acidic (black arrow) granules within the cytoplasm (*black arrow*), containing major basic protein (which induces mast cell and basophil degranulation), eosinophil cationic protein (a ribonuclease with antiviral activity) [7], and other proteins that play a role in innate immune defenses [8]

contribute to the total concentration of growth factors by either directly releasing growth factors themselves [9] or by stimulating platelet release of growth factors [10]. Leukocytes have been reported to have productive (anabolic) effects on cells [11] and help kill bacteria within the wound site [10, 12, 13]. Other researchers [14] have claimed that leukocytes, particularly neutrophils, release harmful enzymes that dissolve proteins and the structural components of the wounded tissue, which one might hypothesize could hinder functional wound healing. Interestingly, one group of researchers have found that transgenic mice lacking both macrophages and functional neutrophils are able to heal scarlessly, as in the embryo [15, 16], and posit that while macrophages are normally crucial coordinators of the repair process, acting both as professional phagocytes to clear wound debris as well as a major source of wound growth factor signals, it may be possible to achieve scarless healing if they are completely eliminated. There is research ongoing in the fields of plastic surgery and dermatology trying to harness this information [17]. It is likely that each of the cell types within the wound site has a combination of anabolic and catabolic functions and that it is the careful orchestration and balance of all the cells that leads to functional wound healing.

Immediately following tissue injury, neutrophils begin infiltrating the wound site and remain the predominant cell type in the wound for the first 24–48 h. Neutrophils release additional growth factors but are also responsible for clearing tissue debris and getting rid of any infectious pathogens that might be present in the wound site. Neutrophil concentration within the wound site increases to a maximum at approximately 24 h after injury. After this first day, the neutrophils begin to undergo a process of programmed cell death, or apoptosis, and they are cleared from the wound by the macrophages.

Macrophages originate from monocytes circulating in the blood. Stimulation by the growth factors in the wound site causes the monocytes to leave the circulation and invade the wound site. Once they migrate into the wound, they are called macrophages. Macrophages replace neutrophils as the most abundant cell type in the wound by 2 days after injury and bind to extracellular matrix (ECM) through cell surface integrin receptors. While the highest concentration of macrophages is seen during the initial inflammatory phase, significant numbers of macrophages are found in the wound during all stages of the wound healing process [18]. Macrophages remain active for several weeks after injury, phagocytosing ECM and promoting wound debridement [19]. They are present in the granulation tissue that is gradually replaced by collagen and elastin produced by fibroblasts and they act to continuously remodel the scar tissue.

T lymphocytes also migrate into the wound during the inflammatory phase, attracted to the wound site by IL-1, and are present in the wound starting approximately 72 h following injury. The next sections will review these cell types in the order in which they appear in the wound site.

Granulocytes (Neutrophils, Eosinophils, and Basophils)

When a tissue is wounded, the platelets set off the coagulation and inflammatory cascades. The next major cell type that joins the action are the granulocytes. Granulocytes include neutrophils, eosinophils, and basophils. In blood, or in any wound site, the neutrophils are by far the dominant granulocyte type, typically comprising over 90 % of the granulocytes present in the site. The migration of neutrophils into the wound site of a connective tissue occurs within hours, with the peak concentration occurring at 24–48 h after injury and ceasing a few days after injury. The neutrophils have a short lifespan (up to 3 days in tissues), and their main role appears to be to engulf (phagocytose) any cell or tissue debris within and around the wound and to release enzymes and reactive oxygen species (ROS) to kill pathogens. The short lifespan of neutrophils initially led to their being thought of as only minimal contributors to wound healing. However, more recent awareness of the multiple cytokines (IL-4, IL-8, TNF-α) these cells release in the first day of wound healing has led to a greater respect for their role in the overall wound healing process [20].

Interestingly, neutrophils are a key player in not only the initiation but also the termination of acute inflammation [21, 22]. Shortly after invading the wound, the

neutrophils begin to die through programmed cell death, a process called apoptosis. The apoptotic process not only prevents the release of the potentially damaging contents of the neutrophils, like the ROS, but it also tips off macrophages to ingest them through engagement of "death receptors" [23, 24]. The neutrophils start to shrivel up as they undergo apoptosis and they become targets for macrophages to clean up. The phagocytosis of apoptotic neutrophils by the macrophages has been thought to cause the macrophages to shift from a tissue debridement function to a reparative function, which has been thought to be the key to setting off the shift from the inflammatory stage to the proliferative and later stages of wound healing in the site of injury. Macrophage phagocytosis of apoptotic polymorphonuclear neutrophils (PMN) is a main stimulus for their secretion of TGF-β1 [25, 26], a critical cytokine for collagen production and wound healing. In vitro studies have shown that ingestion of apoptotic cells by macrophages results in an anti-inflammatory effect and suppression of proinflammatory mediators. Furthermore, injecting apoptotic neutrophils into a wound site in the body has been shown to enhance the resolution of acute inflammation [25]. These effects probably result from the interactions between macrophages and T lymphocytes that we will explore in more detail further on.

Eosinophils are often small in number in the wound site, as they are generally involved in combating parasites and in allergic reactions as opposed to wound healing, but these cells are known to produce VEGF, PDGF, TGF-α, TGF-β, and a variety of interleukins in acute inflammatory responses. Eosinophils have been found to have the ability to participate in tissue remodeling in the body ("in vivo") by producing TGF-α and TGF-β, which promote cell proliferation and blood vessel formation, and improving the structural organization of the wound site [27]. Eosinophils have been found to persist in a wound site longer than other granulocytes, which might mean they could have a greater role in the remodeling phase than other cell types [28]. Furthermore, the eosinophil has long been recognized as a source of plasminogen [29], which catalyzes the breakdown of fibrin, a critical step in the process of wound healing. The plasminogen-plasmin system has been recognized as a major player in each phase of the wound healing process. Plasmin, the active form of plasminogen, activates MMPs and TGF-β, which stimulates the expressions of collagen, fibronectin, tissue inhibitor of metalloproteinase-1 (TIMP-1), and plasminogen activator inhibitor-1 (PAI-1) [30].

There has been little focused study of the role of basophils in wound healing, but it has been recognized that basophils migrate into wound sites upon injury and secrete proinflammatory mediators. When basophils get activated, they release histamine, heparin, elastase, and leukotrienes, as well as a variety of cytokines and other proteoglycans and proteolytic enzymes. Histamine and leukotrienes produced by basophils make the blood vessel walls easier for cells to migrate though, so that they can enter the wound site. This can indirectly influence wound repair by allowing white blood cells including monocytes to move more easily from the blood vessels into the wound site.

Monocytes and Macrophages

Monocytes are another white blood cell type that is present in the circulation. Monocytes (Fig. 6.6) are known to originate in the bone marrow and are subsequently released into the peripheral blood, where they circulate for several days before entering tissues where they become macrophages. Monocytes are attracted to wound sites, and they migrate through the blood vessel wall into the provisional scaffold of the wound. Once in the wound site, the monocytes differentiate into macrophages. Macrophages replace neutrophils as the most abundant cell type in the wound by 2 days after injury and are often active for several weeks after injury, "cleaning up" the wound by consuming or phagocytosing cellular debris and pathogens. Once the macrophages have ingested certain cellular debris (e.g., the apoptotic neutrophils described above), they switch from a "demolition" role to a "rebuilding" role. The role of the macrophage as a "professional antigen-presenting cell (APC)" is likely to be important in the shift from the inflammatory phase to the "rebuilding" (proliferative and remodeling) phases of wound healing, in that macrophages process and present fragments of proteins from the cells that they ingest – whether foreign as in the case of bacteria or native as in the case of neutrophils – to CD4+ T lymphocytes, thereby activating the T cells to develop into different effector cells, including regulatory T cells (T_{reg} cells, formerly known as suppressor T cells) that dampen inflammation. Macrophages also produce multiple cytokines, which change over time depending on the activation state of the cells. During the proliferative phase, for instance, these include IL-6, FGF, EGF, TGF-β, and PDGF [31], which promote fibroblast infiltration of the wound site and fibroblast production of collagen to begin to regenerate the collagen tissue that was injured.

"Classically activated" or "M1" macrophages are characterized by enhanced microbicidal or tumoricidal capacity, owing to a high capacity to present antigens and produce high levels of the cytokines IL-12 and IL-23, as well as toxic intermediates including nitric oxide (NO) and reactive oxygen intermediates (ROI) [7]. These are likely the phenotype encountered early in wound healing, during the inflammatory phase. In contrast, several subcategories of "alternatively activated" or "M2" macrophages that suppress immune responses (called "regulatory macrophages" by some authors) and regulate tissue repair (called "wound healing macrophages" by some authors) have been proposed [7]. These are likely the phenotypes encountered later on as wound healing progresses. A shift in macrophage phenotype in the wound site from M1 "classical activation" to M2 "alternative activation" has been recognized as a key step in the resolution of inflammation and is brought on by the phagocytosis of apoptotic neutrophils by these cells [21, 22]. There is some controversy about the classification system for the different phenotypes of macrophages, though, as recent work has indicated that macrophages exist not as distinct subpopulations but rather as a continuum, with macrophages present in wound sites sharing traits with both M1 and M2 phenotypes and with a temporal shift in the phenotype of macrophages in the healing wound [8].

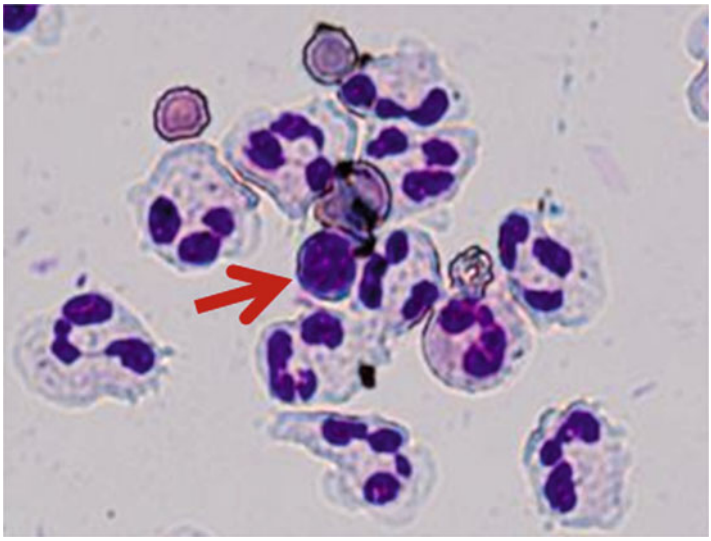

Fig. 6.6 Peripheral smear of a monocyte and neutrophils. Monocytes (*red arrow*) are characterized by their kidney-shaped nuclei and neutral staining under hemotoxylin and eosin staining

Lymphocytes

After the macrophages, lymphocytes become the dominant white blood cell type in the healing wound. In addition to the roles of the two classes of lymphocytes in the adaptive immune system, with T lymphocytes largely responsible for effector mechanisms of cell-mediated immunity and B cells responsible for humoral (antibody) responses, they likely play distinct roles in wound healing, as well. In particular, T lymphocytes are attracted to the wound site by the presence of IL-1 [32] and are first seen approximately 72 h after injury. T lymphocytes then subsequently control the activity of fibroblasts during normal wound healing [33]. As wound healing progresses, there is an increase in the number of CD8+ regulatory T lymphocytes, a subpopulation of T lymphocytes, formerly known as T suppressor lymphocytes, that are thought to be responsible for winding down T cell-mediated immunity toward the end of an immune reaction [34].

The role of B lymphocytes in wound healing has not been examined in great detail and some authors have claimed that B lymphocytes are unlikely to play a significant role in the regulation of wound healing [34–36]. However, B cells have been shown to migrate to sites of tissue inflammation following wounding, differentiate into phagocytes, and secrete interleukin-10 (IL-10), an anti-inflammatory

cytokine [37]. Studies of human dermal wounds have shown that there is an increase in B lymphocyte presence in the wound site during the first week after injury [34]. Furthermore, when splenectomized mice have their B cell populations restored, their wound healing capabilities return to the same levels as before they had their B cells depleted [38]. Together these data suggest a greater role for B cells in wound healing than previously recognized.

The Proliferative Phase

As the inflammatory phase is winding down, the wound site is invaded by not only blood cells but also by fibroblasts. The infiltration of the wound site by fibroblasts is the hallmark of the proliferative phase of wound healing. Fibroblasts are the working cells of skin, ligaments, and tendons. These cells produce the major structural proteins of the ligaments and tendons (primarily collagen) and are responsible for making tissue with strength enough to withstand typical forces on the ligament or tendon with normal activities. During the proliferative phase, these fibroblasts produce granulation tissue within the wound, replacing the provisional scaffold of the blood clot with tissue that has blood vessels and collagen matrix. During this phase, the fibroblasts produce additional collagen (both type III and type I) and the scar gets stronger.

Thus, the main outcome of the proliferative phase is to transform the biologically active, but mechanically weak, provisional scaffold of fibrin, platelets, white blood cells, and red blood cells into a mechanically strong structure which can withstand physiologic loads. The main processes of this phase are fibroblast infiltration, fibroblast proliferation, blood vessel and nerve ingrowth into the wound, and collagen production. The principal cell in this process is the fibroblast and its role will be described in greater detail in the section below.

Fibroblasts

Fibroblasts are the workhorse cells of most soft connective tissues – including skin, ligament, and tendon. These cells can range in shape from cells with round nuclei and relatively compact cytoplasm to spindle-shaped cells with extremely long cytoplasmic processes. The main function of these cells is to produce collagen, and while these cells are able to perform this function even in the presence of a limited blood supply, they typically can produce more collagen when a better blood supply is present [39].

The rate of formation of the collagenous scar tissue appears to be dependent on interaction of fibroblast integrin receptors with fibrin [40], thus the initial fibrin clot has a critical function as a source of fibroblast-stimulating proteins and cytokines.

After fibroblasts migrate into the fibrin clot, they begin to lay down a tissue composed mainly of collagen and elastin fibers, with proteoglycans and glycoproteins in between the fibers, replacing the fibrin clot as it is degraded [31]. When collagen density in the wound reaches a certain threshold, fibroblast proliferation and collagen synthesis are suppressed [19] and the remodeling process begins.

Wound fibroblasts synthesize more collagen than non-wound fibroblasts and also proliferate less and actively carry out matrix contraction [41]. The cytokine-rich wound environment no doubt plays a role in the differences in the phenotypes of fibroblasts isolated from wounds versus non-wound fibroblasts, but the mediators of these differences have only been partially characterized [42, 43]. For instance, lactate, which accumulates in significant amounts in healing wounds over time, has been found to be a potent regulator of collagen synthesis, through a mechanism involving ADP ribosylation [44]. It has also been found that interleukin-4 (IL-4), the Th2 cytokine secreted by T lymphocytes that has been implicated in the development of "alternatively activated" M2 macrophages, also stimulates fibroblasts associated with the proliferative phase of healing in a dose-, cell-, and time-dependent manner [45]. Systemic administration of IL-4 to rats prior to and after rupture of their medial collateral ligaments results in a time-dependent effect on fibroblast proliferation; treatment of the rats with IV IL-4 for 5 days post-injury results in decreased wound size and type III collagen and increased type I procollagen, indicating a more regenerative early healing in response to IL-4 treatment. However, continued treatment with IL-4 until day 11 slowed overall healing, antagonizing the early benefits seen. This is consistent with the notion that successful wound healing demands a balance between catabolic inflammatory processes and anabolic proliferative processes.

The Remodeling Phase

After the replacement of granulation tissue with early matrix, wound contraction and scar tissue remodeling occurs. The largely unorganized collagen formed rapidly during the proliferative phase is gradually broken down by matrix metalloproteinases (MMPs) and replaced with collagen fibers aligned along the lines of mechanical stress. The net collagen content in the wound is the result of a balance between this collagen breakdown and the new collagen synthesis. TGF-β and PDGF, produced by the cells in the preliminary scaffold, mediate the synthesis of new collagen and the breakdown of old collagen, respectively [31]. TGF-β and PDGF also potentiate differentiation of fibroblasts into myofibroblasts, which facilitate wound closure. Myofibroblasts, characterized by increased smooth muscle actin expression as compared to fibroblasts [39], align themselves along the borders of extracellular matrix (ECM) to generate a constrictive force and close the wound margins [19] in the second week post-injury.

Wound strength depends on both the quantity and quality of the collagen. A maturing wound goes through a characteristic pattern of ECM deposition:

fibronectin and type III collagen constitute the early scaffold, which is replaced by GAGs and proteoglycans and finally by type I collagen. By several weeks after the injury, the amount of collagen in the wound reaches a plateau, but the tensile strength continues to increase for several months [46] because of fibril organization and cross linking (via lysyl oxidases [47]).

Summary

Wound healing progresses through a series of overlapping stages that can be generalized to all soft tissue wounds. But tissue repair can also be seen to represent a juxtaposition of two distinct forces: anabolism (tissue formation) and catabolism (tissue remodeling). Rather than the false impression of temporally distinct stages that operate independently, tissue repair can be understood within the context of a balance between the anabolic and catabolic effects of the cell populations and chemical mediators involved. Different cell types are active in different stages of the wound healing process (Fig. 6.7), and each may play a different role in different

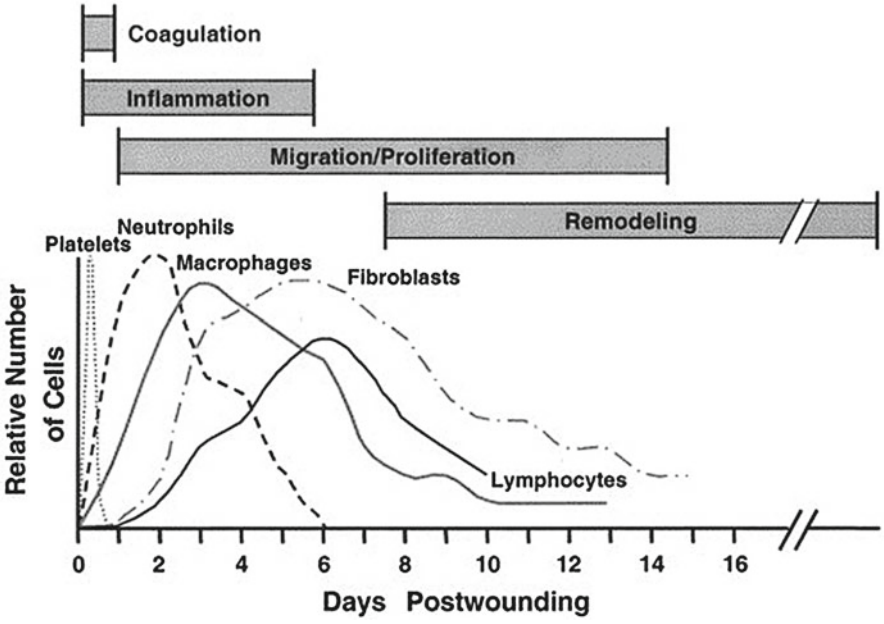

Fig. 6.7 Timing of cellular involvement in wound healing. As illustrated here, the various cell types found in blood participate in different parts of the wound healing process and some play different roles in the different phases of wound healing (Reprinted from Park and Barbul [35], with permission from Elsevier)

stages of wound healing. We have learned a great deal about this complex process, but the sheer number of cells and growth factors involved ensure this will be a field for healthy research for decades to come.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR052772 and RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 7
The ACL Response to Injury

Martha M. Murray

As we have seen in the prior chapters, the ACL fails to heal after injury, even with primary repair. Thus, current clinical treatments typically involve removing the ruptured ACL and replacing it with a graft of tendon, an operation called an ACL reconstruction. While ACL reconstruction is an excellent operation for restoring the gross stability of the knee, some issues could be improved. For example, ACL reconstruction requires harvesting of other tissues from the knee, a procedure with its own associated morbidities. In addition, the surgical procedure of ACL reconstruction also requires removing the native ACL tissue to make room for the graft, particularly for "anatomic" ACL reconstruction, where visualization of the bone of the insertion sites is advocated, and which requires the complete removal of the original torn ACL remnants. Removal of the remnants also eliminates the proprioceptive nerve fibers contained within the tissue and thus the patient loses the proprioceptive function of the ligament and its contribution of joint proprioception. No studies of ACL reconstruction have demonstrated regrowth of the nerve fibers into the ACL graft. Lastly, ACL reconstruction replaces a complex, fan-shaped bundle of 17 different ligament fascicles with one or two bundles of tendon fibers. One or more of these issues may be the reason that patients remain at a high risk for osteoarthritis after an ACL tear despite ACL reconstruction – as high as 78 % of patients will have radiographic signs of arthritis at only 14 years after surgery [1]. For a 14-year-old teenager with an ACL tear, that is a striking and troubling statistic.

As we thought about ACL injuries and their current treatment, we wondered whether getting the ligament to heal, rather than replacing it with a tendon graft, might someday offer a better treatment alternative. However, as we saw in Chap. 2, prior attempts at suture repair of the ACL have failed dismally, with rates of failure

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_7,

of healing (nonunion) and increases in structural laxity of the ACL, even with surgical repair, ranging from 40 to 100 % [2–4]. This is in contrast to rates of nonunion seen in extra-articular tissues (i.e., the medial collateral ligament) where reported rates are less than 5 % [5]. The lack of functional healing seen in the ACL after suture repair has been previously attributed to the "hostile" environment of synovial fluid [6, 7], to alterations in the cellular metabolism after injury [8, 9], and to intrinsic cell deficiencies [10–16]. Which mechanisms were most dominant were unclear.

If we are going to attempt to engineer an improved ACL repair strategy, we first need to understand why it does not heal in the first place. To begin to answer this question, we retrieved human ACL tissue from patients undergoing ACL reconstruction (where as we said earlier, the torn ACL is routinely removed from the knee and discarded). We began to study the harvested tissues, and the cells within them, to help identify what the problems were for ACL healing in patients. We looked at the cell behaviors of the ACL cells in 2-D and 3-D models in vitro and will summarize those results in this chapter.

ACL Cell Migration After Injury

As we know from the basic science of wound healing discussed in Chap. 6, one key cellular characteristic that ACL cells must have if they are to participate in a successful wound healing process is the ability to migrate, particularly to migrate into an adjacent scaffold (i.e., the provisional scaffold across the wound site). To study whether ACL cells had this ability, we obtained tissue from both intact and ruptured human ACLs. We cut the tissue into small pieces, or explants, and put these into culture on tissue culture wells (two-dimensional culture) as well as onto collagen scaffolds (three-dimensional culture) to study how the cells within the pieces of the ACL tissue would migrate out of the injured tissue onto a flat surface or 3D scaffold. Our major questions were whether the cells would grow out of the tissue onto a culture plate or scaffold or whether they would simply die inside the tissue.

When we performed this study using intact ACL tissue from human patients undergoing total knee arthroplasty (where the intact ACL is routinely removed and discarded), we found that the cells from the intact ACLs were able to migrate out of the tissue and onto a tissue culture plate (Fig. 7.1). To look at this for the two-dimensional cultures, we cut the retrieved ACL tissue into small pieces and cultured them on a tissue culture dish. We were able to observe the cells migrating out of the tissue and recorded the area of outgrowth every 3 days using an inverted microscope and transparent grid sheet. The migration of cells out of the tissue started at an average of 10 days after injury and the rate of outgrowth was 0.25 mm/day [17].

We then performed a similar experiment for tissues obtained after an ACL tear (from patients undergoing ACL reconstruction). We again measured the outgrowth

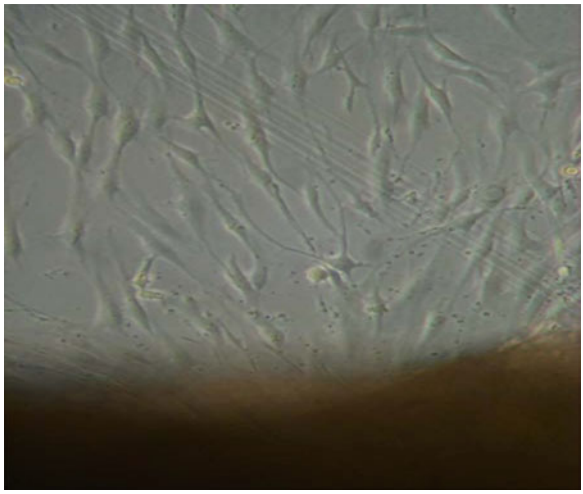

Fig. 7.1 Cellular outgrowth from an ACL explant (*dark area*). The fibroblastic cells can be seen streaming from the tissue by 1 week after injury and tissue explantation. In studying human ACL tissue, we found that ACL cells would actively migrate out of the tissue within a few days of culture

for the two-dimensional cultures by measuring the area of the plate covered with outgrowing cells under the microscope three times per week. For the ACL tissue that had been injured previously, cells were first noted to be migrating onto the culture plate as early as 3 days and an average of 1 week after the tissue was cut and the cultures started. The cell outgrowth not only started earlier from the injured samples, it also had a similar rate of outgrowth (0.29 mm/day) to that seen from the intact ACL explants (0.25 mm/day). Interestingly, the tissue pieces that had the most blood vessels in the tissue also had a larger area of outgrowing cells when this was measured at 2 weeks after the cultures were started. The highest rates of migration onto the plate were seen from the pieces of tissue taken right at the site of rupture of the ligament [18].

The results of this experiment demonstrated that ACL fibroblasts within a torn ACL are able to grow out of the torn tissue onto an adjacent structure or into an adjacent wound site. Thus, they have the ability to migrate if structure is provided. However, most ligament wounds are three-dimensional volumes of space, rather than 2-D culture plates. Thus, our next step was to culture the ACL tissue on 3-D scaffolds to see if the ACL fibroblasts would also crawl into the three-dimensional scaffolds.

For the three-dimensional cultures, we placed the tissue pieces onto a collagen scaffold and then looked to see how many cells had moved into the scaffold at 1, 2, 3, and 4 weeks of culture. By 1 week, there were already cells from the ACL tissue that had migrated into the scaffolds, and the number of cells in the scaffolds increased each week. As in the two-dimensional cultures, the highest rates of migration onto the plate were seen from the pieces of tissue taken right at the site of rupture of the

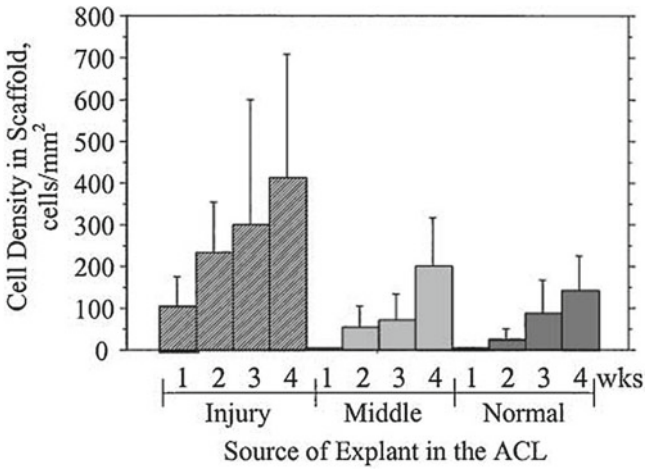

Fig. 7.2 Bar chart showing the cell number density in the collagen scaffolds as a function of explant location and time in culture (values are mean ± SEM). The results of this study told us that ACL fibroblasts could crawl into an adjacent three-dimensional scaffold and that cells at the site of an ACL injury were programmed to do this especially well (Reprinted from Murray and Spector [18], with permission from Elsevier)

ligament (Fig. 7.2). These data demonstrate that cells in the human ACL retained their ability to migrate into an adjacent collagen-based scaffold in vitro, weeks after complete rupture [18].

ACL Cell Proliferation After Injury

Cell proliferation is a key biologic process that typically occurs after injury in tissues that successfully heal. One of the hypotheses for the failure of the ACL to heal was the inability of ACL cells to proliferate after injury (possibly due to contact with the detrimental synovial fluid). To assess this cell behavior for human ACL fibroblasts, we looked at the ACL cell numbers in human ACL tissue retrieved at the time of surgery to assess whether there were more cells or less in the ruptured ACL in the weeks and months after rupture.

In this experiment, we found that the cell number within the ruptured human ACL increased after injury, to a peak between 16 and 20 weeks after rupture ($p < 0.005$; Fig. 7.3), and then decreased between 20 and 52 weeks after the injury [19]. Thus, we found that rather than the cells decreasing in number after injury, they were actually proliferating within the human ACL in the first 4 months. This suggests that at least initially, there is a productive, proliferative cell response within the human ACL.

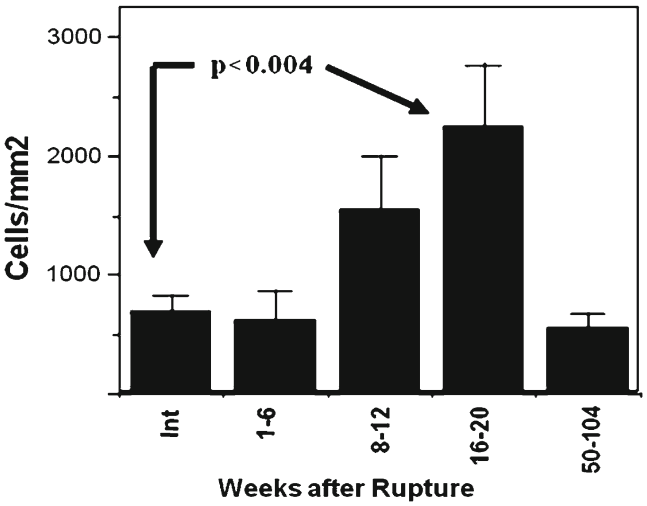

Fig. 7.3 Cell number within the injured human ACL as a function of time after injury. A significant increase in cell number is observed between the time of injury and 16–20 months after injury ($p < 0.001$) and then a decrease is seen after that time point (Used with permission from Murray et al. [19])

ACL Cell Collagen Production After Injury

Once we determined that ACL cells were able to migrate into a wound site, proliferate within the wounded tissue, and proliferate within a simulated wound scaffold, we wanted to know if ACL cells were able to produce collagen after the ACL was injured. Collagen is the main structural protein of the ACL, and to fix any damaged sections, fibroblasts must produce collagen. Thus, if this function is lost after an ACL tear, healing could be significantly impaired, even if the other cellular processes were effective.

To look at collagen production by cells within the ACL after injury, Spindler et al. examined pieces of injured ACL tissue obtained from patients undergoing ACL reconstruction. They performed in situ hybridization to look for gene expression of collagen, collagenase, and tissue inhibitors of metalloproteinases (TIMPs). The tissues were studied at time points up to 1 year after injury [20]. mRNA expression of type I collagen was detected in all specimens, was equally distributed throughout the remnants, and remained evident even 1 year after injury. In contrast, none of the matrix-degrading enzymes (collagenase or gelatinase) were expressed at substantial levels at any time point. The conclusion of this experiment was that fibroblasts within the ACL remnants remained metabolically active up to 1 year after injury. Other investigators have also confirmed type I gene collagen expression in a rabbit partial ACL injury model at time points up to 1 month from injury (the longest time point studie) [10]. The findings of these studies tell us that ACL cells are able to also make collagen, even a year or more after the initial injury.

Changes in Vascularity of the ACL After Injury

So, if the ACL cells can migrate, proliferate, and make collagen after the ACL is torn, why won't the ACL heal after injury? For decades, another hypothesis was that the failure of the ACL to heal was due to the lack of sufficient blood vessels within the ACL substance. To study this assumption, we examined the numbers of blood vessels within the ACL, both before and after injury. Interestingly, we found that the ACL was vascular and that the number of blood vessels within the ACL actually increased after injury, peaking at approximately 4 months for the torn human ACL [19].

The density of blood vessels within the injured ACL also changes after injury, with a peak in blood vessel number seen at 16–20 weeks after injury [19] (Fig. 7.4). The blood vessel density appeared to decrease with distance from the rupture site. The neovascularization observed in the ACL remnants was similar to that reported in studies of healing of other connective tissues, including the MCL [21] and tendons [22, 23]. This finding is also critical to the development of future methods of facilitating ACL healing and regeneration. The pronounced neovascularization and cell proliferation observed in the ACL remnants suggests that harnessing of these responses, and their extension into the gap between the ruptured ligament ends, may provide a cell-biology-based method of repair of the ACL, with the ligament remnants providing the cellular constituents of the repair tissue [19].

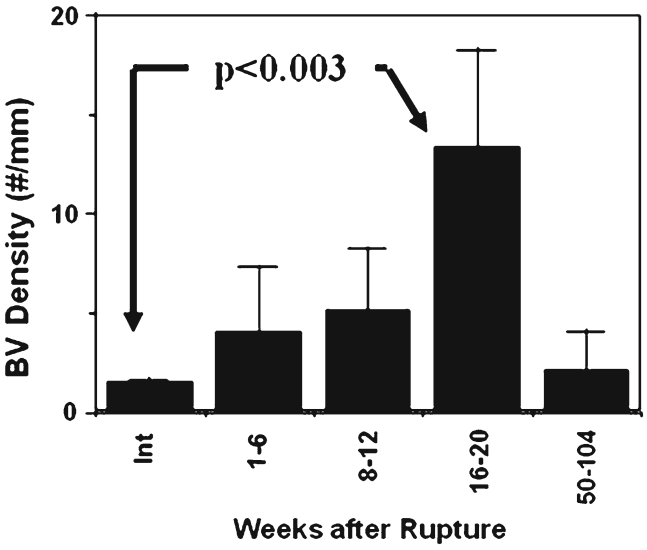

Fig. 7.4 Blood vessel number within the injured human ACL as a function of time after injury. A significant increase in blood vessel density is observed between the time of injury and 16–20 months after injury ($p < 0.001$) and then a decrease is seen after that time point (Used with permission from Murray et al. [19])

With so many of the basic cellular functions appearing to work well in the injured ACL, we then turned to study the integrated response of all of these processes in an attempt to define the mechanism of failure of ACL healing.

Histologic Phases of the Response to Injury for the ACL

To better understand what is happening within the ACL tissue as a whole, we took torn human ACLs from patients undergoing ACL reconstructive surgery and looked at a whole slice of the tissue from the rupture site to the bony insertion site microscopically [19]. As patients have surgery on the ACL at times from injury ranging from 1 week to 2 years, we were able to collect groups of ligaments at monthly intervals after surgery and look at the changes in the ligament at various time points [19]. What we found was that the ruptured human ACL undergoes four histologic phases after rupture: an inflammatory phase, an epiligamentous reparative phase, a proliferative phase, and a remodeling phase [19]. These phases were similar to those seen in tissues that heal successfully, with the exception of the epiligamentous reparative phase as noted below.

The Inflammatory Phase for the ACL

Within the first few weeks after the rupture, the synovial fluid is a rust-colored, viscous material containing no blood clot and can easily be suctioned or aspirated from the knee. The ACL tissue itself is swollen and friable and the synovial and epiligamentous tissue is grossly disrupted. No connection between the two torn ends of the ligament was noted (Fig. 7.5). When the tissue was examined microscopically, there was an initial influx of inflammatory cells which gradually subsided, leaving fibroblasts as the predominant cell type after a week or so.

The Epiligamentous Regeneration Phase

Between 3 and 8 weeks after rupture, the epiligamentous and synovial layers covered over the ACL remnant (see Fig. 7.5). No tissue bridged the gap between the tibial and femoral stumps of the ACL, although some of the tibial stumps adhered to the adjacent posterior cruciate ligament (PCL). The cell density within the midsubstance of the ligament was unchanged from the intact state. However, there was an increase in cell number and blood vessel density in the epiligament or tissue that was surrounding the ACL.

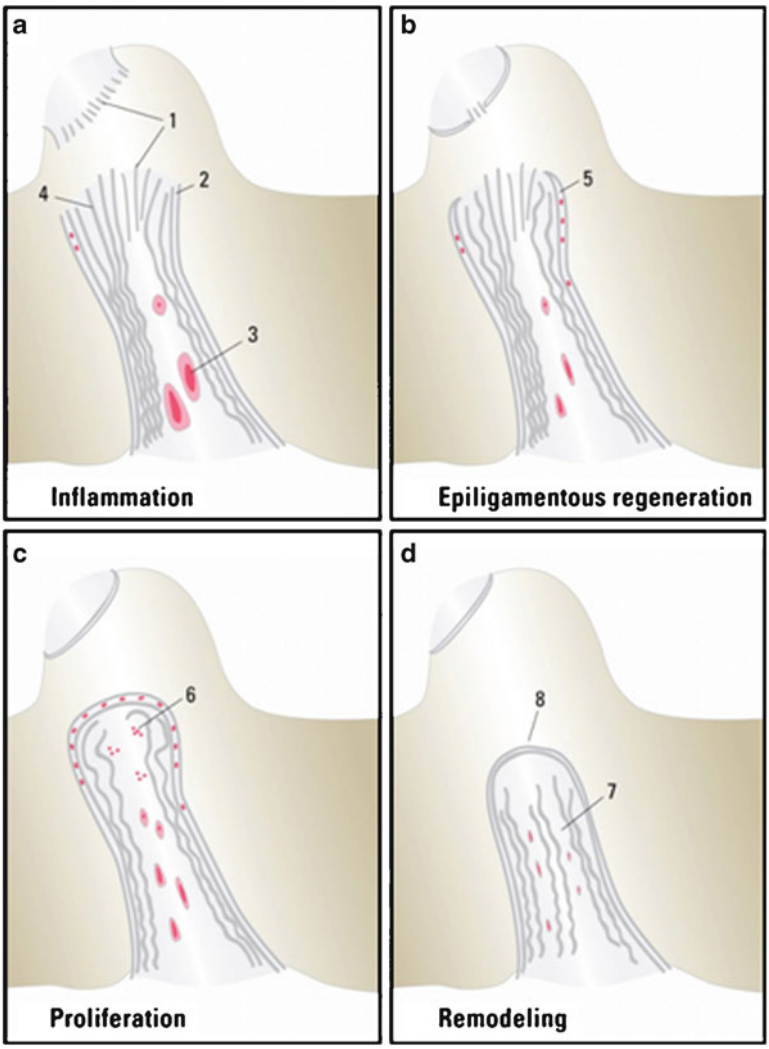

Fig. 7.5 The four histologic phases of the ACL response to injury. **a**) The inflammatory phase is characterized by friable ends of tissue (1), disruption of the epiligament and synovial covering of the ligament (2), intimal hyperplasia of the vessels (3), and loss of the organized crimp structure (4). **b**) The epiligamentous regeneration phase involves a gradual covering of the ligament with vascularized tissue (or epiligament) and synovial tissue (5). **c**) The proliferative phase has increased in both cell number and capillaries (6) and **d**) the final remodeling phase is characterized by a decrease in cell number density and blood vessel density (7) and by retraction of the ligament remnant (8) (Used with permission from Murray et al. [19])

The Proliferative Phase for the ACL

By 8 weeks after rupture, the distal remnant of the anterior cruciate ligament was completely encapsulated by a synovial sheath, and again, no tissue was visible between the proximal and distal ligament remnants. During this period, cell density and blood vessel density were noted to be increasing in and among the collagen bundles within the ligament itself. The peak cell number density was seen at 16–20 weeks after injury. The cells were still disorganized. Vascular capillary buds were seen, along with anastomoses of vessels close to the torn end forming a diffuse network of immature capillaries.

The Remodeling Phase for the ACL

During this phase, the cell number and vessel number decreased within the remnants, and again, no tissue was seen to connect the two ends. The fibroblasts became more aligned and a more axial alignment of the collagen fascicles was seen.

The response to injury in the ACL was similar to that reported for other dense connective tissues such as skin or tendon as described in Chap. 22, with three important exceptions that are likely interrelated: (1) the formation of a synovial cell layer on the surface of the torn ligament, (2) the lack of any tissue crossing or bridging the rupture site, and (3) the presence of an epiligamentous reparative phase that lasts for 8–12 weeks.

Summary

In summary, the ACL has a surprisingly productive response to injury. The cells within and around the tissue proliferate after injury, they are capable of producing collagen and other extracellular matrix molecules, and they are capable of migrating onto an adjacent provisional scaffold. However, for the torn ACL, there is no provisional scaffold bridging the two ends of the tissue. This may be a key mechanism behind the failure of the ACL to heal and will be discussed in more detail in Chap. 8.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR052772 and RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 8
The Biology of Impaired Healing of Joint Tissues

Martha M. Murray

Orthopedic surgeons struggle mightily with getting certain tissues to heal. Some tissues are relatively easy – the medial collateral ligament of the knee (MCL) routinely heals after 6 weeks of brace treatment [1–4]. Long bone diaphyseal fractures heal uneventfully 94–100 % of the time with standard treatments [5]. In contrast, the ACL fails to heal, even with suture repair, in over 90 % of patients [6–8]. The rotator cuff tendons are thought to have failure rates as high as 95 % after repair [9]. The meniscus has a failure rate of as high as 45 % after repair [10]. Articular cartilage is known for its complete inability to repair itself. Why do some tissues heal easily, even without surgery, while others do not heal, even with our best surgical attempts at repair?

Interestingly, all of the tissues listed above which have difficulty healing live within the joint environment or "intra-articular" (IA), while those that heal uneventfully are outside the joint, or "extra-articular" (EA). We hypothesized that this difference in environment might be the key to the difference in healing potential between tissues. To begin to investigate this question, we selected two comparable tissues, the ACL and MCL. Because the ACL is intra-articular (IA) and the MCL is extra-articular (EA) we looked at differences in their behavior to determine what would make one ligament heal, while the other would not. We first reviewed a series of studies examining the differences in healing response between the ACL (which fails to heal) and the MCL and patellar tendon (which heal uneventfully) and then conducted a few studies of our own. We summarize these findings in this chapter.

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_8,

ACL Versus MCL Healing

The histologic response to injury for the intra-articular ACL and the extra-articular MCL has been described by many groups of investigators. Prior descriptions of the histologic response have focused on three components: (1) characterization of the cellular response, in terms of inflammatory cells and fibroblast infiltration; (2) the formation of extracellular matrix, predominantly focusing on the production and arrangement of type I collagen; and (3) characterization of the vascular response, both in terms of number of vessels in the wound site and vessel type.

Characterization of the Cellular Response to Injury in EA and IA Ligaments

The cellular response to injury in ligament wounds has been described using five basic criteria: (1) the presence of inflammatory cells, (2) the presence and number of fibroblasts, (3) the nuclear aspect ratio of the fibroblasts, (4) the nuclear orientation of the fibroblasts, and (5) the arrangement of the cells. In both intra-articular and extra-articular ligament wounds, inflammatory cells are present in the first few weeks after injury [8, 11–14], with the inflammatory response seen to persist in intra-articular injuries up to 12 weeks after injury [8]. The number of fibroblasts within the wound site increases in the first 3 weeks after injury in both extra-articular ligament wounds [11, 12, 15, 16] and intra-articular ligament wounds [7, 17], at which point the cell numbers begin to decrease [7, 11, 14, 16]. However, the tissue remains hypercellular at 6 weeks [12, 15, 16, 18], 3 months [11, 16, 18], and 40 weeks [11] after injury in extra-articular ligaments and up to 2 years after injury in intra-articular ligaments [8, 19]. The nuclear aspect ratio (NAR) of the fibroblasts also changes with time in both types of wounds as the early fibroblasts are noted to have plump nuclei [12, 15], while at 3 [14, 20] and 6 weeks [12], longer and more slender fibroblasts are present. Increased alignment of the fibroblasts with the longitudinal axis of the ligament wounds is seen at 6 weeks [11, 12, 14] and 3 months [8, 11] after injury. In addition, the columns of cells have been noted to be shorter and less organized in healing ligament wounds when compared to normal ligament tissue [16].

Characterization of the Formation of Collagen in Extra-articular Versus Intra-articular Wounds

The formation of a mature collagenous structure in the wound site of a healing ligament has been described using three main criteria: (1) collagen organization into bundles, (2) orientation of those bundles, and (3) the presence of crimp within the

bundles. In extra-articular ligaments, the initial wound site is filled with hemorrhage or blood clot that is quickly replaced with disorganized collagen fibers [12, 13]. Orientation of the collagen fibers begins to appear in extra-articular ligament wounds by 3 weeks. Ligament strength returns as this organization improves over the next few months [11, 12, 15, 18]. The formation of wider collagen fascicles and development of crimp is seen after collagen orientation occurs [15]. In partial [8, 14, 21], or complete [8] ACL defects, filling of the wound is limited to the edge of the defect [21] where a gelatinous [7] or pannus-like [14] material is observed. Where this limited material is present, it gradually becomes replaced with disorganized collagen fibers followed by collagen fibers with increasing alignment with the longitudinal axis of the ligament [8, 14]. In ACL wounds, the formation of crimped fibers is seen after alignment of the collagen into fascicles [14]; however, this response is limited to the small area where an initial gelatinous material is seen at the wound edge.

Characterization of the Revascularization Response in Intra-articular Versus Extra-articular Ligaments

Revascularization of ligament wounds has been described using three main criteria: (1) the density of blood vessels within the wound, (2) the orientation of the new blood vessels with longitudinal axis of ligament, and (3) the development of larger vessels through the wound site. After extra-articular or intra-articular ligament injury, blood vessels are initially absent from the wound site. Revascularization is seen as early as 1 week [8, 11] after injury in small animals and 3 weeks after injury [12] in goats. The number of vessels initially increases, and during this period of revascularization, no increase in wound strength is seen [17, 22]. This phase is followed by a period of decrease in the number of vessels in the wound [12, 22], during which time the strength of the healing ligament increases [12]. By 3 months in small animal models, the blood vessel development is thought to be nearly complete [21]. In larger animal models, vessel organization is seen to improve between 3 and 9 months after injury [22].

Epiligament and Endoligament Response to Injury

The fascicles of ligament and tendon are surrounded individually by a cellular and vascular tissue called endoligament or endotenon. The entire ligament or tendon is also covered by an additional layer of tissue called the epiligament or epitenon. The epiligamentous layer is noted to be an active site for cellular proliferation after injury for both extra-articular [13] and intra-articular [8, 14, 17, 21] ligament injuries. The endotenon is known to play a role in tendon healing [23, 24]; however, the role of the endoligament in ligament healing is less well characterized.

ACL Versus MCL in a Head-to-Head Test

The previous sections compared ACL and MCL healing from different studies. We were particularly interested in finding differences in ACL and MCL healing in an attempt to determine the reason for the failure of the ACL to heal. To begin to investigate this question, we conducted a series of studies examining the differences in healing response between the ACL (which fails to heal) and the MCL and patellar tendon (which heal uneventfully) in the same knee. In this series of studies, we examined the hypothesis that the histologic response to injury would be different for tissues inside the joint (IA) and tissues outside of the joint (EA).

To test this hypothesis, we created central wounds in the ACL and MCL of an in vivo model and looked at the response in and around the wound site over the first 6 weeks of recovery. ACL and MCL wounds were created by using a 3.5-mm blade and making a slit in the middle of each ligament (Fig. 8.1). The resulting wound was 3.5 mm in width by 100 μm in height. The biologic response was characterized within the defects in IA (MCL and PL) and EA (ACL) ligaments over a 6-week period using immunohistochemistry for a selected set of molecules associated with dermal and ligament healing [14, 25–33], as well as a histologic scoring system developed to incorporate the qualitative observations of ligament healing parameters published by previous investigators. We developed the Ligament Maturity Index (which incorporated all of the findings noted above as indicators of ligament healing; Table 8.1) to score and compare the ligament responses.

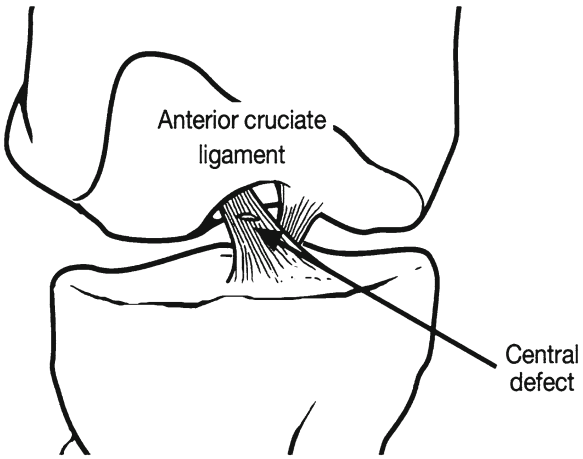

Fig. 8.1 Schematic of the central defect model of ACL injury. An ACL wound is created using a 3.5-mm blade and making a slit in the middle of each ligament. The resulting wound was 3.5 mm in width by 100 μm in height. This resulted in an ACL defect which was mechanically stabilized by the adjacent intact ACL fascicles, allowing for assessment of the biologic healing response without the additional factor of mechanical instability as a first step in developing a technique for bio-enhanced ACL repair. (Used with permission from Murray et al. [4])

Table 8.1 Criteria used to generate the composite Ligament Tissue Maturity Index for EA and IA wound sites and adjacent transected fascicles

Ligament Tissue Maturity Index (Total = 28)	Cellularity Subscore (total = 10)	Presence of inflammatory cells	
		Necrosis	0 points
		Polymorphonuclear cells	1 point
		No inflammatory cells	2 points
		Number of fibroblasts	
		None	0 points
		More than 2x normal ligament	1 point
		Less than 2x normal ligament	2 points
		Nuclear aspect ratio (NAR) of fibroblasts	
		No cells	0 points
		Avg NAR less than 2	1 point
		Avg NAR greater than 2	2 points
		Orientation: Long axis of nucleus parallel with normal fascicles	
		No cells	0 points
		Less than 30% of cells oriented	1 point
		More than 30% of cells oriented	2 points
		Arrangement of cells into columns	
		No cells	0 points
		Cells in columns of 2 to 3	1 point
		Cells in columns of more than 3	2 points
	Collagen Subscore (total = 12)	Width of bundles	
		No bundles	0 points
		Width less than 50 microns	2 points
		Width greater than 50 microns	4 points
		Bundle orientation	
		No orientation	0 points
		Presence of bundles perpendicular to long axis of ligament	2 points
		Presence of bundles parallel to long axis of ligament	4 points
		Crimp	
		None present	0 points
		Crimp length < 0.5 normal length	2 points
		Crimp with normal length present	4 points
	Vascularity Subscore (total = 6)	Density of blood vessels	
		None present	0 points
		Twice as many as normal present	1 point
		Less than twice normal present	2 points
		Orientation of vessels with long axis of ligament	
		No vessels oriented	0 points
		Less than 30% oriented	1 point
		More than 30% oriented	2 points
		Vessel maturity	
		No vessels seen	0 points
		Capillaries only present	1 point
		Arterioles present	2 points

Extra-articular Wound Healing

What we found was that extra-articular wounds had an orderly progression of events that led to wound healing, similar to what we have discussed in Chap. 6. The extra-articular wounds were filled with cells and a scaffold at all time points (3, 7, 21, and 42 days). The defects were distinguishable from the surrounding tissue on hematoxylin and eosin staining at 3 and 7 days by the presence of inflammatory cells and red blood cells. Immunohistochemistry at 3 and 7 days revealed the presence of fibrinogen, fibronectin, PDGF-A, TGF-b, and FGF within the wound at this time point. Procollagen I was not identified in the wound site (Fig. 8.2). By 3 weeks after injury, increased fibroblast density was seen within the defect; however, there was no evidence of crimp within the wound site. By 6 weeks, the defects were far less visible with hematoxyin and eosin staining alone. The wound site, as detected by the presence of fibrinogen, had changed in geography from a transverse wound to a complex geometry with increased cellularity seen in the endoligamentous tissue adjacent to the wound site as well as the wound site itself. This resulted in a stellate lesion within the ligament that had increased cellularity, vascularity, and expression of PDGF-A, TGF-b, FGF2, procollagen I, and von Willebrand's factor (Fig. 8.3).

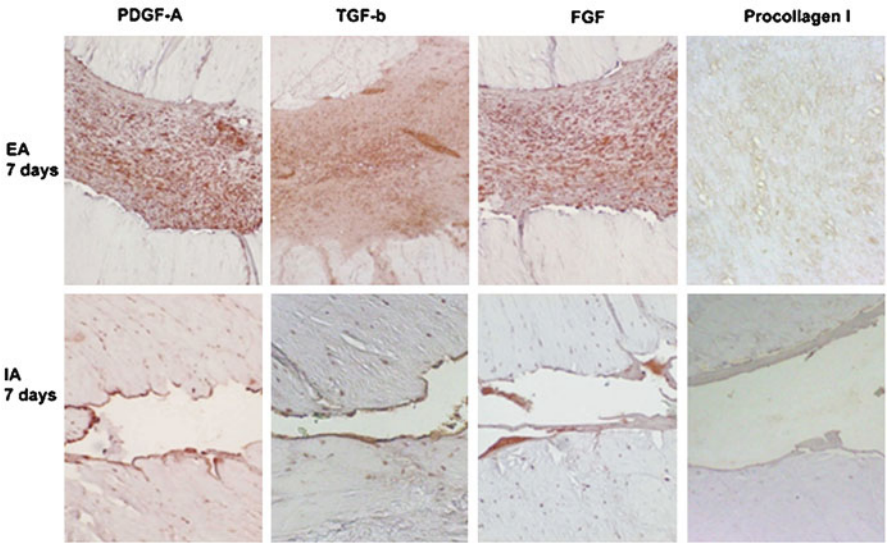

Fig. 8.2 Photomicrographs of EA (*top row*) and IA (*bottom row*) ligament 7 days after wounding. All photomicrographs taken at 10×, immunohistochemistry where *red* designates a positive presence of the molecule of interest (from *left to right columns*: PDGF-A, TGF-b, FGF-2, and procollagen I). The EA ligament wounds demonstrate a relatively uniform and intense staining for PDGF, TGF, and FGF throughout the wound site, where the IA ligament wounds are relatively empty of any substratum (Used with permission from Murray et al. [34])

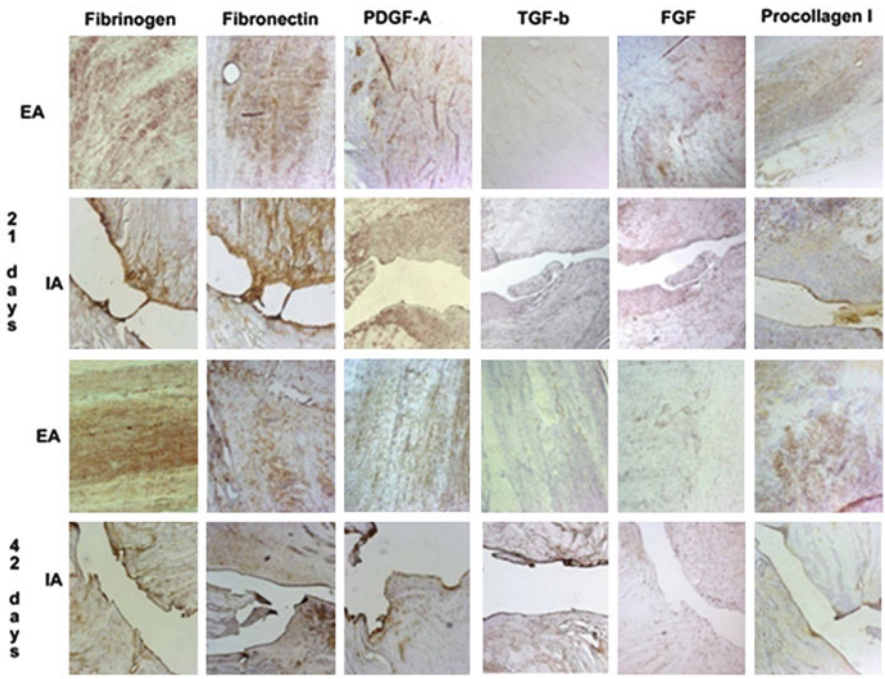

Fig. 8.3 Photomicrographs of extra-articular (EA; *first and third rows*), untreated intra-articular (IA; *second and fourth rows*) 21 and 42 days after wounding. All photomicrographs taken at 10×. Immunohistochemistry (where *brown* or *red* denotes a positive presence of the molecule of interest) revealed active and functional wound sites in the extra-articular wounds at both time points; however, the untreated IA ligament wounds remain relatively empty of any substratum (Used with permission from Murray et al. [34])

Intra-articular Wound Healing

In contrast, the wound site was essentially empty in all of the intra-articular wounds (see Figs. 8.2 and 8.3). Immunohistochemistry revealed a paucity of fibrinogen, fibronectin, PDGF-A, TGF-b, and FGF within the wound site compared with the EA wounds (see Fig. 8.2) and minimal presence of these proteins in the adjacent fascicles. No evidence of TGF-b or von Willebrand's factor were seen in the IA wounds at 3 weeks, and a relative paucity of fibrinogen, fibronectin, PDGF-A, TGF-b, and FGF was seen within the IA wounds in comparison with the EA wounds (see Fig. 8.3). At 6 weeks, von Willebrand's factor was still absent from the IA wounds and the decreased presence of the other markers again observed (see Fig. 8.3). Polarized light microscopy demonstrated loss of crimp in the ends of the transected fascicles at the 3- and 6-week time points.

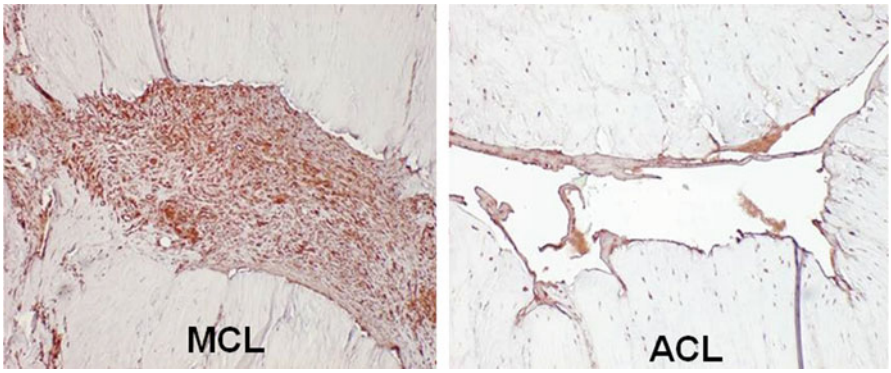

Fig. 8.4 The primary defect for healing of intra-articular injuries. Wounds for tissues outside of the joint (like the MCL) fill with a bioactive fibrin clot after injury as is seen in the left-hand picture. In contrast, wounds inside the joint (intra-articular, like the ACL) fail to form this provisional scaffold and, therefore, are missing a key component of successful wound healing. The wound remains open, and healing cannot occur (Used with permission from Murray and Spindler [35])

One of the major findings in the work summarized in the previous chapter and in this chapter was the premature loss of the provisional scaffold between the two ends of the torn ACL – a problem not seen in the extra-articular MCL (Fig. 8.4). Prevention of clot formation within the joint was likely advantageous during human evolution. A human who had an injury and had clot form within the knee joint would likely fibrose the joint and make ambulation and running difficult (and thus food finding and escape from the saber tooth tiger more challenging), while a human who had the ability to prevent clot formation within the joint would have better motion short term and thus likely have a greater survival rate.

However, now that we are living longer and asking more of our joints as a result, this premature loss of the provisional scaffold or bridge (or the failure of it to form at all) becomes more problematic. Now that life expectancy approaches a century, longer term effects of knee instability become more important. Failure of the ACL to heal leads to degenerative joint disease within several decades of injury. Other tissues that live within joints (meniscus, rotator cuff, cartilage) also have relatively high failure rates of healing, even with surgical repair. These tissues all have one thing in common – they live in the synovial fluid environment and are subject to the failure of a provisional scaffold to form.

Thus, we came to our general hypothesis: tissues within joints fail to heal due to the premature loss of provisional scaffolding between the two ends of the tissue (see Fig. 8.2). This could be a huge deterrent to tissue healing. If we look at the following schematic for MCL vs ACL injury, we can see why this is. When the MCL tears (top row of Fig. 8.2), blood/fibrin clot forms between the two torn ends of the ligament. Over time, surrounding fibroblasts move into the fibrin clot, gradually replacing it with a collagenous scar tissue. This scar is by no means normal (i.e., does

not have the regularity and collagen organization of the original ligament), but it is functionally adequate and the organization of the ligament improves over time. In contrast, after the ACL tears (bottom row of Fig. 8.2), no blood or fibrin clot forms between the two ruptured ends of the tissue, and thus there is no structure for surrounding fibroblasts or other reparative cells to migrate into and remodel. Without this structure, there is no healing, and eventual failure of any suture repair we may have attempted.

Why No Provisional Scaffold Formation Within the Joint?

Perhaps the most striking finding for the ruptured ACL tissue was a complete lack of bridging between the two torn ends of the ligament. In connective tissues that heal, such as the MCL, a fibrin clot forms that is invaded by fibroblasts and is gradually replaced by collagen fibers (Fig. 8.5). This has been demonstrated to be instrumental in the healing process in both tendon [36, 37] and the medial collateral ligament [38]. When the MCL is injured, bleeding from the two ends forms a fibrin clot or scaffold between the two ruptured ends. This scaffold, or bridge, serves as a place for reparative cells to set up shop and knit the ligament back together (see Fig. 8.5). However, prior work by Harrold and his group [39] has demonstrated that fibrin clot does not form in the intra-articular milieu, likely due to enzymes that break down clot in the synovial fluid [40]. This fits in with what we observe clinically in human patients as well – when a patient gets an injury that results in bleeding in the knee, we can pull the fluid off as a rust-colored hemarthrosis. There is no fibrin or blood clot within the joint that clog the needle, the synovial fluid has prevented its formation.

But why does this clot not form? Interestingly, the synovial fluid of joints always has a protein called plasminogen circulating within the fluid. Plasminogen is an

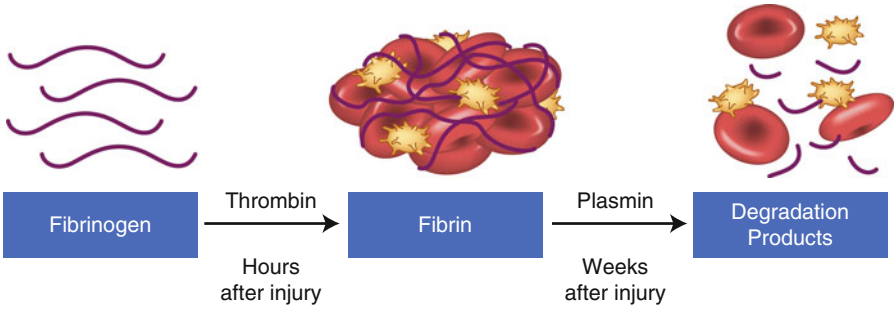

Fig. 8.5 The provisional clot formation process for tissues outside of joints. When the tissue is injured, fibrinogen is cleaved by thrombin into fibrin, a protein which is able to form a sticky clot that traps platelets in the wound site and serves as a provisional scaffold, filling the wound (Used with permission from Murray and Spindler [35])

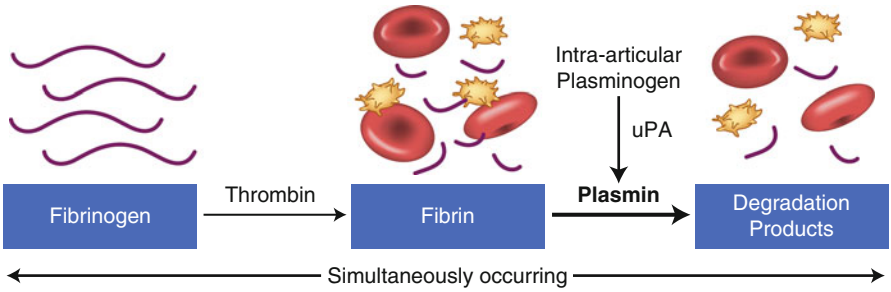

Fig. 8.6 The early wound healing process for tissues within joints (intra-articular, like the ACL). For these tissues, when the injury occurs, there is an immediate increase in the uPA, an enzyme which converts the inactive plasminogen circulating in the joint into plasmin. The plasmin then degrades the fibrin clot as quickly as it forms, thus effectively preventing the development of the fibrin clot, or provisional scaffold, within the wound site (Used with permission from Murray and Spindler [35])

innocuous, inactive protein. However, plasminogen can be cleaved by an enzyme (plasminogen activator) into its active form, plasmin. Plasmin is a potent dissolver of fibrin clot (that is why tissue plasminogen activator is used to dissolve blood clots in heart vessels as a therapy). After injury to a joint, the cells lining the joint (synoviocytes) upregulate their production of urokinase plasminogen activator, and this converts the inactive plasminogen into its active form of plasmin (Fig. 8.6). The fibrin that is trying to form to create a clot within the ACL wound site is bathed in a solution of plasmin that dissolves the fibrin clot before it can fill the wound site. Thus, fibrin clot is unable to form the provisional scaffolding for the ACL wound the way it can for tissues outside of joints that are not exposed to the plasmin bath.

Summary

Tissues that are exposed to synovial fluid when they tear (ACL, rotator cuff, meniscus, articular cartilage) may all suffer from this premature loss of provisional scaffold, and without this scaffold, the tissues will not reunite and any suture repair is likely to eventually fail as the sutures fatigue and break. However, identification of this mechanism also gives us something to work toward – what if we could design a substitute scaffold that we could place between the torn tissue ends to stimulate healing? This will be the topic of the next two book sections on engineering a solution for ACL injury.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR052772 and RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Part III
Translational Medicine: In Vitro and In Vivo Models

Chapter 9
Translational Medicine

Patrick Vavken

What Is Translational Medicine?

Translational Medicine is a building block of a larger approach to medicine called evidence-based medicine [1–3]. In evidence-based medicine, the "current best available knowledge is applied to clinical decision making," and translational medicine is the tool meant to bring this "best available knowledge" into the clinical arena [4, 5]. Typically, translational medicine has been described as the "bench to bedside" approach to medicine, where findings and insights from the laboratory's bench are brought to the bedside to help the patient and physician to achieve the best possible outcome in a given clinical situation (Fig. 9.1). A frequently cited example is the use of losartan in the prevention of aortic aneurysms. Hal Dietz at Johns Hopkins University found that this blood pressure medication effectively prevented aortic aneurysm formation in mice with Marfan syndrome [6, 7]. Now this drug is successfully used in clinical trials to prevent this deadly problem in children.

More recently, the definition of translational medicine has been expanded to include a "bedside to bench" approach [8–10]. For example, the National Institutes of Health Clinical Center rebranded its "Bench to Bedside Award" in 2007 as "Bedside to Bench Award" to, quote, place the emphasis of the back and forth cycle of "bedside to bench and back" (http://www.cc.nih.gov/ccc/btb/). Through the end of 2012, this center has distributed almost $50 million in support of translational research [11, 12].

The reason for this change to "bedside to bench" is a certain dissociation of basic science from clinical medicine. In some cases, the basic science researcher is studying fundamental mechanisms on a gene expression level for cells in a petri dish,

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_9,

Fig. 9.1 Outlines the two-way principle of translational research, which aims at the incorporation of basic science and clinical problems into a joint approach

which may not be obviously applicable to a clinical patient with a disease. For example, the mechanisms of cell migration defined so elegantly for two-dimensional petri dish cultures turns out to be irrelevant when the cells are living in a three-dimensional environment. The scientist may not interact with patients or clinicians at all, which can make understanding the clinical needs more difficult. In turn, clinicians caring for patients see the problems that need to be solved to improve patient care but may not know about basic science advances that could be used to help solve these problems. More often than not, it is physicians at the bedside or patients and their relatives, asking such questions as "Isn't there something better that we can do?" This has lead to an increased flow of clinical questions back to the laboratory to find specific answers, but these questions can get lost in the disconnect that lies between the clinical realm and the arena of scientific discovery.

The ideal situation is a constant, cyclical flow of basic research data from the bench to the bedside, where these data are tested for clinical effectiveness, and a subsequent return of this clinical information back to the bench to further understand the basic science. Currently, legions of scientists and clinicians are involved in developing and implementing such cycles using unprecedented amounts of private and public research funding – an exciting development which is likely to significantly change the way we practice medicine.

So Why Do We Need Translational Medicine?

Medical research is being produced on an industrial scale. The online databases that collect medical publications add millions of new articles annually (e.g., http://www.ncbi.nlm.nih.gov/pubmed/). Further articles in science and technology are produced at a similar rate. In addition, while astounding and groundbreaking findings in basic science are being produced on a daily basis, it seems that the number of significant improvements in medical treatments proceeds at a much slower pace. At the same time, purely clinical research can suffer from a lack of understanding of basic science progress.

The last 30 years offer three excellent examples of the bench to bedside concept and the difficulties that are encountered when attempting to translate new technologies to clinical practice. The first one is gene therapy. In the 1970s, genes and

cloning were considered the keys to unlocking a door to a disease-free future. Countless research papers were published on how to get cells to express certain genes in vitro, and science promised answers to problems ranging from the treatment of congenital heart disease to a better understanding of the extinction of dinosaurs. By 2000, there were about 400 clinical trials for patients being treated with gene therapy [13, 14]. However, today (and almost 30 years of intensive research in gene therapy later), the only clinical gene therapy that exists – and only approved in China – is Gendicine, a gene therapy approach to fight some forms of head and neck tumors [15]. While this is obviously a complex issue, a few reasons for the failure of gene therapy have been identified. Primarily, diseases such as heart disease, hypertension, diabetes, and Alzheimer have multigenetic causes, i.e., more than one gene causes the problem. Secondly, the effect of gene therapy is often short-lived. Therapeutic DNA is introduced into cells, but not integrated into the genome. Thus, the DNA disappears over time as cells divide and die. Third, introducing therapeutic DNA can initiate an immune response that targets and preferentially removes the "treated" cells. The same is true for the viral vectors that are commonly used to introduce therapeutic DNA.

While most of these problems could be circumvented, gene therapy suffered a severe setback in 1999 [16, 17]. Jesse Gelsinger, an 18-year-old patient with ornithine transcarbamylase deficiency, a serious genetic disease that destroys the liver and in its full-blown version is fatal at birth, volunteered to join a clinical trial of a gene therapy developed at the University of Pennsylvania. Four days after receiving an adenoviral vector carrying the therapeutic DNA, Gelsinger suffered from a massive immune response leading to multiple organ failure and brain death. Seventeen patients had been successfully treated before Mr. Gelsinger, but since his unexpected demise, gene therapy has come under closer scrutiny, particularly for treatment of nonlethal diseases [18, 28]

Another example is the use of stem cells. In the 1980s, the use of both embryonic and then adult stem cells was thought to hail the onset of a new medical revolution. Over 150,000 articles have been collected in the US National Library of Medicine since 1980 on this topic, but stem cell research has produced relatively few new treatments to date. Problems such as death of the carefully cultured cells when they were reimplanted into the body were likely responsible for the end of clinical improvements on testing of these cells in orthopedic applications [19, 20]. In addition, removal of cells from the body and then later reimplantation is costly and requires painstaking care at each step to ensure quality and safety of the end product. To date, the cost-benefit analysis has not yet justified the use of stem cells for orthopedic applications.

Finally, "Tissue Engineering and Regenerative Medicine," or TERM, entered the field in the 1990s [21]. It was believed that tissue engineering would fare better than its precursors, mostly because it could involve simpler materials and cell sources to avoid the more complicated issues previously encountered with modifying genes and obtaining embryonic stem cells [29]. To date, tissue engineering has resulted in a few orthopedic applications, for example, autologous chondrocyte implantation [22],

but again, the clinical use of this technology has lagged behind the great basic science research bank.

However, what held back gene therapy, stem cells, tissue engineering and many other scientific achievements is not "bad" or imperfect science. The problem is and has been that basic science and clinical day-to-day reality exist in parallel and not as a sequence. Outstanding basic science is conducted, but often by individuals who are not fully aware of clinical needs and issues surrounding patient care. At the same time, clinicians, faced with pressing health care issues, do not have access to knowledge of scientific methods to answer their problems. Thus, translational research and medicine are acutely needed. In this chapter, we will describe how such translation is achieved, what the main barriers are, and what translational medicine does for the ACL and orthopedics in general.

How to Translate in Medicine

Originally, it was thought that the aims of translational medicine could be materialized by sending scientists to the operating room and surgeons to the lab. However, given the highly specialized nature of both of these occupations, this was only partly fruitful. As both orthopedic surgery and basic science research in orthopedics have become more complex, it has become more and more difficult for the two cultures to communicate and exchange ideas. Even the languages are different – "RNASeq" and "siRNAs" are words not typically in an orthopedist's vocabulary, while the difference between ACL repair and ACL reconstruction may not be evident to a scientist. Therefore, the language barriers may be significant and difficult to overcome.

Another approach might be to train individuals to be knowledgeable and conversant in both arenas. This "translational researcher" could serve as an interpreter between clinical and basic scientist, preferably having experience and "language proficiency" on both sides of the divide. It is no stretch to imagine the language barriers and cultural differences that would exist in a conversation between physicians, biologists, and engineers, for example. Thus, the job of a translational researcher would not be easy, but it would be potentially very rewarding to help create new solutions for clinical problems from basic science research. The key to success is the ability to understand the potential clinical impact of new discoveries and the basic science needed to answer clinical problems.

To assist the researchers in translating their work from the bench to bedside, three "clinical trial" phases are required (Fig. 9.2) (http://www.fda.gov/drugs/resourcesforyou/consumers/ucm143534.htm)

In phase 1, a nonclinical concept or finding from basic science is applied in a preliminary trial to establish the efficacy and safety, i.e., whether it performs better than what is currently available without causing additional complications. Before phase 1 can be started, approval of the new treatment or medication must be obtained from the FDA, or similar institutions if outside the USA. In the USA, this is typically an investigational device exemption (IDE) or an investigational new drug

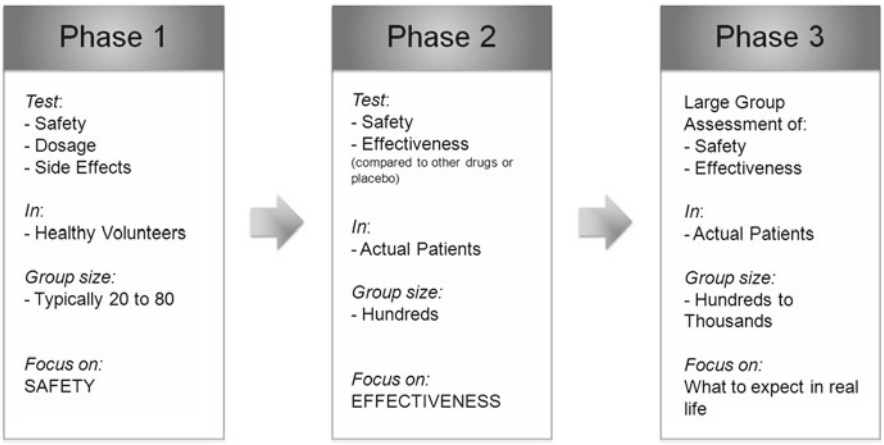

Fig. 9.2 Once a new treatment or drug moves from preclinical development into clinical trials, it has to go through three phases that test safety and effectiveness. After successful completion of the three phases, the drug or treatment will go to the FDA for approval. The time of clinical application after approval is often referred to as "phase four," when widespread use is documented for quality control purposes

exemption (IND), both of which require extensive preclinical testing to justify the transfer of basic science knowledge to the first-in-human study. Phase 1 trials are often times done in a very limited, very specific, and diligently controlled population.

Once safety is established, phase 2 is designed to focus on evaluating the effectiveness of how this treatment will perform. This is typically a larger number of patients who can be followed as a cohort or in a randomized clinical trial. Phase 3 trials evaluate how the new treatment performs in "real-life", with more surgeons performing the procedure than those initially trained specifically for the Phase 1 and 2 studies. In such a real-life setting, the inclusion and exclusion criteria for patient recruitment are expanded. This is important to make sure that the treatment works in the entire target population. It also incorporates other factors since patients do not always adhere to treatment guidelines all the time; medications are lost, forgotten, or used incorrectly, and treatments are combined. At the same time, cost-effectiveness of the new treatment is analyzed to see if the additional cost will provide additional benefit compared to what treatments are already available.

The most important fact that should be taken from this chapter is that translational medicine, i.e., bench to bedside, is actually a multiple-step process and should better be referred to as "bench to research bedside to all bedsides." The National Institutes of Health (NIH) Clinical Research Roundtable stresses that translational research has two blocks, T1 and T2 [23]. T1 is the transfer of basic science knowledge to the bedside in the form of a clinical trial. T2, may be more importantly but less appreciated, is the transfer of knowledge from such clinical trials onward to day-to-day medical practice (Fig. 9.3).

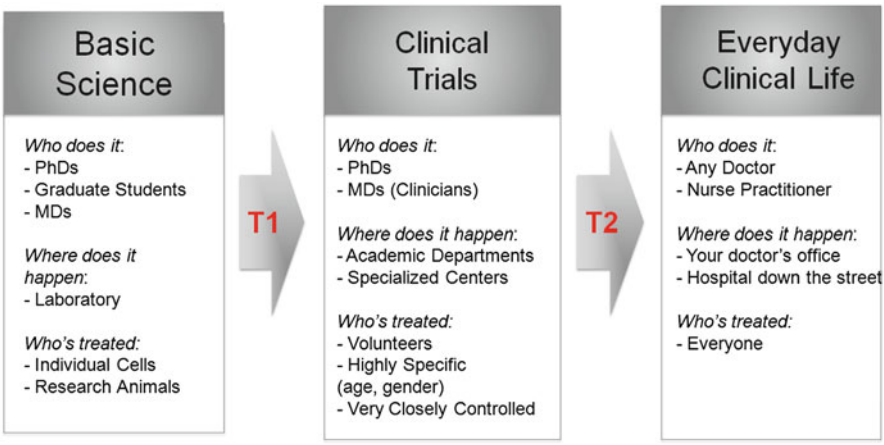

Fig. 9.3 The T1–T2 dilemma: Frequently, the T1 translation from basic science to clinical trials at academic centers is successfully done, but development efforts falter at the T2 transition from academic centers to everyday clinical reality

Barriers to Translation

Numerous barriers exist in translational medicine. The most obvious one is the language barrier between clinicians and the experts from the various fields of science that are involved. Daniel Federman, the Carl W. Walter Distinguished Professor of Medicine at Harvard Medical School and a leading voice in translational medicine, famously said "as in the translation of a language, getting the word right is relatively easy but getting the meaning of a word is much more difficult" [24]. This can already be seen when looking at the definition of translational medicine – is it bench-to-bed, bed-to-bench, T1 and T2, etc.

Another barrier to successfully conducting translational medicine is the regulatory framework. The FDA provides a justified, yet often formidable, challenge to any researcher [25–27]. For example, large amounts of preclinical data are required to ensure safety and efficacy to justify the use of the new treatment in the first few patients. Lastly, time is a barrier of paramount importance. By the time a new cutting-edge, state-of-the-art treatment has gone through all phases of testing and approval, more often than not, years have passed and the treatment is already outdated before it has been fully approved [25]. However, this does not mean that thorough testing of new treatments and medication is not justified and necessary [24].

Lastly, translational medicine does not always fit into the classic allocation of responsibilities in science. This can best be understood by looking at it from the perspective of the involved parties. PhDs and basic scientists depend on grant money for their livelihood. The chances of obtaining a grant, a promotion, or a tenured position, depends on the timely and frequent publication of new findings and data. Translational medicine is a slow and expensive science that does not provide

much new information, since it focuses on the application of available knowledge rather than on the generation of new knowledge. As such, it may be career suicide for a scientist to take on a translational project which will require years of "non-novel" work to complete, with possibly one paper at the end with multiple authors. Petri dish research is more reproducible, less expensive, and a more reliable generator of publications and novel findings. The first priority of clinicians, in turn, is the medical care of their patients and all of the administrative work that accompanies that position. While they may be interested in using a new treatment, they may not have the time, energy or expertise to help translate the new treatment from the bench to bedside. One way of getting both surgeons and scientists involved in translational medicine is providing the funding necessary to compensate the costs and efforts associated with translational medicine, but, again, most funding agencies have been focused on research that produces new data, while translational medicine was considered treatment development and as such part of the obligation of the industry.

Finally, industry may be one place where translation of medical findings could flourish. However, in recent years, major orthopedic companies are more interested in technologies that have the first-in-human data available for safety and efficacy. Like surgeons and scientists, the "valley of death" in getting from the basic science studies to the first-in-human studies is not a desirable place for industry either due to the prohibitive cost barriers.

Conclusion

In summary, translational medicine, or the science that aims at merging basic science and everyday clinical reality to improve patient outcomes, is much needed. Numerous barriers exist, but fortunately there are ways to overcome them, and since the importance of translational medicine has been widely accepted, there is, hopefully, enough support to do so. Encouragement and facilitation of both scientists and surgeons to be able to cross the "valley of death" to the phase I clinical trials may help develop more effective and new technologies that could benefit future patients.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR047910 and RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 10
In Vitro Models of ACL Injury

Patrick Vavken and Braden C. Fleming

The History of In Vitro Studies

In vitro is Latin for "within glass," as opposed to in vivo, which is Latin for "within the living." Glass refers to the glassware that was used initially in the early days of cell culture. In the 1890s, Leo Loeb moved from the University of Zurich Medical School to Chicago where he conducted research in a rented room behind a bookstore for his doctoral thesis on skin transplantation [1]. He demonstrated that the survival of cells isolated from blood and connective tissue in serum and plasma "within glass" was possible [2]. However, other researchers still had difficulties maintaining and manipulating cells in such culture.

One big problem was solved in 1927. On September 3rd, Alexander Fleming, a Scottish biologist known both for his brilliance as a researcher and his untidy nature, returned to his laboratory from a monthlong summer holiday. At the time he was doing research on a type of bacteria called staphylococci, and to his horror, he realized, when entering his lab, that he had left his bacterial cultures out before leaving a month earlier. During the hot and humid weeks of August, plenty of fungus had grown on one of his culture dishes, and oddly enough, all the bacteria immediately around this fungus had died. He was able to grow this mold and isolate its secretion, which he called "mold juice," but when the time came to share his findings, he decided to rename it after the strain of mold it came from: penicillin [3].

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_10,

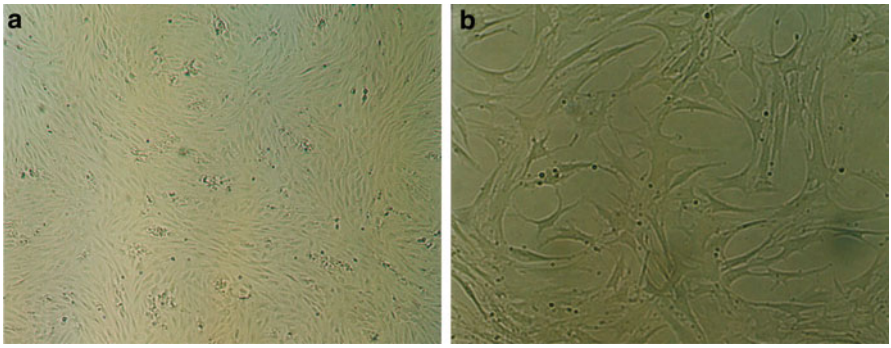


Fig. 10.1 *In vitro cell culture*. Illustrates ACL cell in culture at 100× (**a**) and 400× (**b**). ACL cells can be maintained and studied in cell culture. They typically show an elongated shape with stellated ends

The identification of a substance that could prevent the cultured cells from becoming infected was a great step forward for clinical research and basic science.

In 1955, Harry Eagle of Johns Hopkins University had already established himself as a leading pathologist. His 1937 monograph of the serodiagnosis of syphilis was considered the reference work in the field, and his research on blood coagulation and bacterial growth was recognized as groundbreaking [4–6]. However, having just turned 50, his focus of interest shifted to the culture of cells [7, 8]. Since the 1940s, techniques had been developed to isolate cells from tissue and keep them alive in culture for a little while. Eagle was able to devise, through a series of experiments, a mixture of salts, amino acids, vitamins, and carbohydrates that sustained cell viability and growth. This "cell culture medium" allowed researchers to grow cells and unlocked the potential to study cell function. Today, almost 60 years later, every lab on the planet holds a stock of some modification of the same cell culture medium.

Since the days of Leo Loeb, Alexander Fleming, and Harry Eagle, cell biology has made astounding breakthroughs, and cell culture has become an essential instrument in modern medical and basic science. However, many of the inventions of the early pioneers are still in use. In current standards, cells are grown in plastic dishes rather than glass (vitro) ones, but they are still nourished with "Eagle's Medium," usually supplemented with specific vitamins and proteins for specific cell lines and antibiotics. ACL cells, for example, are typically grown in Dulbecco's Modified Eagle's Medium (DMEM) with Vitamin C, to support collagen synthesis, an antibiotic and some mix of growth factors such as serum from blood (Fig. 10.1).

In vitro research has a number of advantages: First of all, cell culture offers scientist with the opportunity to analyze basic biological processes outside the hugely complex interactions that control and govern all body functions. Secondly, this can be done with fairly simple methods, or at least methods that follow simple principles. Lastly, compared with human or animal trials, in vitro research is less time consuming, less space consuming, and less expensive. Naturally, the applicability of

the findings of cell-based research is somewhat limited, but these findings are the foundation of further experiments in more complex models or living organisms.

In Vitro Models: What to Look For

In vitro wound models can be used for a number of purposes. The general, overall ability of a cell type to fill a defect can be assessed. The beneficial and detrimental effects of growth factors, chemicals, and materials on wound healing can be tested. The chronology of the individual steps of healing can be observed and described. All of the above have been studied in ACL healing [9].

For ACL cells to fill in a defect with new tissue, three major biologic mechanisms need to take place. First, the cells have to be able to move, or migrate, into the defect. Once the cells have migrated into the defect, they have to be able to proliferate and then to produce collagen to fill the defect. Of course, there are innumerable other endpoints and assays, a full listing of which would fill more pages than this book, but for the purpose of this chapter, we will stick with to three endpoints: migration, proliferation, and collagen production.

Migration

Migration is the ability of a cells to move. The mechanisms of migration are not fully understood yet, but it has been observed that the cell uses its cytoskeleton of actin to push a part of itself (the "leading front") forward in the direction of migration. This projection, or leading front, subsequently pulls the rest of the cell body forward. This way cells can move at speeds of up to 10 μm per minute. Usually, cells move in response to external stimuli, a process called chemotaxis.

Cell migration can be measured in different ways. One can simply observe the distance a cell travels over time. At speeds in the range of micrometers/minute, this is usually done by recording the cell's position on a grid every few minutes using time-lapse films. The outcome is called "mean displacement" and is usually reported in micrometers. Measuring mean displacements is time consuming, requires substantial equipment, and is also associated with considerable variability (Fig. 10.2).

An alternative, simple, and inexpensive approach is the use of cell migration assays. These assays are based on two chambers stacked on top of each other with a permeable membrane in between them. The top chamber contains the cells to be assessed, while the bottom chamber contains a chemoattractant (i.e., a growth factor or chemical that attracts the cells). Over a given time, for example, 24 h, cells are allowed to migrate at their own pace into the bottom chamber, following the cues of the chemoattractant. The pore size of the membrane in between the chambers can be chosen to allow only migration of cells of a certain size and thus a certain type as desired. At the end of the migration period, the number of cells in the bottom

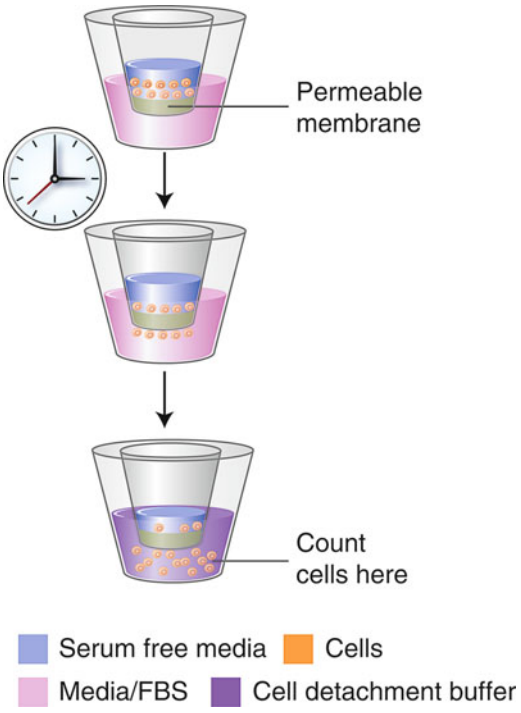


Fig. 10.2 *Principle of a migration assay*. The migration assay tests the ability of cell type to cross a permeable membrane. A suspension of cells to be tested is placed in the top chamber of a two-chamber system that is separated by a permeable membrane. In the bottom chamber, an attractant, usually a chemical to be tested, is placed. Over time, the cells migrate from the top into the bottom chamber, at a rate that is dependent on the strength of the attractant, the migratory potential of the cells, and the pore size of the membrane. After a predefined time period, the number of cells in the bottom chamber is counted

chamber is counted to assess the extent of migration. For an experiment, the chemoattractants in the bottom chamber could be varied in terms of content or concentration. An attractant of known strength can be used as a positive control, together with a negative control without chemoattractive effect, such as saline. A growth factor or chemical to be tested can be used in a third chamber and compared to these positive and negative controls.

Proliferation

Proliferation is cell growth in the context of cell division. Cell division occurs during the cell cycle, a continuous and repetitive process of duplication of cellular organelles followed by the separation of a cell into two daughter cells. The rate of such doublings is affected by the presence of nutrients, temperature, and external chemical cues. Since cells divide, every new generation doubles the population, that is, growth is exponential (at least in theory, since older generations die and thus, the actual number of cells is lower).

Cell proliferation can be quantified in a number of ways. The most straightforward one is to count cells under a microscope with the help of a cell-staining method to improve visibility. Naturally, this method is limited by the number of cells one

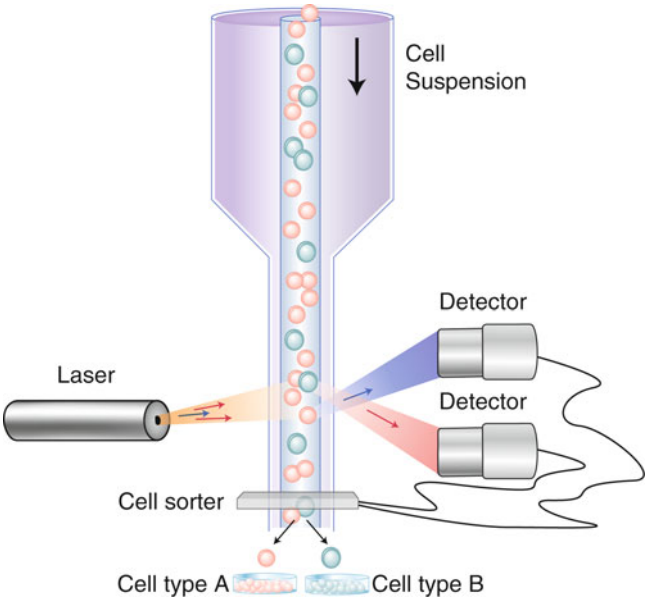


Fig. 10.3 *Principle of flow cytometry*. Flow cytometry is used to identify, count, and/or sort cells using laser-based biomarker detection. Briefly, a cell suspension is guided through a laser beam. The laser is scattered by the cells, and both back and side scatter, as well as laser specific biomarkers, are registered by detectors, which identify and count cells with specific scatter characteristics. As an additional step, the detectors can be synchronized with cell sorters, which are electromagnets that pull cells into different collection tubes

wishes to count. The commonly used petri dish of 10 cm diameter can contain millions of cells, and hand counting becomes impractical. Today, cell counting can also be automated in a process called flow cytometry. With flow cytometry, the cells are labeled with a fluorescent tag and suspended in liquid. Within the flow cytometer, the liquid is streamed through a light beam. The suspended cells and their fluorescent tags scatter the light beam, and the scattered light is collected and provides a measure of the number of labeled cells that have passed through the machine. Based on cell size or surface markers, flow cytometry systems can even differentiate between different cell types, live and dead cells, and levels of cell activation or differentiation (Fig. 10.3).

A somewhat simpler approach is not to count cells but rather to measure the amount of DNA in a sample and divide that number by the known DNA content per cell. Generally, the amount of DNA is 4–8 picograms (10^{-12} g) per mammalian cell. Thus, a doubling of DNA content in a cell suspension is a surrogate for a doubling of the cell number in this suspension. DNA content, in turn, can be measured with commercially available kits. DNA can be measured in different ways. For example, DNA absorbs ultraviolet light at a wavelength of 260 nm. This characteristic is used in spectromorphometric analysis, where ultraviolet light passes through a sample and the amount of UV light absorbed corresponds to the content of DNA in the

sample following a rule called the Beer Lambert Law [10, 11]. However, a major disadvantage of the 260 nm absorption method is the effect of contaminants in the sample. Commercial assays use a dye that binds to DNA, or even to subtypes of DNA, to avoid such biases.

Additional techniques measure cell constituents other than DNA. The so-called MTT assay, for example, measures mitochondrial activity. Mitochondria are small organelles within cells that produce energy. MTT, or 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, is a yellow dye that is changed to purple by mitochondria [12]. Since the number of mitochondria is stable in cells, the amount of purple dye corresponds indirectly to the cell number. However, the amount of purple dye is also affected by mitochondrial activity. Many tissue engineering techniques described in this book require the use growth factors and/or platelet-rich plasma (PRP) to release growth factors to enhance cellular and mitochondrial activity. Thus, the MTT assay can be falsely high in such experiments, and the DNA measurements as described above produce more reliable results.

Biosynthesis

A third parameter of interest is biosynthesis, or the production of molecules and proteins by cells. In order to produce a certain protein, the corresponding *DNA* sequence is transcribed into *mRNA*. This mRNA is then translated into *amino acids* that are assembled to a *protein*. The products of these individual steps can be quantified.

The best-known technique to quantify DNA or mRNA is by polymerase chain reaction or PCR. Briefly, PCR amplifies DNA or mRNA to measureable levels. Asking if a type of mRNA or DNA is present process called qualitative PCR. Alternatively, a known amount of "mRNA standard" can be added and used as a gauge to see how much of a target mRNA is present in a sample – this process is called quantitative PCR. But since DNA/mRNA transcription is only the first step in cellular protein production, a positive PCR result is only a surrogate, not final proof of protein expression by a cell.

The next step in biosynthesis is amino acid production. The key elements of an amino acid are carbon, hydrogen, oxygen, and nitrogen atoms. These atoms can be radioactively labeled and added to the nourishing medium of a cell culture. If the cells in this culture produce amino acids, they will use these radioactive atoms, and the level of radioactivity will correspond to the amount of newly produced amino acids.

Lastly, a protein of interest can be quantified directly. For ACL-related research, this protein is almost exclusively type I collagen. Direct quantification of a protein can be achieved through photometry, typically with the use of a color that binds to proteins in general, or a specific protein. Antibodies can be used in enzyme-linked immunosorbent assays (ELISA) to attach a color to a protein and thus to measure it with photometry.

In Vitro Studies and the ACL

For a long time, physicians were startled by the fact the ACL does not heal spontaneously and sometimes not even after surgery, whereas the collateral ligaments of the knee – only an inch away – heal without surgery after a short period of protection in a brace. A key to unlock this problem was to compare the cells from both ligaments. ACL and medial collateral ligament cells were compared for proliferation and biosynthetic activity using some of the methods described above. It was anticipated that differences in cell behavior would explain the differences in healing seen clinically. Thus, cells were isolated from ACL and MCL tissue, and cell proliferation, cellular matrix production, and cell migration of both cell sources were compared. However, no differences between cell sources were found [13–16]. Later on, it was determined that the reason for the lack of wound healing in the ACL was the lack of a clot (i.e., provisional scaffold) to initially fill the defect that cells can crawl into rather than the inability of cells to function intra-articularly [17–19] (Fig. 10.4).

New scientific approaches to treat ACL injuries focus on enhanced healing with biological stimuli. The basis for this was shown with in vitro studies using ACL wound models. These models can be broadly categorized into three groups. First, cells alone can be cultured to observe the effect of growth factors or other environmental cues on those cells. Second, cells can be cultured in three-dimensional cultures, for example, seeded on a biomaterial, to better simulate their orientation in a tissue. Lastly, complex in vitro cultures, which combine cells, compound biomaterials, and biomechanical stimuli, can be designed to better recapitulate in vivo tissue function. Examples of such in vitro wound models are given below.

	MCL	ACL
Cell Proliferation	Yes	Yes
ECM Production	Yes	Yes
Cell Migration	Yes	Yes
Bridge over wound site?	Yes	NO

Fig. 10.4 *Differences between ACL and MCL cell behavior*. In an effort to identify why the ACL does not heal, while the MCL, a few centimeters away, does, cells from both tissues were compared for their proliferative and biosynthetic activity, as well as their potential to migrate. However, no differences in cell behavior were found that could explain the differences in healing capacity. Yet in further assessment, it was shown that there is a lack of defect bridging in ACL tears, prohibiting defect filling and cell migration into the filled defect

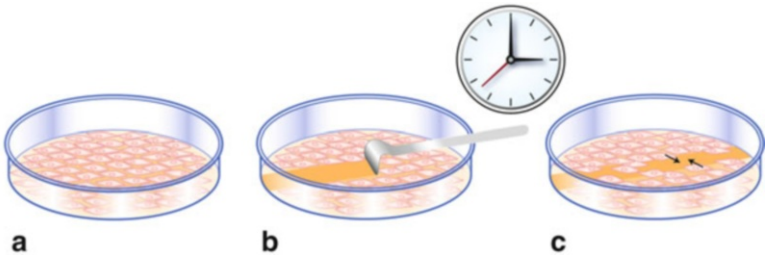


Fig. 10.5 *The scratch test*. The scratch test is a test of cell migration and can be used as a model of wound healing. Cells are grown in a confluent monolayer (**a**). A tool of known dimension is used to create a "scratch" of predetermined size in this cell layer (**b**). Subsequently, closure of this scratch is observed over time (**c**)

Wound Models: Cells Alone

The simplest ACL wound models are cultures of cells alone. ACL cells can be easily isolated and maintained in culture. Subsequently, these cells can be exposed to various agents, such as growth factors, nutrients, and different levels of oxygen, and their response to these agents was evaluated using the measurement assays described above.

Beyond the assessment of cellular response, simple assays have been devised to study wound healing in cell cultures. The best-known test is the "scratch assay" [20]. Briefly, cells are grown in a petri dish to confluence. Subsequently, the cell cover is scratched to simulate a wound. Closure of the scratch, that is, healing of the wound, is observed under a microscope [21]. Successful closure depends on the cell's ability to migrate and multiply. Again the effect of various factors on the speed of scratch closure can be measured (Fig. 10.5).

A major disadvantage of such cultures is that they are two-dimensional, that is, they grow on the surface of a cell culture vessel, such as a petri dish, in a monolayer. However, three-dimensionality is an essential stimulus for ACL cell function (i.e., ACLs are not flat structures), and thus, monolayers cannot reproduce normal physiology. Thus, 2-D cultures are not perfectly representative of how cells behave in the body. Nevertheless, 2-D cultures are valuable tools to begin to quantify the cellular response to exogenous factors.

Wound Models: Cells and Biomaterials

For the purposes of tissue engineering, 3-D wound models are indispensable. Such models are intended to bridge the gap between the conditions in a monolayer culture and the conditions in real life in a human patient or a test animal [22]. The most

important reason, as mentioned above, is the fact that mesenchymal cells need to grow 3-dimensionally to achieve full differentiation [23, 24]. Countless 3-D culture systems exist as well as online registries collecting data from these systems (e.g., www.3dcellculture.com). More precisely, these techniques typically model ligament development and identify potential ligament treatments rather than simulate ligament wounds.

Mesenchymal cells, which include cartilage cells, bone cells, and ligament cells, can be grown in so-called high-density cultures [25]. In such culture systems, cells are grown in large clusters suspended in a nutrient medium. The condensation of mesenchymal cells is actually the trigger of limb development in embryonic development [26, 27]. High-density cultures mimic these conditions in vitro and are thus most commonly used for studying the development of ligaments, cartilage, or bone. Ligament and tendon cells have been shown to be able to survive in such high-density culture and to spontaneously produce type I collagen and scleraxis, a tendon-specific marker, in large quantities [28]. It was also shown that high-density cultures of uncommitted adult stem cells with ligament cells result in differentiation of these stem cells into ligament cells, too [29] (Fig. 10.6).

Other 3-D wound models require biomaterials that provide structure. Choosing the appropriate material is the first decision point in developing a 3-D culture model. The criteria to choose a biomaterial include various factors, such as using a natural or synthetic material, a scaffold made from one source material or various materials. Material parameters, such as micro-architecture, porosity, hydrophilia, electric conductivity, and many more, are known to influence cell behavior and must be considered [30, 31]. A description of the currently available materials, both biological and synthetic, is far beyond the scope of this book, and we refer all interested to pertinent publications [32, 33]. For ACL research, scaffolds made from type I collagen are being used widely, since type I collagen is the main constituent of the ACL [9, 34].

An excellent biomaterial-based wound model for ACL injuries is the actual ACL itself. Two pieces of ACL can be kept in a cell culture, and migration of cells across the gap in between and the filling of this “defect” can be studied. This model has been repeatedly used to test candidate biomaterials for ACL repair by placing them in between two pieces of ACL tissue and analyzing cellular remodeling of the candidate material. An additional beneficial factor is that the ACL pieces provide cells, identical to those found *in situ* for the in vivo wound, for the wound healing model.

Complex Wound Models

Additional levels of complexity can be added to the models described above to better mimic the real-life situation. Among the most essential clues, which are not readily provided in the in vitro environment, is biomechanical stimulation to

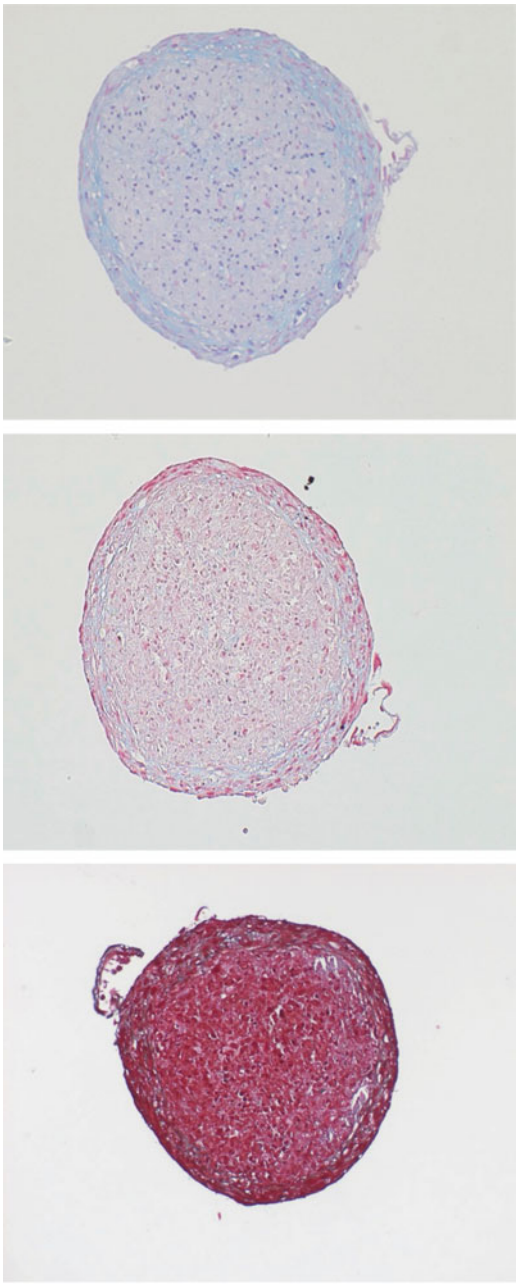


Fig. 10.6 *High-density cell culture*. High-density cultures are used to stimulate differentiation of mesenchymal cells by reproducing the conditions of embryonal development. In this figure, we see mesenchymal stem cells from the bone marrow of adult patient that have been grown in high-density culture without further addition of growth factors or environmental stimuli for 21 days. The three panels show histology with Alcian, Azan, and Safranin-O staining, specific for collagen and glycosaminoglycan production. After 21 days in high-density culture, we can see a dense cellular core with a compact rim of extracellular matrix formed by collagen and glycosaminoglycans, as it is seen in embryonic limb development

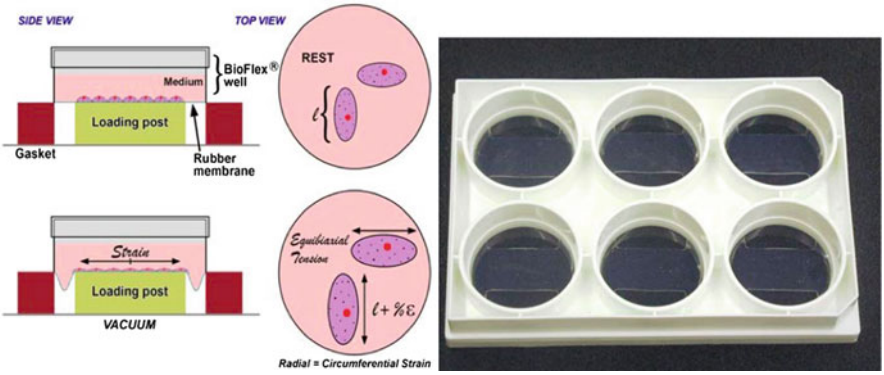


Fig. 10.7 *Flexcell bioreactor for 2-D cultures*. Simple bioreactors can be used for 2-D culture models. In this case, a 6-well plate (seen *right*) is put on a vacuum generator with central posts. A vacuum is generated at a given frequency and strength and sucks the flexible membrane into the gap between the loading post and the gasket. This tensions the membrane, and this tensile stress is translated onto the cells as a radial strain (FX5000, FlexcellR International Corporation, Hillsborough, NC, with permission)

regulate cell behavior. Wolff's law describes the influence of biomechanical stimuli on cell differentiation among the mesenchymal cell types. Julius Wolff, a surgeon and anatomist of the nineteenth century, originally described this law in 1892 for bone, stating that this tissue will remodel to adapt to extraneous stress and strain. Subsequently, this law has been expanded to include other tissues, stating that mesenchymal stem cells will differentiate into bone under pressure, cartilage under shear, and ligaments under tension. As such, biomechanical stimulation is an essential part of ligament healing, but for the longest time was only available as a factor in animal models. Techniques and machines have been developed to add such stimuli to in vitro cultures. For example, cells can be cultured on flexible membranes. Pistons or vacuum underneath membranes or tensioners on the diagonal ends of the membrane can be designed to stretch cells under cell culture conditions (Fig. 10.7). It has been shown repeatedly that ACL cells in culture respond to tension or stretch by increasing the expression of type I and type III collagen mRNA [35]. While the exact conditions leading to a maximum cell response are still elusive, it has been shown that uniaxial cyclic stretch of 10 cycles per minute with 10 % length difference increased mRNA expression in ACL cells via the expression of TGF-beta 1 from the ligament cells themselves [36]. An excellent review of the issue of biomechanical stimulation in tissue engineering of ligaments is given by Benhardt et al. in their recent paper in Tissue Engineering in 2009 [37]. The more interested reader might refer to this publication.

The addition of biomechanical simulation to cell culture was a huge step forward for in vitro research, because it effectively re-created one of the most essential factors relevant to the in vivo conditions. Thus, in vitro studies of ligament and tendon

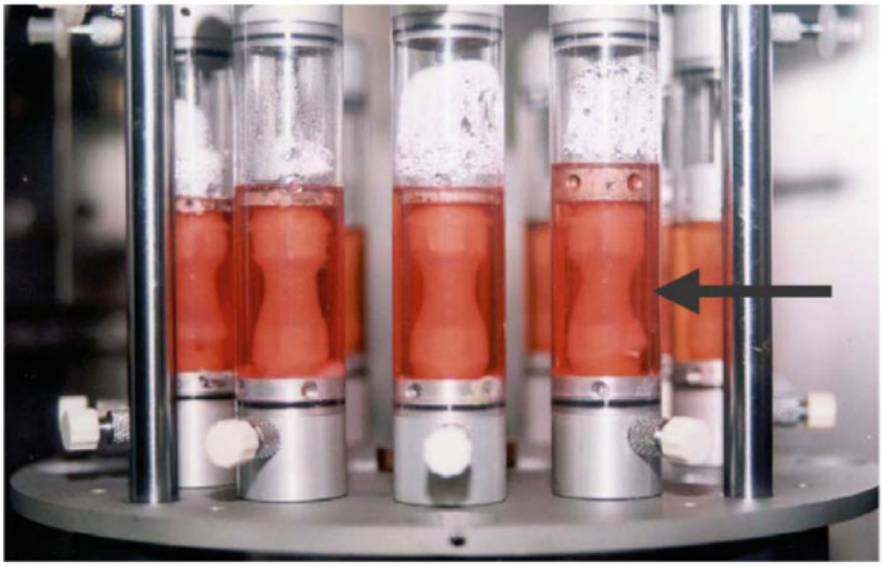
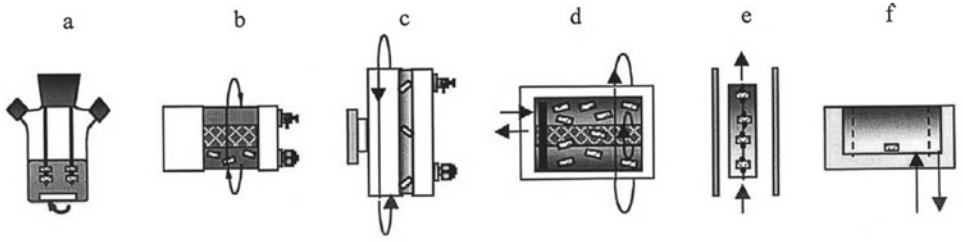


Fig. 10.8 Ligament bioreactor design. This figure shows a bioreactor that is specifically made for anterior cruciate ligament tissue engineering. The *black arrow* indicates a collagen biomaterial that is suspended between two bone blocks in culture medium (*red liquid*). The bioreactor exerts longitudinal and torsional stress. This setup has been shown to stimulate ACL cell growth in culture (from Altman et al. [38], with permission)

healing have become more reliable and more applicable to a human situation. Along with the introduction of a mechanical stimulation into routine tissue engineering methodology, bioreactors systems have been developed. Hailing from the flexible membranes described above, most of the new bioreactors regulate biomechanical simulation as one of the key function, while allowing researchers to control medium flow rates, pH, and oxygen concentration in addition to multiaxial biomechanical stimulation. Vunjak Novakovic et al. developed such bioreactors that allow controlling all the above-mentioned parameters. In a bioreactor specifically designed for ACL tissue engineering (Fig. 10.8), Altman et al. were able to show that over 14 days in culture, biomechanically stimulated cells produced significantly more type I and type III collagen as well as the tenascin-C when compared to unstimulated cells [38].

Another improvement in the use of bioreactors is increased seeding efficiency [39]. Typically, in static seeding, cells are the sets on top of the biomaterial and allowed to crawl into the scaffold over time. However, such static seeding leads to the creation of cell clusters on top off, or in the topmost layer, of a biomaterial and no further penetration of the cells into the scaffold. Using either medium flow or rotation, bioreactors are able to push cells into a biomaterial, thus enhancing seeding efficiency and cellular penetration, resulting in a uniformly seeded biomaterial (Fig. 10.9).



Cultivation vessel	Spinner flask (static or mixed)	Rotating vessel (STLV)	Rotating vessel (HARV)	Rotating wall perfused vessel (RWPV)	Perfused column	Perfused chamber
Operating volume (cm ^{3})	120	110	100	125	15	3, 10 or 30
Engineered tissues (5 mm diameter x 2 mm thick discs)	Fixed in place; up to 12 per vessel	Freely settling; up to 12 per vessel	Freely settling; up to 8 per vessel	Freely settling; up to 10 per vessel	Fixed in place; up to 4 per vessel	Fixed in place; up to 5 per vessel
Operating mode						
(a) medium exchange	Batch-wise (3 cm^{3}per tissue per day)	Batch-wise (3 cm^{3}per tissue per day)	Batch-wise (3 cm^{3}per tissue per day)	Periodic medium recirculation (80 cm^{3}every 6 hrs) and exchange (5-10 cm^{3}per tissue per day)	Continuous medium recirculation (2-3 cm^3/hr) and exchange (2.5-15 cm^{3}per tissue per day)	Continuous medium recirculation (0.1 - 30 cm^3/min) and exchange (1-30 cm^{3}per tissue per day)
(b) gas exchange	Continuous, via surface aeration	Continuous, via an internal membrane	Continuous, via an internal membrane	Continuous, via an external membrane	Continuous, via an external gas exchanger	Continuous, via an external gas exchanger
Mixing mechanism	Static flask: none Mixed flask: magnetic stirring	Construct settling in rotational flow	Construct settling in rotational flow	In microgravity: rotation of inner and outer cylinders at same or different rates; viscous pumping In unit gravity: vessel rotation as a solid body	Recirculating flow	Recirculating flow
Fluid flow	Static flask: none Mixed flask: turbulent	Laminar	Laminar	Laminar	Laminar	Laminar
Mass transfer in bulk medium	Static flask: molecular diffusion Mixed flask: turbulent convection	Convection (due to construct settling)	Convection (due to construct settling)	Convection (due to differential viscous pumping or construct settling)	Convection (due to recirculation)	Convection (due to recirculation)
References	Vunjak-Novakovic et al. (1996, 1998, 1999a) Freed and Vunjak-Novakovic (1997b) Carrier et al. (1999) Bursac et al. (1999)	Freed and Vunjak-Novakovic (1995, 1997a) Freed et al. (1998) Vunjak-Novakovic et al. (1999a) Riesle et al. 91998) Carrier et al. (1999) Fleitzel et al. (1998)	Vunjak-Novakovic et al. (1997a) Carrier et al. (1999) Papadaki et al. (1999)	Freed et al. (1997)	Williams et al. (1998) Obradovic et al. 91997)	Vunjak-Novakovic et al. (1999b) Searby et al. 91998)

Fig. 10.9 *Bioreactor designs.* This figure shows common bioreactor designs together with a summary of their operating conditions: (**a**) static flask with spinner, (**b**) slow-turning lateral vessel (*STLV*), (**c**) high-aspect ratio vessel (*HARV*), (**d**) rotating-wall perfused vessel (*RWPV*), (**e**) perfused column, and (**f**) perfused chamber (from Martin et al. [40]), with permission

Conclusion

In vitro models of wound healing are the first step and basis in research and development of ACL treatments. Numerous models of various levels of complexity exist that can be tailored to the research question at hand. However, great care must be taken in interpreting the results of these studies as even with the most complex in vitro assays, the in vivo situation is not replicated.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 11
In Vivo Models of ACL Injury (Central Defect, Porcine, Ovine, Canine)

Benedikt Lorenz Proffen and Martha M. Murray

Introduction

Anterior cruciate ligament (ACL) injuries are among the most common ligament injuries in the knee, occurring in 400,000 patients a year in the USA alone [1]. A rising number of children and adolescents have been recently reported to sustain this type of injury [2], possibly due to the trend towards certain injury prone activities and a higher intensity during sports for these age groups. Since the ACL plays a crucial role in the stabilization of the knee, especially during turning and twisting motions, the loss of the ACL results in joint instability and secondary damage to the menisci and articular cartilage, leading ultimately to early onset osteoarthritis [3]. Therefore, surgery is often recommended for the treatment of ACL injuries. The current gold standard of surgical treatment is ACL reconstruction, during which the torn ACL is removed and an autologous or allogeneic tendon graft is implanted. This procedure restores knee stability to a certain point, but it is unable to restore the geometry and proprioceptive function of the ACL [4, 5]. It also fails to reestablish the native knee kinematics [6]. Results of systematic long-term follow-up studies of ACL reconstruction imply that this dysfunction may be the reason for joint degeneration in the long term [7]. In addition to the long-term complication of osteoarthritis, ACL reconstruction, unfortunately, also has a relatively high re-rupture rate (as high as 30 %), specifically in young, active patients [8]. In addition, for skeletally immature patients, transphyseal drilling for ACL tunnels is a potential risk factor for growth arrests of the leg – leading to a shorter leg or angular deformities [9]. Thus, new treatment options are of interest.

A rather simple option would be to repair instead of replace the ACL, thus maintaining its complex cellular architecture, insertion sites, and proprioceptive nerve

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_11,

receptors [10]. As mentioned in the previous chapter, our lab has discovered interesting and promising in vitro results. Fibroblasts grown in combination with platelet-rich plasma and collagen in cell culture showed increased proliferation and synthesis of extracellular matrix – just what we would desire in a healing ACL.

However, before human patients can benefit from such data, translational work in large animal models is often necessary to help us as researchers to understand how these basic biologic processes interact in the joint. Often, what is found in vitro is completely different in vivo, and thus, watching and measuring what happens in the living joint is a critical next step in developing a new technology. This is why large animal studies often are part of regulatory requirements and principles of translational medicine [11]. Large animal models provide a uniform, experimental platform for development and evaluation of the effectiveness and safety of novel treatments [12]. Most commonly, sheep, goats, pigs, dogs, and rabbits have been used as models of the human knee to test implants or to discover determinants of disease progression [13–17]. Ideally it is preferable to perform these pre-clinical studies in joints of similar size to the human.

In vivo testing of new ACL treatments in animal models has considerable strengths compared to in in vitro testing. Factors that do not play a role in vitro but are important in vivo include mechanical stress, nutritional supply through the vasculature, and innervation factors, as well as *in situ* cellular responses and overall animal responses, including local and systemic inflammatory factors. Unlike clinical trials in humans, animal studies can be designed to closely examine outcomes with biomechanical testing and histological examination of the healing tissue – studies that cannot be performed in human subjects. Animal studies can also be used to develop noninvasive measures, such as MRI, to predict the biomechanical or histological outcome of studies for use in future human trials.

The weaknesses of in vivo studies in animals include the greater variability between the specimens than in vitro models, the variability in environment and mechanical loading (such as our inability to control post-operative rehabilitation), and the cost of the models (particularly large animal models). Additionally, the majority of animal models walk on four feet rather than two – which makes gait and knee motion slightly different to the human condition [18].

Differences in the Histology (Cell and Vessel Distribution in the Tissue) between Human ACLs and Large Animal Models for ACL Injury (Rabbit, Canine, Porcine, Ovine)

The most common large animal models for the knee joint are the rabbit, dog, pig, sheep, and cow. To choose the most similar model to the human knee joint, the animals should be compared micro- as well as macroscopically. Since the method of ACL repair mostly relies on the enhancement of the wound-healing abilities of the human body, it may be important to select an animal model with similar cellular distributions to that of the human anterior cruciate ligament.

Therefore, we conducted a descriptive histological study comparing the cell and vessel distribution of normal human anterior cruciate ligaments with that of three animal models and anterior cruciate ligaments from osteoarthritic human knees [19, 20]. The histology of each of the anterior cruciate ligament sources was reported in terms of cell number density, expression of alpha-smooth muscle actin, blood vessel density, and cell nuclear morphology using standardized histomorphometric techniques. The normal human anterior cruciate ligament was similar to the canine anterior cruciate ligament and the anterior cruciate ligament from patients with osteoarthritis with respect to cell density, blood vessel density, and cell nuclear shape. The normal anterior cruciate ligament had significantly fewer vessels than the bovine anterior cruciate ligament and rounder cells than the bovine and ovine anterior cruciate ligaments. In summary, there was significant interspecies variation in the histology of the anterior cruciate ligament, with the canine anterior cruciate ligament being the most similar to the human anterior cruciate ligament. The pig ACL was not studied here but has recently become increasingly popular.

Interspecies Differences in Gross Anatomy and Biomechanics of the Knee

Whereas the biological compatibility to the human ACL is important for having similar results on the cellular level and wound healing, long-term results are also heavily influenced by the gross anatomy and biomechanics of the knee joint among the different species (Table 11.1). First of all, no animal model mirrors the human knee anatomy perfectly. Furthermore, all animal models are quadrupeds and therefore do not walk upright, which results in a proportionally lesser body weight bearing per limb as well as an extension deficit of around 30° throughout all large animal models. These differences between animal and human knees led to another study in our lab in which we compared the passive range of motion as well as the anatomy and sizes of the intra-articular structures of the human knee to rabbit, dog, pig, sheep, goat, and cow knees [18].

For this study, the passive range of motion was measured using a goniometer by palpating the femur and the tibia, aligning the arms of the goniometer along these bones, and recording the angles in full extension and full flexion of the hind limbs. The intra-articular structure sizes of the ACL, PCL, notch width, and menisci were measured using calipers and afterwards normalized to the tibial plateau width before comparing them among the species.

Table 11.1 Tensile strength of healthy ACLs in different species

Maximum load of healthy ACL	
Mouse	30 N
Rat	30–32 N
Rabbit	303 N
Dog	1,129 N
Sheep	723–2,280 N
Goat	1,553–1,926 N
Pig	713–1,800 N

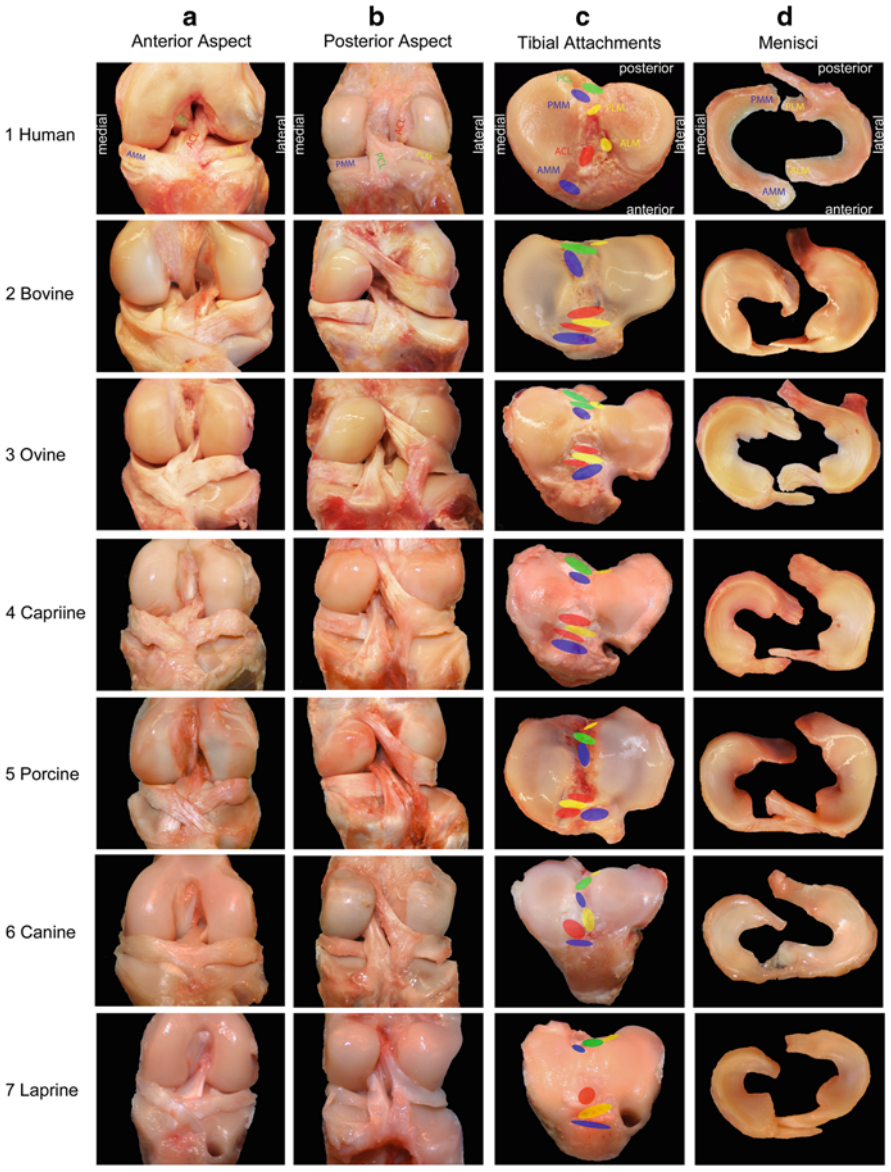

Fig. 11.1 Different aspects of the knees of the seven species. *Column A* shows the anterior aspect of the knees with the medial side being on the left and the lateral side on the right. Noticeable is the small band attaching the intermeniscal ligaments in the canine knee to the tibial plateau (*6A*). The anterior attachment of the lateral meniscus courses between the anteromedial and posterolateral bundles of the ACL in the bovine, ovine, and porcine knees (*2A*, *3A*, *5A*). *Column B* represents

Statistically significant differences in both the range of motion and intra-articular structure sizes normalized by the tibial plateau width were found among all the species. The most important result concerning the passive range of motion was that only the human knee was able to attain full extension. After normalization of the measured structures, the pig ACL was found to be significantly longer than the human counterpart; the sheep and the pig PCL was longer than the human. The human intercondylar notch, where the ACL resides, was significantly wider in all the animals, and the human menisci were narrower than those of the cow and pig [18].

Qualitative anatomical inspection revealed that the tibial insertion site of the ACL was split by the anterior lateral meniscus attachment in the cow, sheep, and pig, but not in the human knee (Fig. 11.1). The sheep PCL had two distinct tibial insertion sites, while all the other knees had only one. Furthermore, only in human knees, both lateral meniscal attachments were located more centrally than the medial meniscal attachments.

In summary, it is therefore important to note that despite the relatively preserved dimensions of the cruciate ligaments, menisci, and intercondylar notch among human and animals, the structural differences in the cruciate ligament attachment sites, morphology of the menisci, and biomechanics of the knee between humans and animals are important to consider when selecting an animal model.

Animal Models for ACL Injury and Treatment

Mouse and Rat Models

The obvious advantages of a rodent model are the well-analyzed genetics, the regular and plentiful availability of age and gender specific animals, and the low price. Despite these positive facts, the size of the knee makes it very challenging to use

Fig. 11.1 (continued) the posterior aspects of the knees. Salient in all knees is behind the PCL passing medial meniscus attachment. In the human knees, the posterior meniscofemoral ligament inserts more inferiorly on the medial femoral condyle (*1B*). Note the posterior thickening of the meniscotibial coronary ligament between the lateral meniscus and tibial plateau in the ovine, canine, caprine, and lapine knees (*3B*, *4B*, *6B*, *7B*). *Column C* shows the different tibial attachments of the knees. Notice the splitting of the ACL insertion by the anterior LM in the bovine, ovine, and porcine knees (*2C*, *3C*, *5C*) and the LM attachments being central to the MM attachments in the human knee (*1C*). *Column D* shows the morphology of the menisci with the medial meniscus on the left, the lateral meniscus on the right, and the anterior horns facing down. In the human knee (*1D*), the posterior horn of the lateral meniscus attaches anteriorly to that of the medial meniscus, a feature not seen in any of the other six species examined (*ACL* anterior cruciate ligament, *ALM* anterior lateral meniscus, *AMM* anterior medial meniscus, *PCL* posterior cruciate ligament, *PLM* posterior lateral meniscus, *PMM* posterior medial meniscus)

human ACL surgical techniques, the biomechanics of gait differ from what we see for human walking, and healing of the ACL is less likely to be dependent on the generation of a vascular supply as the smaller mouse or rat ligament may be able to sustain itself via the diffusion of nutrients.

The rat model has also been used to study the effects of technique on ACL reconstruction results (Table 11.2). The semitendinosus tendon as a free tendon graft ACL reconstruction in a rat model was used by Kadonishi et al. They enhanced the tendon-bone healing of the graft by injecting matrix proteins from developing porcine teeth and thus significantly increased the number of Sharpey's fibers at the tendon-bone interface and the mean load to failure over the nonenhanced control group after 6 weeks of healing [21]. Brophy et al. used the flexor digitorum longus tendon to reconstruct the ACL and subsequently subjected the graft to either cyclic loading or immobilization for 4 weeks after surgery. The biomechanical results showed no difference, but the cyclically loaded group had more signs of inflammation and less bone-tendon healing histologically [22]. A similar study, also using the flexor digitorum longus tendon to compare immediate versus delayed (10 days) postsurgical strain found a significantly stronger graft in the delayed strain group [23].

Hays et al. found that the depletion of macrophages at the tendon-bone interface with Clondronate (which selectively induces macrophage apoptosis) resulted in a greater degree of interface remodeling between tendon and bone, and a significant increase in load to failure and stiffness [24]. The role of macrophages in tendon to bone healing after ACL reconstruction was also highlighted by Dagher et al., who found a decreased number of macrophages at the healing interface of tendon to bone after 4 weeks in specimens that had been immobilized right after surgery, although there were no significant differences in the maximum load or stiffness [25].

The rat model has also been used to study the effects of ACL repair techniques. Kanaya et al. used a partial defect model to demonstrate that injecting mesenchymal stem cells (MSCs) into the defect could enhance healing. While the untreated defects were still empty after 4 weeks, the gap in the defects treated with MSCs was covered with healing tissue and had a higher ultimate failure load [26]. Oe et al. found that the injection of MSCs into the partial defect resulted in restoration of the normal tensile strength compared to the intact ACL (29 N vs. 32 N) after only 4 weeks of healing [27].

Rabbit

The rabbit is the model most commonly used for ACL reconstruction and repair (Table 11.3). Compared to the mouse, the size of the rabbit allows the researcher to see and work with the ACL relatively well. The purchase costs for rabbits are low, and therefore, this model is relatively affordable and allows for greater study sample sizes, which may lead to a more reliable and statistically significant results (assuming that the procedure can be accurately recapitulated in an animal of this size). These features make the rabbit an attractive model for conducting studies investigating new methods for ACL injury. The downside of the rabbit model is that the size

Table 11.2 Biomechanical data for different reconstruction methods in rats

Reconstruction technique			Failure load (N) after weeks (w)					
Graft choice		Fixation (additions)	Intact	0 week	4 weeks	6 weeks	12 weeks	Reference
Semitendinosus	Autograft	Periosteum			8	5	7	Kadonishi et al. *JBJS Br*. 2012 [21]
		Periosteum (EMD)			10	17	9	
Flexor digitorum longus	Autograft	Periosteum			9			Brophy et al. *JBJS*. 2011 [22]
		Periosteum			14			Bedi et al. *JBJS*. 2010 [23]
		Periosteum				14		Hays et al. *JBJS*. 2008 [24]
		Periosteum + immobilization			14			Dagher et al. *CORR*. 2008 [25]
ACL partial defect		Mesenchymal stem cells	100 %	55 %	70 %			Kanaya et al. *Arthroscopy*. 2007 [26]
		Bone marrow concentrate	32		29			Oe et al. *Stem Cells D*. 2011 [27]

Table 11.3 Biomechanical data for different reconstruction methods in rabbits

Reconstruction technique			Failure load (N) after weeks (w)					
Graft choice		Fixation (additions)	Intact	4 weeks	8 weeks	12 weeks	52 weeks	Reference
Semitendinosus tendon	Autograft (single)	Bone bridge			28			Bhatia et al. *AJSM*. 2012 [33]
		Anchor screw		49	34			Hashimoto et al. *JOR*. 2011 [38]
		Anchor screw (BMP2)		72	66			
		Endobutton			20			Tischer et al. *TissEng A*. 2010 [34]
		Endobutton (triton X + dermal fibroblasts)			61			
		Periosteum				72		Yeh et al. *JOR*. 2007 [114]
		Periosteum (HBO)				97		
	(Quadrupled)	Bone bridge (unbraided)	303				146	Xu et al. *KSSTAA*. 2010 [31]
		Bone bridge (braided)					106	
	Frozen allograft	Bone bridge (irradiated)			31			Bhatia et al. *AJSM*. 2012 [33]
Long digital extensor tendon	Autograft	Periosteum			43	38		Pan et al. *Int Orth*. 2009 [40]
		Periosteum (RBX)			65	57		
		Periosteum (CPC-BMP)			79	53		Pan et al. *JOR*. 2011 [41]
		Periosteum (CPC-BMP)			60	51		
Achilles tendon	Frozen allograft irradiated	Periosteum (MSC)		16		30	91	Wei et al. *BBRC*. 2011 [37]
		Periosteum (MSC-TGFβ1)		23		43	123	
		Periosteum (MSC-VEGF)		17		31	95	
	Frozen allograft	Periosteum (MSC-TGFβ/VEGF)		17		32	135	
		Periosteum (MSC)		26	38			Soon et al. *AJSM*. 2007 [35]
		Periosteum		19	24			
Other	Silk scaffold	Anchor screw	132		26	9	7	Fan et al. *Biomaterials*. 2008 [42]
		Anchor screw (MSC)			46	25	25	

BMP bone morphogenetic protein, *MSC* mesenchymal stem cell, *HBO* hyperbaric oxygen, *RBX* recombined bone xenograft, *ICPC* injectable calcium phosphate cement

of the ACL is still far smaller than the human ACL, and the activity of a few hops intermittently as opposed to a more sustained gait make the mechanical environment somewhat different to the human condition. Nevertheless, the rabbit is used as an in vivo model for ACL reconstruction.

The rabbit model has been used to study the effects of ACL reconstruction on immature patients with open growth plates. The problem of harming the growth plate in skeletally immature patients by drilling a bone tunnel through the open growth plate during ACL reconstruction was investigated in a rabbit model by Babb et al. [28]. They used radiographs to compare limb length and tibial and femoral angles of rabbits treated with a semitendinosus tendon autograft with or without the addition of mesenchymal stem cells from the bone marrow to the healthy contralateral side. After 20 weeks, the no-cell group had tibial and femoral length discrepancies of −8 and −6 mm and angular differences of −12° and −10°, while the stem cell group had significantly smaller changes of length (−1 and +1.5 mm) and angles (0° and 4°).

Lui et al. [29] analyzed the tendon-bone integration at the femoral and tibial side at 6 and 12 weeks after ACL reconstruction with the semitendinosus tendon. Using micro-CT and histology, they found higher bone density, smaller tunnel diameters, and better graft-bone integration on the femoral side.

Nikolaou et al. used semitendinosus tendon grafts to determine the effect of a single- versus double-bundle technique in rabbits. They found significantly less anterior tibial shift in the double-bundle implants after 3 months than in the single-bundle knees [30].

Four-stranded semitendinosus grafts were implanted either braided or unbraided by Xu et al. and analyzed histologically and biomechanically after 52 weeks. Braided grafts showed 67 % of the strength of the normal ACL after 52 weeks, whereas the unbraided grafts only reached 36 % [31].

Xie et al. [32] reconstructed the ACL using a semitendinosus tendon autograft and compared remnant dissected, remnant preserved, and sham operated outcomes, and found that preserving the ACL remnant significantly increased collagen1-alpha1, collagen3-alpha1, and transforming growth factor beta1 (TGF-β1) (suggesting greater collagen production in the graft) at 6 weeks and vascular endothelial growth factor gene expression at 12 weeks after surgery.

In addition, the processing of allo- and autografts and the subsequent effect on graft performance has also been studied in rabbits. Bhatia et al. [33] used 48 New Zealand white rabbits to investigate the use of low-dose irradiated (1.2 MRad) semitendinosus allografts in comparison to nonirradiated allografts and autografts. While they did not find any significant statistical differences in the maximum load after 8 weeks, the histology showed a higher cellularity in the autografts compared to the allografts. Tischer et al. found that decellularizing and repopulating autografts with autologous dermal cells resulted in grafts containing greater numbers of inflammatory cells during histological analysis and were significantly weaker (19.7 ± 20.3 N) than untreated autografts (61.2 ± 31.2 N) [34].

The effects of adding stem cells, genetically modified cells, and growth factors to ACL grafts have been studied in the rabbit model as well. The addition of MSCs to fresh-frozen Achilles tendon allografts was found to result in more organization

of the tendon-bone interface and the ultimate load to failure was 56 % higher (37 N vs. 24 N) [35]. Li et al. [36] found that transduction of the MSCs with a gene for PDGF resulted in hypervascularization of the allograft tissue when implanted, but a more normal cell density by 12 weeks. Wei et al. [37] found that tranducing the MSCs with TGF-β1 increased the tensile strength of the graft at 6 and 12 weeks, while transfection with both TGB-β1 and VEGF resulted in the highest ultimate failure load among all groups.

Hashimoto et al. [38] demonstrated that the injection of bone morphogenetic protein 2 (BMP 2) into tendons 6 weeks prior to harvest resulted in a bone plug at the end of the tendon. This tendon was then used for an autograft ACL reconstruction. After 8 weeks, CT scanning and histology revealed a superior integration of the BMP 2-treated graft within the tunnel and a superior tensile strength of the entire graft (66.1 ± 17.5 N and 34.1 ± 10.4 N).

The rabbit model has commonly been used to study the integration of soft tissue grafts to the bone tunnel. Kanazawa et al. [39] found the tendon-to-bone healing process very similar to endochondral ossification. In the early phase, type-III collagen was formed in the outer layer of the graft and then matured into Sharpey-like fibers. Pan et al. [40] found that pretreating the bone tunnels with cancellous bone and BMP resulted in better bone-tendon integration and higher graft maximum loads (56.84 ± 16.81 N compared to 38.29 ± 11.97 N) after using the long digital extensor tendon as a soft tissue graft. They also reported similar findings for pretreatment of the tunnel with a mixture of calcium phosphate cement and BMP [41]. Yeh et al. demonstrated that hyperbaric oxygen treatment could also be used to improve the tendon-bone integration after 18 weeks, as seen by more fibrocartilage formation in the tendon-bone interface, more neovascularization, and denser, bigger and more organized collagen fibers in the electron microscope evaluation. This also resulted in a higher pullout strength for the hyperbaric oxygen (HBO)-treated specimens (96 N vs. 72 N).

The rabbit model has also been used to study synthetic grafts. Fan et al. demonstrated that use of a silk scaffold seeded with MSCs resulted in a regenerated ligament with fibroblast-like cells producing extracellular matrix components type I collagen, type III collagen, and tenascin-C and a tendon-bone interface with the typical four zones (bone, mineralized fibrocartilage, fibrocartilage, ligament) after 24 weeks. The tensile strength of the scaffold with MSC yielded only 18.7 % of the native ACL after 24 weeks but was still significantly higher than the no-cell control group (5.1 %) [42].

The Canine Model

The anterior cruciate ligament in dogs is more accurately referred to as the cranial cruciate ligament. Cranial cruciate ligament insufficiency is one of the most common causes of lameness in dogs. Since the rupture of this ligament and the subsequent treatment of it in dogs itself has a huge economic impact – experts estimate the costs of treating canine cruciate ligament injuries in the USA at over one billion

dollars [43] – research using the canine knee for developing techniques of the ACL rupture is not typically aimed to test techniques for the human patient, but to develop and test new techniques for this companion animal.

Cruciate ligament insufficiency in the dog will lead to meniscal injuries and osteoarthritis, comparable to the human patient [44]. Therefore, a treatment of the ACL rupture which can minimize this disease progression would be of great interest. There are various treatment options currently available to treat cruciate ligament insufficiency in canines, including reconstruction of the cranial cruciate ligament with autogenous tissue [45], allografts [46], or synthetic materials [47]. However, most of the techniques have not been found to be effective in maintaining stability of the canine knee or minimizing osteoarthritis [48]. As a result, other techniques have also been successfully introduced to treat the cruciate-deficient canine stifle joint. These techniques include tibial plateau leveling osteotomy [49], tibial tubercle advancement techniques, and extracapsular tethering techniques. The osteotomies are proposed to level the canine tibial plateau; however, the human tibial plateau is typically level already, so these types of procedures are not intrinsically translational. Extracapsular tethering has been tried in humans and not found to be particularly effective either (see Chap. 2). Thus, use of the canine knee as a model for the anterior cruciate ligament injury in the human knee may add additional difficulties not present in the human knee and may be a less useful translational model as a result.

However, the canine model does share the problems of intra-articular healing with the human model. One initial study demonstrated that cutting the central 40 % of the ACL fibers using a special blade resulted in a persistent defect and loss of mechanical strength of the ligament [50]. This suggested this model might be useful in studying the stimulation of healing, as little functional healing occurs spontaneously in this model.

The canine model has been used to study techniques of ACL reconstruction (Table 11.4). Tomita et al. found that the use of a bone-patellar tendon-bone graft resulted in a stronger graft than a soft tissue graft at 3 weeks after grafting, but that the difference disappeared by 6 weeks. For both graft types, failure was at the graft-tunnel interface at 3 weeks, and at the bone plug-tendon interface of the B-PT-B graft, but midsubstance for the soft tissue graft (double flexor tendon – FT) [51]. Qi et al. found that Achilles tendon grafts implanted 20 mm in the tibial bone tunnel had a higher load after 6 weeks than grafts with only 5 mm implant depth [52]. Goertzen et al. [53] found that irradiation (2.5 MRad) resulted in no difference in the ultimate failure load of the grafts after 12 months, although the irradiated grafts were slightly hypervascular compared to the nonirradiated grafts.

The canine model has also been used to study biologic augmentation of an ACL graft. Huangfu et al. showed that injection of tricalcium phosphate into the bone tunnel before placing a soft tissue graft resulted in Sharpey's fibers, fibrocartilage, and calcified cartilage appearing earlier at the tendon-bone interface and increased the pullout strength of the graft [14]. Yamazaki et al. demonstrated similar findings with the injection of TGF-β1, with enhanced formation of Sharpey-like fibers and improved pullout strength (188 N vs. 99 N) [54]. The processes of vascularization and reinnervation after ACL reconstruction have also been characterized for the canine model. Tanaka reported the growth of blood vessels into a patellar tendon

Table 11.4 Biomechanical data for different reconstruction methods in the dog model

Reconstruction technique			Failure load (N) after months (m)						
Graft choice		Fixation	Intact	Graft	1.5 months	3 months	6 months	12 months	Reference
Achilles tendon	Autograft	Endobutton (T+F)				103			Qi et al. *Arthroscopy*. 2011 [52]
Patellar tendon (PT)	Autograft (tendon split)	Anchor screw			241	259			Tomita et al. *Arthroscopy*. 2001 [51]
Flexor digitorum longus tendon	Autograft (single)	Endobutton		450	47				Huangfu et al. *Arthroscopy*. 2007 [14]
		Endobutton+TCP			75				
		Endobutton			99				Yamazaki et al. *Arthroscopy*. 2005 [54]
		Endobutton+TGF-beta			182				
	Autograft (double)	Anchor screw				328			Tomita et al. *Arthroscopy*. 2001 [51]
ACL	Allograft	Interference screw (T+F)	1,129			387	557	780	Goertzen et al. *Arthroscopy*. 1994 [53]
	Allograft irradiated					582	536	718	

F femur, *T* tibia, *TCP* tricalcium phosphate, *TGF* transforming growth factor

autograft 2 weeks after implantation [55]. Free nerve endings and mechanoreceptors were found near the surface of the allograft and at the bony attachments in bone-ACL-bone allografts 12 months after implantation in a study by Goertzen et al. [56]. A final study demonstrated that the use of a collagen-platelet composite in a central defect in the canine ACL resulted in better fill of the defect and improved biomechanical properties of the ligament at 6 weeks after treatment [57].

The Ovine Model (Sheep)

Sheep and goats both are ruminants and popular animal models for research on the ACL (Tables 11.5 and 11.6). Both animals have a relatively low activity level, and among the described animal models, they also have the straightest resting position of the hind limbs. However, for studies which involve the preparation of platelet-rich plasma (PRP), the sheep model can be more difficult, as the standard techniques for PRP preparation do not work in the sheep due to the similarity in size of the red blood cells and platelets. For the sheep model, biomechanics, molecular aspects [58], vascular [59], and neural anatomy [60, 61], as well as proprioception [62] of the healthy ACL are well described. Most of these studies were conducted in mature sheep.

The sheep has been used primarily as a model to study ACL reconstruction techniques (see Table 11.5). In most sheep studies, the superficial flexor digitorum tendon is the graft of choice. This graft is easy to obtain and has sufficient length to perform as an analogous graft to the hamstring in humans. Studies using this graft evaluated and compared fixation techniques with Endobutton, absorbable interference screws [63], intercondylar screws [64], cross-pin fixation [65], and bone plugs [66]. Less frequently, other free tendon grafts like the Achilles tendon [65], fascia lata [67], or the long digital extensor tendon [68] have also been studied. Various tendon grafts have been used to compare allografts (tendons used from a donor sheep) and autografts (tendons from the same animal) [69].

For fixation techniques of tendon grafts, the sheep has been used to evaluate Endobutton, cross-pin fixation, and interference screw fixation. Hunt et al. found no difference in graft performance after fixation with interference screws or Endobutton after 24 weeks [63]. Zantop et al. found an initial 25 % greater tensile strength when the graft was fixed using cross-pin fixation (338 N vs. 228 N), and after 6 weeks of implantation, the cross-pin fixed graft strength was still higher (172 N vs. 42 N).

The sheep model has been used to test the strength of various graft types. At time of implantation ("Time 0"), the superficial digital flexor tendon, split Achilles tendon, and double lateral digital extensor tendons have the highest tensile strength, with failure loads of over 1,000 N [63, 64, 70], comparable to the intact sheep ACL tensile strength which has been reported to range from 720 N [64] to 1,800 N [66] depending on sheep breed and size.

Over time, the strength of all implanted ACL grafts decreases, and only one publication states that the strength of the long digital extensor tendon graft approached the time-zero value 1 year after implantation [68] (see Table 11.5). For the majority

Table 11.5 Biomechanical data for different reconstruction/repair methods in the sheep model

Reconstruction technique			Failure load (N) after months (m)						
Graft choice		Fixation	Intact	Graft	0 month	3 months	6 months	12 months	Reference
Superior digital flexor tendon (SDF)	Autograft	Endobutton (F) & sutured bone bridge (T)	1,612			375			Schmidt et al. *Cell Tissue Bank.* |2012 [72]
		Endobutton (F) & sutured bone bridge (T)	1,671			392		632	Scheffler et al. *Arthroscopy*. 2008 [69]
		Interference screw (F & T)	1,513	1,120		237	314	685	Hunt et al. *Arch Orthop*. 2005 [63]
		Endobutton (F) & sutured bone bridge (T)				302	466		
		Microporous β-TCP (F & T)	1,764		256	233	808		Mayr et al. *Arthroscopy*. 2009 [66]
		Endobutton(F), washer (T)	759			316	523		Meller et al. *Arthroscopy*. 2008 [73]
	Frozen allograft	Endobutton (F) & sutured bone bridge (T)				281		307	Scheffler et al. *Arthroscopy*. 2008 [69]
	E-beam allograft	Endobutton (F) & sutured bone bridge (T)	1,612			291			Schmidt et al. *Cell Tissue Bank.* 2012 [72]
						63			
Semitendinosus tendon	Autograft (double)	Bone screw (T) & Endobutton (F)	1,500			301			Kondo et al. *AJSM*. 2012 [74]
		Bone screw (T) & Endobutton (F) + synovium cells/TGF-β				572			
	Autograft (quadr.)	Interference screw	1,249		200	200			Roy et al. *Arthroscopy*. 2010 [75]
		Interference screw + autograft bone			350	190			

Achilles tendon	Autograft (tendon split)	Cross-pin fixation (F & T)			337	172			Zantop et al. *Arthroscopy*. 2007 [65]
		Interference screw (F & T)			228	42			
		Interference screw (F & T)	1,513	1,120	267	237	314	685	Weiler et al. *Arthroscopy*. 2002 [70]
Fascia lata	Autograft + PLLA	Richards fixation staples (F & T)	1,425			124	296	295	Laitinen et al. *Arch Orthop*. 1993 [67]
Double lateral digital extensor (DLET)	Autograft	Endobutton (F) & interference (T)				580	1,100		Shaw et al. *ORS*. 2010 [68]
		Transcondylar screw (F) & interference (T)	723	1,140	1,033	188	584		Milano et al. *Arthroscopy*. 2005 [64]
	Allograft	Endobutton (F) & interference screw(T)				400	600		Shaw et al. *ORS*. 2010 [68]
Patellar tendon (PT)	Autograft (tendon split)	Set screw (F) & interference screw (T)	723	830	608	185	603		Milano et al. *Arthroscopy*. 2005 [64]
		Microporous β-TCP (F & T)	1,764		198	599	714		Mayr et al. *Arthroscopy*. 2009 [66]
		Interference (F & T) & PDS augmentation	1,516					901	Holzmueller et al. *Unfallch*. 1992 [115]
ACL Repair	Frozen allograft	Interference screw & Endobutton (F & T)	1,398	1,398	263	267		360	Jaskulka et al. *Unfallch*. 1997 [71]
		No augmentation	2,280			0			Richter et al. *J Mater Sci*. 1997 [72]
		Absorbable suture				237			
		Nonabsorbable suture				253			
		Absorbable suture + Endobutton + CPC	1,600			121	343		Proffen et al. 2012 [116]

F femur, *T* tibia, *b-TCP* beta- tricalcium phosphate, *PLLA* polylactic acid, *PDS* polydioxanone suture, *CPC* extracellular matrix-platelet composite

Table 11.6 Biomechanical data for different reconstruction and repair methods in the goat model

Reconstruction technique			Failure load (N) after months (m)					
Graft choice		Fixation	Intact	0 month	3 months	6 months	12 months	Reference
Achilles tendon	Autograft (tendon split)	Interference screw (F & T) + collagen hydrogel	1,608		107			Spindler et al. *JOR*. 2009 [17]
		Interference screw (F & T) + collagen-platelet composite	1,926		139			
		Endobutton (F) & anchor screw (T)	462		180			Zantop et al. *AJSM*. 2008 [65]
Patellar tendon (PT)	Autograft (tendon split)	Bone pegs		98	346	512	542	Buma et al. *Int Orthop*. 2004 [81]
		Bone pegs + PDS augmentation		441	421	482	755	
		Staple	1,553		178			Abramowitch et al. *JOR*. 2003 [83]
		Interference screw			847			Cummings et al. *JOR*. 2002 [84]
	Allograft	Press fit		215				Musahl et al. *Knee*. 2003 [86]
		Interference screw		328				

F femur, *T* tibia, *PDS* polydioxanone suture

of studies, the grafts drop to 25–50 % of their original strength (15–30 % of intact ACL strength) at 3 months after implantation, then increase in strength over the next 9 months. Grafts typically achieve 50 % of the implantation strength at 1 year (33 % of the intact ACL strength; see Table 11.5). In comparing graft types, Milano et al. found that while the use of a double lateral digital extensor tendon graft had a higher implantation strength than a patellar tendon graft (1,140 N vs. 830 N), the differences at 3 months and 6 months after implantation were negligible (see Table 11.5) [64]. Mayr et al. compared the flexor digitorum superficialis tendon graft to the patellar tendon graft after press-fit fixation in the bone-tunnel with a microporous beta-tricalcium phosphate cylinder. While initially there was no difference between the two treatment groups in ultimate load to failure (256 vs. 198), the patellar tendon grafts had a significantly higher strength after 3 months (599 N vs. 233 N), but at 6 months, the free tendon grafts were equally strong (808 N vs. 714 N).

The sheep has been used to study the effects of choosing allograft or autograft. Scheffler et al. reported that autografts had a mean strength 25 % greater than the allografts at 3 months after implantation and 100 % higher at 12 months (632 N compared to 307 N) [69]. Shaw et al. reported similar findings where the autograft was 33 % stronger at 3 months and almost twice as strong at 6 months after implantation [68]. Jaskulka et al. implanted bone-tendon-bone patellar tendon allografts which were untreated and freshly frozen. The allografts reached a maximal strength of around 300 N after 12 months [71]. Schmidt et al. found the use of e-beam irradiation for sterilization resulted in a substantial loss of maximum load to failure compared to nonirradiated tissues [72].

The sheep has also been used as a model for testing ACL reconstruction in skeletally immature animals. Meller et al. used immature sheep to examine the healing of a transphyseal reconstruction of the ACL with an ipsilateral flexor digitorum tendon [73] across open physes. Histologically, a transient hypertrophy of the physeal tissue at the passing site of the graft was visible.

The sheep has also been used as a model for testing biologic enhancements of ACL grafts. Kondo et al. showed that application of synovial-derived cells grown in TGF-β-enriched media resulted in an increase in the maximum load to failure of the implanted graft after 12 weeks of healing (572 vs. 216 vs. 301 N) [74]. Roy et al. augmented their quadruple semitendinosus autograft with an autologous bone augmentation but did not find any benefit in this technique [75].

The sheep model has also been used to study the effects of primary suture repair. Primary suture repair of the torn ACL in the sheep model produces a decrease in the mechanical properties when compared to ACL reconstruction. Richter et al. showed that repair of a proximal ACL transection resulted in strength of only 230 N after 13 weeks in vivo [76]. Similar results were seen with suture repair using an extracellular matrix-platelet composite [77] where the maximum load at six 6 months averaged 343 ±157 N. While these results were still lower than the contralateral healthy intact ACL (1,600 N) and most of the results reported in the literature for several ACL reconstruction procedures in sheep, they were comparable to the use of a free Achilles tendon autograft reconstruction in a study of Weiler et al. [70] as well as the use of a superior digital flexor tendon allograft in a study of Scheffler et al. [69]. Noteworthy, the bio-enhanced ACL repairs significantly increased their strength over

the 6-month period, while all the auto- and allografts did not show any improvement from the time-zero value to the 6-month data (see Table 11.5).

The Caprine Model (Goat)

The goat is another very popular large animal model for research in ACL reconstruction or repair (see Table 11.6). The specific anatomy of the knee [18], biomechanics of the knee during motion [78] as well as the different ACL bundles [79] have been described. However, for studies which involve the preparation of PRP, the goat model can be more difficult, as the standard techniques for PRP preparation do not work in the goat due to the similarity in size of the red blood cells and platelets.

The goat has been used primarily as a model to study ACL reconstruction techniques with various tendon grafts. Zantop et al. found no difference in pullout strength for 15 versus 25 mm of free tendon graft in the tunnel after 6 and 12 weeks [80]. Buma et al. found no benefit of augmenting a bone-tendon-bone graft with a nonabsorbable suture at 12 months (542 N vs. 755 N) [81]. Cummings et al. compared patellar tendon autografts with initially different widths (4 and 7 mm) but did not find significant mechanical differences after 6 weeks [82].

The goat has also been used to evaluate different initial fixation techniques and tensioning of the ACL graft. Abramowitch et al. found no effect of tensioning the graft during implantation at 6 weeks after surgery [83]. Cummings found similar results after only 2 weeks [84]. Fleming et al. used cadaver goat knees to find the optimal initial tension and tensioning position during the graft fixation for the lowest laxity and found 60 N at 30° extension as superior [85]. Musahl et al. demonstrated that interference screw fixation had a higher pullout strength than bone plugs placed using a press-fit technique [86].

The goat has also been used as a model for testing biologic enhancements of ACL grafts. Mutsusaki et al. [87] found that soaking the graft in calcium phosphate before implantation led to a smaller gap between the tendon and the bone and less tibial tunnel enlargement.

The goat model has also been used to study biological/synthetical grafts. Collagen scaffold grafts were implanted into goat knees as a tissue-engineering approach to ACL reconstruction in several studies and showed good cell ingrowth, vascularization, and collagen fiber organization after 3–6 months [88, 89]. An earlier experiment with cross-linked collagen fibers as ACL substitutes in goats failed to provide enough stability after 6 weeks [90].

The goat model has also been used to study the effects of primary suture repair. Fisher et al. investigated the use of genetically altered pig extracellular matrix wrapped around a suture-repaired goat ACL, which resulted in higher stiffness and volume of the repair after 12 weeks in comparison to the ACL where only sutures were used [91].

Our lab used the goat model for two studies using the patellar tendon graft for reconstruction of the ACL. In the first study, we hypothesized that use of an extracellular matrix platelet-rich plasma scaffold would decrease postoperative laxity as well as improve the biomechanical properties of the graft. After 6 weeks, AP laxity

was significantly lower in the group treated with an extracellular matrix-blood composite, whereas no other biomechanical property varied significantly between these two groups. There was also a positive correlation between serum platelet concentration and AP laxity ($R^2=0.643$; $p=0.009$), maximum load ($R^2=0.691$; $p=0.006$), and graft stiffness ($R^2=0.840$; $p<0.001$) [17].

The second study investigated the predictability of graft structural properties as well as AP laxity by use of magnetic resonance imaging. In that study, a significant relationship was found between the failure load and the graft volume as well as graft volume and the linear stiffness of the graft after 6 weeks of healing. Anterior-posterior laxity correlated with the graft volume after normalization with the T2 relaxation time. This study showed that it may be possible to predict structural properties of the graft by using a noninvasive measure such as magnetic resonance image analysis [92].

The Porcine Model

The pig has become our model of choice for ACL reconstruction and repair. The porcine model has not been widely used due to the difficulty in controlling joint motion postoperatively. This has not been found to adversely affect the treatment of suture repair evaluated in our preliminary studies. The biomechanical similarities of human knees and pig knees [94], as well as the similarities in the hematologic systems [93] have led us to continue with use of this animal model (Table 11.7). The anatomy [18], biomechanics [94], and hematologic [93] characteristics make it more analogous to humans in comparison with other animal models. Unlike the goat model, the porcine model may be better to study articular cartilage health following ACL injury and its treatment as pigs are not susceptible to the Caprine Encephalitis Arthritis Virus, which is common in goats and can cause arthritic changes in the joint.

Two different porcine breeds are commonly used. One is the Yorkshire, the common farm pig, which is a readily available but fast-growing animal, and thus more suited for studies of young animals and studies which only last a few months. The second breed is the Yucatan mini-pig, which is a smaller breed, reaching only 50–70 kg with a proper diet. This breed is more suitable for studies of skeletally mature animals or for long-term studies where the steady weight is preferable to the rapid weight gain of the Yorkshires. Unfortunately, these animals are also much more expensive and often need to be raised for the desired experiment, which can add years on to the length of a study.

The pig model has been used to study ACL reconstruction technique, particular for results at time zero. Debandi et al. compared the *in situ* forces of different bundle reconstruction techniques and saw the best imitation of healthy ACL *in situ* forces to be reestablished by an anatomic three-tunnel double-bundle reconstruction [95]. The flexor digitorum profundus tendon was used by Meuffels et al. to compare a double versus a single tibial tunnel reconstruction and found no biomechanical differences between the two techniques [96].

The pig model has been used to study fixation devices for ACL reconstruction, again, primarily at time zero. Bohn et al. used porcine knees to prove that the

Table 11.7 Biomechanical data for different reconstruction and repair methods in the pig model

Reconstruction technique			Failure load (N) after months (m)					
Graft choice		Fixation	Intact	Graft	0 month	3 months	6 months	Reference
Semitendinosus	Autograft	Bioscrew			536			Adam et al. *AJSM*. 2004 [105]
Patellar tendon (PT)	Autograft (tendon split)	Metal screw + pin	1,091	1,140	1,021		1,140	Milano et al. *Knee Surg Sports Traum*. 2007 [118]
		Bio-absorbable screw			658			Adam et al. *AJSM*. 2004 [105]
	Allograft	Interference screw	948			248		Vavken et al. *Arthroscopy*. 2012 [111]
		Interference screw	1,100			310		Fleming et al. *AJSM*. 2009 [107]
		Interference screw + PRP5x				484		
Long digital extensor	Autograft	Endobutton			969			Bohn et al. *Scand J Med*. 2011 [97]
		Interference screw			708			
Flexor digitorum profundus	Autograft	Interference screw			440			Meuffels et al. *Arthroscopy*. 2010 [96]
Other	Silk scaffold	Endobutton	800					Fan et al. *Arthroscopy*. 2008 [42]
		Endobutton + MSC						
Repair	Primary	Suture				322		Fleming et al. *JOR*. 2010 [108]
		Suture + collagen-based scaffold				367		
		Suture	713		30	80		Joshi et al. *AJSM*. 2009 [110]
	Bio-enhanced	Endobutton, PRP 1x	948			210		Vavken et al. *Arthroscopy*. 2012 [111]
		Endobutton, PRP 3x				362		Mastrangelo et al. *JOR*. 2011 [113]
		Endobutton, PRP 5x				291		
		Suture, PRP 5x				60		Murray et al. *Arthroscopy*. 2010 [117]
		Endobutton, PRP 5x				267		
		Endobutton, PRP 1x	713		47	126		Joshi et al. *AJSM*. 2009 [110]

PRP platelet-rich plasma, *1x* platelets in the PRP are equal to that of the systemic blood, *3x* platelets in the PRP are three times that of the systemic blood, *5x* platelets in the PRP are five times as high as that in the systemic blood, *MSC* mesenchymal stem cell

femoral fixation of a long digital extensor tendon by Endobutton was superior to an interference screw [97]. The pig model was also used for testing several fixation methods of free tendon autografts during in vitro testing [98–100] with Kousa et al. conducting the most comprehensive study comparing six different devices for the fixation of a hamstring tendon graft [101, 102]. The patellar tendon graft as a bone-tendon-bone graft was also investigated in the pig model. Milano et al. compared different femoral fixation techniques (absorbable screw, metal screw, absorbable pin, combination pin, and metal screw) and found the combination of pin and metal screw superior to the other fixation techniques [103]. Dargel et al. showed that the technique of tibial bone tunnel dilation compared to tunnel drilling improved the initial graft strength of a press-fit implanted patellar tendon graft [104]. Adam et al. conducted a comparative study and determined that a hamstring graft fixed with a bioscrew has a significantly lower initial strength than a patellar tendon graft fixed with a bio-absorbable screw [105].

The pig has also been used to test fixation techniques for primary repair. For initially stabilizing the knee, different suture positions were compared in an *ex vivo* study. Anterior-posterior laxity of the porcine knee at 60° of flexion was evaluated for five suture repair techniques. Femoral fixation for all repair techniques utilized a suture anchor. Primary repair was performed to either the tibial stump, one of three bony locations in the ACL footprint, or a hybrid bony fixation. It was found that placement of a suture stent across the knee with the suture in the middle third of the tibial ACL stump was able to restore the normal laxity of the knee after an ACL transection, whereas suture repair to the ligament stump did not [106]. This study was followed up with an in vivo study where the suture stent connecting the two bones was compared with a suture going from the femoral bone to the ligament stump, and it was found that the bone-to-bone suture stent (which restored normal anterior-posterior laxity at time of surgery) resulted in significantly improved strength of the healing ACL after 15 weeks of healing [16].

The pig model has also been used to study the effects of biologically based treatment of ACL injuries. Fan et al. implanted a silk scaffold in combination with bone-marrow-derived mesenchymal stem cells into the ACL-deficient porcine knee. They were able to measure a tensile strength of 52 % of the native ACL after 24 weeks of healing [42], a result comparable to porcine autograft (see Table 11.7). The pig model has also been used to evaluate whether the use of an extracellular matrix-platelet composite added to a patellar tendon allograft ACL reconstruction would enhance the biomechanical outcome after 15 weeks. Anterior-posterior laxity as well as maximum load of the reconstruction was significantly improved when using an extracellular matrix-platelet composite [107].

In addition, the first study introducing the bio-enhanced ACL repair method into the pig model was conducted in 2007. Five Yorkshire pigs underwent bilateral ACL transections and subsequently a primary suture repair and a primary suture repair augmented with a platelet-collagen hydrogel. The addition of the platelet-collagen hydrogel proved to enhance the laxity as well as the tensile strength of the repaired ACL after 3 months [77]. In a subsequent study, the primary suture repair was performed with the collagen scaffold alone; no additional blood components were added. After 15 weeks of healing, there was no significant difference between the suture

alone and the suture with the collagen scaffold, leading to the conclusion that the addition of platelets or blood was necessary to enhance the healing of the repaired ACL [108]. Interestingly, a second study demonstrated that ACL repair is not enhanced by the addition of platelet-rich plasma alone either [109], suggesting that both the extracellular matrix carrier and the platelets may be necessary for a successful repair.

We also were able to show in a pig model that the introduction of an extracellular matrix-platelet composite (bio-enhanced ACL repair with a collagen-based scaffold soaked with platelet-rich plasma) to a primary suture repair enhanced ACL healing. In the first study, biologically enhanced repair increased cell density, yield load, and linear stiffness compared to the primary suture group [110]. In a subsequent study, we compared our bio-enhanced ACL repair technique with the commonly used ACL reconstruction with a patellar tendon allograft and untreated ACL transection. After 15 weeks, there was no significant difference in AP laxity or tensile strength between bio-enhanced repair or traditional reconstruction [111].

In another study, young, adolescent, and adult pigs underwent bio-enhanced ACL repair, and we found a higher cellular density during ACL repair at 1, 2, and 4 weeks than in adolescent or adult pig ACL repairs [112].

Finally, a study compared the bio-enhanced ACL repair using either three times or five times the plasma concentration of platelets. After 13 weeks, the reduced platelet concentration group neither showed inferiority in anterior-posterior laxity nor in tensile strength, concluding that in the pig model, lowering the platelet concentration did not harm the outcome of the repair [113].

Summary

Large animal models are a very valuable tool for developing and testing new surgical procedures. Choosing an appropriate large animal model for a certain surgical procedure or development of a new treatment, such as bio-enhanced ACL repair, is essential for the correct interpretation of the outcome and eventual translation into the human model. There are several large animal models commonly used for ACL research; however, comparative gross anatomy, hematology, cartilage integrity, and biomechanics can make certain models preferable. For in vivo research on the ACL repair and reconstruction where blood cells are used to enhance healing, our lab primarily used pigs, which are anatomically, histologically, hematologically, and biomechanically very similar to the human knee. Results for the bio-enhanced ACL repair in these pigs were promising and had biomechanical outcomes similar to the ACL-reconstruction method at 3 months of healing. Comparable results were found in sheep and dogs, which supports a successful implementation of the newly developed method of ACL repair into human medicine.

Acknowledgements Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 12
Tissue Engineering of Ligaments and Tendons

Patrick Vavken and Martha M. Murray

In the past, much of orthopedic surgery has been characterized by surgical repair of tissues that heal effectively and resection and replacement of tissues that do not. Replacement has been with metal, ceramic, or plastic (total joint replacement) or with grafts (ACL reconstruction, meniscal transplant). With the discovery of the cellular and molecular events preventing the tissues within joints to heal, new approaches to specifically address the problems of intra-articular tissue healing have been developed to assist with the move from tissue resection and replacement towards tissue repair and regeneration.

In the late 1990s, a paradigm shift from replacement to regeneration was initiated in general surgery, a trend that quickly spread to orthopedic research and more slowly to orthopedic clinical practice. This new paradigm underscores the enhancement of intrinsic healing. One of the most effective tools in this endeavor is tissue engineering. In a landmark publication, tissue engineering was defined as "interdisciplinary field in which the principles of engineering and the life sciences are applied towards the generation of biologic substitutes aimed at the creation, preservation or restoration of lost organ function" [1]. Briefly, these biological substitutes are made from a combination of three constituents: (1) cells, (2) biomaterial, and (3) signals. Cells can be of any type, including fully differentiated cells, such as cartilage cells (chondrocytes), tendon cells (tenocytes), ligament cells (fibroblasts) or the more flexible progenitor (stem) cells that can become one of many different

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_12,

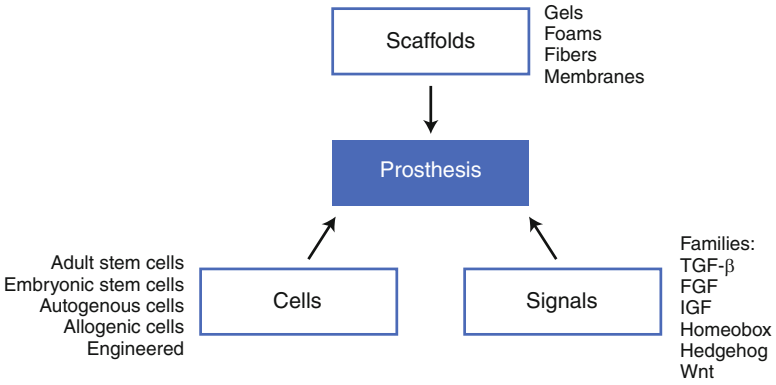

Fig. 12.1 *The triad of tissue engineering*. Tissue engineering rests in three pillars: cells, signals, and biomaterials. Combinations of these three pillars are used to enhance tissue healing, support tissue function, or substitute lost tissues. It is important to remember that constituents from each pillar can have more than one function. Collagen, for example, is a biomaterial but also a signal for platelets to release growth factors. Cells can lump together and form their own biomaterials, or the condensation of cells can act as a signal initiating differentiation

cell types. These cells are usually seeded into a biomaterial (scaffold) that supports cell growth and gives structural stability. Lastly, signals such as growth factors or hormones are used to channel cell differentiation and regulate their biosynthetic activity (Fig. 12.1).

Do We Need Tissue Engineering in ACL Treatment?

Earlier chapters in this book have described the epidemiology of ACL injuries and the problems associated with ACL healing; thus, this content will only be repeated very briefly here. The ACL is quintessential for stability and thus the function of the knee. Tears of the anterior cruciate ligament cause pain and instability and predispose patients to osteoarthritis in long term.

As discussed in Chap. 2, direct repair for the ACL was proposed as a treatment early as 1895, and this technique was further developed well into the 1970s and 1980s [2]. However, a number of studies showed poor outcome after primary repair, and this technique was subsequently abandoned [3, 4]. The current gold standard in ACL treatment is removal of the torn ACL tissue and then replacement with either autologous (patient's own) patellar, hamstring, or quadriceps tendon. Allografts from cadavers, as well as synthetic grafts, are available, but their use is limited by availability and the potential of disease transmission in the former and inflammatory reactions of the foreign body type and medium term failures in the latter. Modern techniques in ACL reconstruction have consistently produced satisfactory results in

joint stability, range of motion, and pain. However, recent studies have presented evidence of relatively high rates of premature osteoarthritis despite ACL reconstruction, even after controlling for other intra-articular damage caused by the initial trauma [5–8].

Given this need for highly effective therapies for these increasingly common diseases, ligament and tendon tissue engineering might very well become a valuable addition to the armamentarium of regenerative medicine in this field.

What Can We Learn from Healthy Ligaments?

When developing a new ACL treatment, be it tissue engineering based or not, it is crucial to familiarize oneself with how a healthy ACL functions. With this as a guide, one can establish targets and directions for a new treatment. The purpose of the ACL is to withstand tensile forces. The healthy ACL supports loads of about 450N during normal activities [9] and will withstand up to 2000N before failing [9]. These mechanical properties are a direct result of the strong tensile characteristics of the aligned collagen protein that makes up the majority of the ligament and resist the tensile loads. Frank and Amiel described dense type I collagen bundles, with additional smaller amounts of type III collagen and glycosaminoglycans in ligaments [9, 10]. On the structural level, both cells and fibers exhibit an undulating pattern, called crimp, which allows for the ligament to stretch up to 6 % before permanent damage starts to happen (Fig. 12.2).

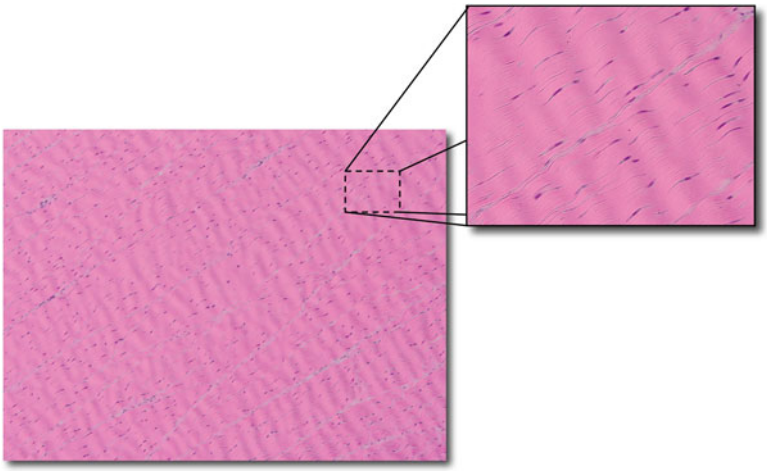

Fig. 12.2 *Crimp*. A healthy ligament exhibits a crimp or a wavy structure that can be seen in light microscopy. This crimp has a major implication in the biomechanics of the tissue. At higher magnification (smaller panel), one can observe that the cells in the tissue follow the crimp closely. Reestablishing crimp is a critical parameter in tissue engineering of ligaments

Healing of ligaments depends on a number of factors. It is commonly accepted that tears of the anterior cruciate ligament will not heal, while tears of the medial collateral ligament heal spontaneously. Rotator cuff tendon tears do not heal while Achilles tendon tears do. Both the ACL and torn rotator cuff tendon are inside the joint (intrasynovial) in humans, and it is thought that they fail to heal due to factors of the intra-articular environment.

A blood clot that forms in the wound site for tissues outside the joint (extrasynovial) serves as a provisional scaffold for inflammatory cell attachment and as a source of stimulatory cytokines from platelet activation. Within this clot, the damaged tissue is absorbed and new tissue is produced. In intrasynovial tissues, the formation of such a blood clot does not occur [11], a fact that is attributed to mechanical factors of the fluid environment as well as biochemical factors such as the presence of activated plasmin in the injured joint. Without a provisional scaffold, the wound site remains empty and the injured ends of the tissue are covered by proliferating synovial cells and retract due to the production of smooth muscle actin-alpha (a contractile protein) in the ligament itself. Bridging the wound site of an intra-articular ligament with a material that could encourage local cell ingrowth and stimulate collagen production in the wound site may be a critical step in healing of tissues within joints.

Tissue engineering is a logical solution for the lack of a scaffold within the ACL wound site. The success of such treatment in vivo should be evaluated by the mechanical strength of the implanted construct over time, which is a function of both the quantitative and qualitative reproduction of cell-matrix interactions. Its clinical success has to be judged in the light of long-term effectiveness at limiting further joint deterioration including cartilage damage, since this is the area of weakness in current treatments.

The composition of the tissue engineering construct, in terms of cells, biomaterials, and signals, should be carefully chosen for any application. Thus, before discussing more complicated tissue engineering-based ACL treatments, a look at the three variables of cells, biomaterials, and signals will be presented.

Cell Sources

Various kinds of cells have been studied for their potential in ACL tissue engineering. An ideal cell source would provide cells with a high proliferation rate and a high biosynthetic activity to build and remodel the ligament as fast and accurately as possible. Fibroblasts of different origins have been extensively studied, following the logic that highly differentiated cells possess all the phenotypic properties necessary to produce and maintain an adequately composed extracellular matrix.

Like currently employed cartilage repair procedures involving scaffolds seeded with chondrocytes, fibroblasts could potentially be obtained for seeding in a ligament scaffold in an initial arthroscopic procedure. Like articular cartilage procedures, this arthroscopy could be performed weeks before the repair procedure. The cells obtained during the initial arthroscopy could be taken to the lab and cultured and stimulated to proliferate until enough cells were present for reimplantation into the injured knee.

Another option would be a one-step technique in which fibroblasts would be isolated in the operating room and directly reimplanted, although low cell numbers might limit such a method. It has been shown by Murray et al. that the fibroblasts from the human ACL are viable long after the initial ACL trauma, and that they are able to migrate into a biomaterial used for tissue engineering-augmented ACL repair [12, 13].

The major problems associated with using differentiated adult fibroblasts are their fairly low proliferation rates and relative scarcity. This has led investigators to consider another cell source: undifferentiated mesenchymal progenitor cells (MPC). These cells can be obtained in relatively high numbers from the bone marrow or other adult tissues. They have a high proliferation rate and the potential to differentiate into multiple different cell types.

However, there is a small but persisting risk of faulty differentiation of MPC that might lead to problems in a clinical application. For example, pluripotential cells implanted to stimulate ligament healing might instead head down the osteogenesis pathway and form spicules of bone within the ligament. This could cause stress risers within the ligamentous tissue and make it easier for the tissue to fail. In addition, the classic way to obtain MPC is a bone marrow biopsy, which is a technically straightforward, yet considerably painful procedure.

Biomaterials

After choosing a cell source, an appropriate scaffolding or biomaterial that satisfies a number of stipulations has to be selected. A suitable scaffold should foster tissue remodeling by providing an environment that stimulates cellular attachment, growth, and biosynthetic activity. Biocompatibility and degradation rates that match tissue remodeling are also likely to be important. Additionally, safety is an important issue, since some biomaterials might provoke inflammatory responses, cause arthrofibrosis, and lead to loss of joint function or systematic adverse reactions. Finally, the biomaterial has to be chosen according to the planned procedure. Tensile strength is less important than enhancement of cellular behavior in primary repair, where the suture repair carries the load, while it is pivotal for scaffolds chosen as an ACL graft.

Natural polymers have a long and successful history in tissue engineering, and collagen is an obvious choice for a tissue-engineered ligament. Collagen, a natural polymer, is and has been in clinical use for decades in suture materials and clotting agents. Its safety profile is well established. Collagen is also used as biomaterial in clinically available tissue engineering methods such as autologous chondrocyte implantation and has been shown to enhance cellular phenotypes in this application [14] (Fig. 12.3). Bovine collagen has been used in multiple studies to establish and sustain fibroblast cultures, yet with somewhat inconsistent results [16, 17]. However, the effect of collagen on cellular behavior depends not only on its mere presence but also on material characteristics such as pore size, cross-linking, and fiber diameter [14, 15]. Other natural polymers that have been studied include hyaluronic acid, fibrin, and chitosan-alginate. Hyaluronic acid is a well-known biomaterial in tissue engineering and has been shown to beneficially influence cellular behavior. Wiig and

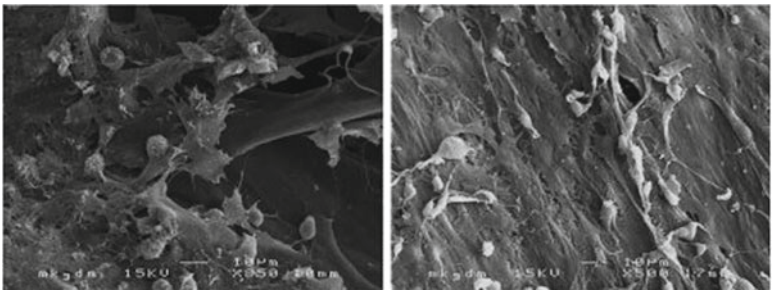

Fig. 12.3 *Collagen biomaterial*. This figure shows an electron microscopy picture of chondrocytes on a collagen matrix. The cells adhere to the surface of the biomaterial and express the same morphology that we have seen in earlier cell culture pictures (cf. Fig. 10.1) (Used with permission from Dorotka et al. [15])

coworkers reported improved healing of a central ACL defect after injection of hyaluronic acid in a rabbit model [18]. Cristino et al. showed mesenchymal progenitor cell growth and differentiation in a modified hyaluronic-acid-based scaffold [19]. Hyaluronic acid has also been shown to have a beneficial effect in the prevention of osteoarthritis in anterior cruciate deficient knees [20, 21]. Fibrin has the advantage of producing a biodegradable scaffold when mixed with thrombin.

Biomaterials like poly(lactic acid), poly(glycolic acid), and other synthetic polymers have been used as suture materials with much success and minimal adverse events. The advantage of these polymers is that their composition can be controlled and adjusted for specific purposes. With modern processing techniques, these polymers can be spun into microfibers, which have been proven to enhance cell attachment by a high area to volume ratio and beneficial properties in mass transport of nutrients [22]. Additionally, these polymers have convincing mechanical properties. Of special interest is silk, which holds a position at the intersection between naturally occurring and synthetically modified materials. Silk in its native form is coated with sericin – a glue-like protein which can cause an immune reaction in humans. Modification of silk by removing the sericin yields an inert, biocompatible material with excellent mechanical properties, similar to the native ACL. Silk has also been used successfully as a scaffold for fibroblasts and shown to enhance fibroblastic differentiation of mesenchymal progenitor cells [23].

Growth Factors and Signaling Molecules

Signaling is the third factor in the triad of tissue engineering. Signaling can be used to direct cellular activity to achieve the desired outcome in terms of cell growth or matrix production. Growth factors that have been associated with cell growth and differentiation were studied initially in an effort to identify factors that would be beneficial in wound repair. Transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) have been shown to improve growth and bioactivity

of fibroblasts [24]. On the other hand, various growth factors, such as TGF and vascular endothelial growth factor (VEGF), have been shown to be produced by fibroblasts themselves and thus have also been investigated for their use in tissue engineering of ligaments and tendons [25, 26]. However, the effects of many growth factors are still not completely understood and how to combine these and optimize their function is difficult to say the least. In one elegant study, the investigators used a carefully chosen growth factor cocktail in tendon repair and were able to get the desired high increase in cell growth. However, this was associated with a mechanically inferior scar [27]. Thus, even careful and thoughtful planning and design may not translate into the desired effect in vivo.

Another very important source of cell stimuli is the physical and mechanical environment of the cells. The structure of the biomaterial selected as the scaffold, in terms of both chemical composition and structural properties, has been shown to stimulate cell attachment, growth, differentiation, and biosynthesis. Mechanical stimuli such as tensile and torsional stress also affect cellular behavior. However, little is known about the details of these effects and the interactions between them. Hence, the directed use of these stimuli in tissue engineering, beyond a general beneficial effect, is not yet possible.

Approaches to Clinical ACL Tissue Engineering

Generally, two philosophies exist in the management of the torn ACL: replacement using a graft or primary repair. Either of these techniques may potentially be enhanced using tissue engineering. For a replacement (also called an "ACL reconstruction"), a graft or synthetic material is used to replace the entire ACL. In this case, cells and scaffolds could be used to enhance the healing of a tendon graft or synthetic ligament to the bone. Advantages of the reconstructive approach are immediate mechanical function and a minimal change from currently used techniques, thus avoiding a steep learning curve. However, the immediate mechanical strength also introduces the potential problem of stress shielding. Stress shielding, where the load is taken by the implanted scaffold rather than the tissue developing within it, can deprive cells of important mechanical stimuli to drive their bioactivity. A bio-artificial ACL has been presented by Goulet et al. [28]. Briefly, this group suggested a complete substitute consisting of two bone plugs connected with a surgical thread. During culture cells attach to this thread and deposit a matrix rich in type I collagen thus building a ligament-like structure (Fig. 12.4). This graft healed well in a goat model and showed tissue ingrowth and vascularization in histology. After 13 months it showed 36 % of the mechanical strength of a normal goat ligament. In a similar approach, Ma et al. from the University of Michigan generated a multiphasic (bone-ligament-bone) ACL construct from bone marrow stem cells (BMSC). Briefly, BMSC were cultured in vitro from either bone or ligament and then combined to a functional graft of approx 80 mm length and 3 mm diameter. These constructs were implanted into sheep after ACL excision and the animals were followed for up to 6 months. At this time, histological and biomechanical

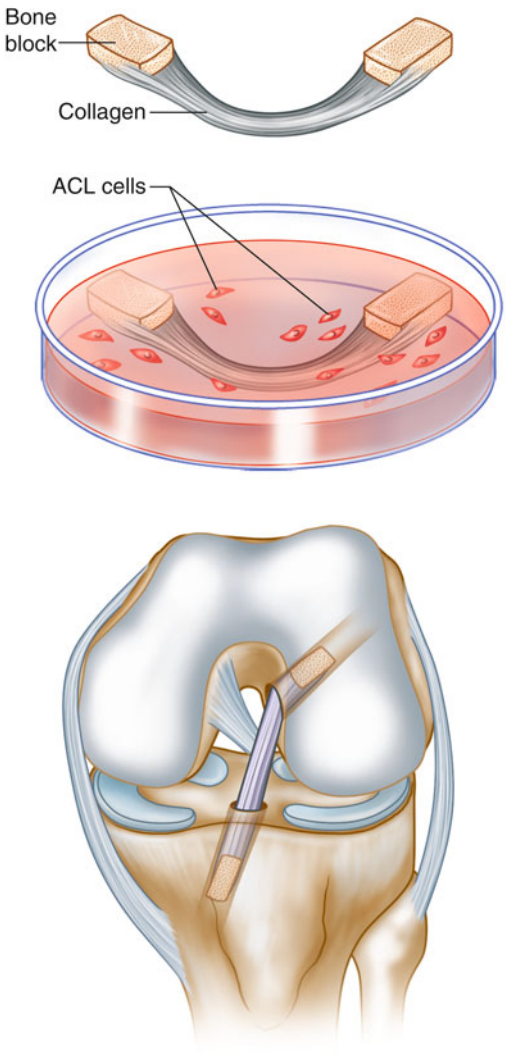

Fig. 12.4 *An artificial ACL.* The *top panel* shows an artificial ACL, that is, collagen fibers attached to bone blocks. In the *middle panel*, this construct is kept in cell culture and ligament cells are cultured on the collagen fibers. Finally, the cell-laden graft is implanted into a goat knee (*lower panel*). Goulet et al. [28]

assessment showed vascularization and innervation of the graft, as well as good bone integration and biomechanics very close to the contralateral, normal knees.

Another approach aims at using tissue engineering to enhance primary repair. The rationale of this approach is that the intricate nature of the ligament insertion, proprioceptive nerves and the complex architecture of the ligament are preserved. Furthermore, the ACL remnants can serve as reservoirs of fibroblasts. Murray and coworkers have described the specifics of such an approach in much detail. In summary, they demonstrated that human fibroblasts remain viable in the ACL stump and are able to migrate into a collagen scaffold as could be used in a bio-enhanced primary repair procedure [12, 13]. Addition of platelet-rich plasma to this scaffold was shown to promote cellular migration and proliferation in a central defect model, thus stimulating healing [29]. Further examination revealed good defect filling in

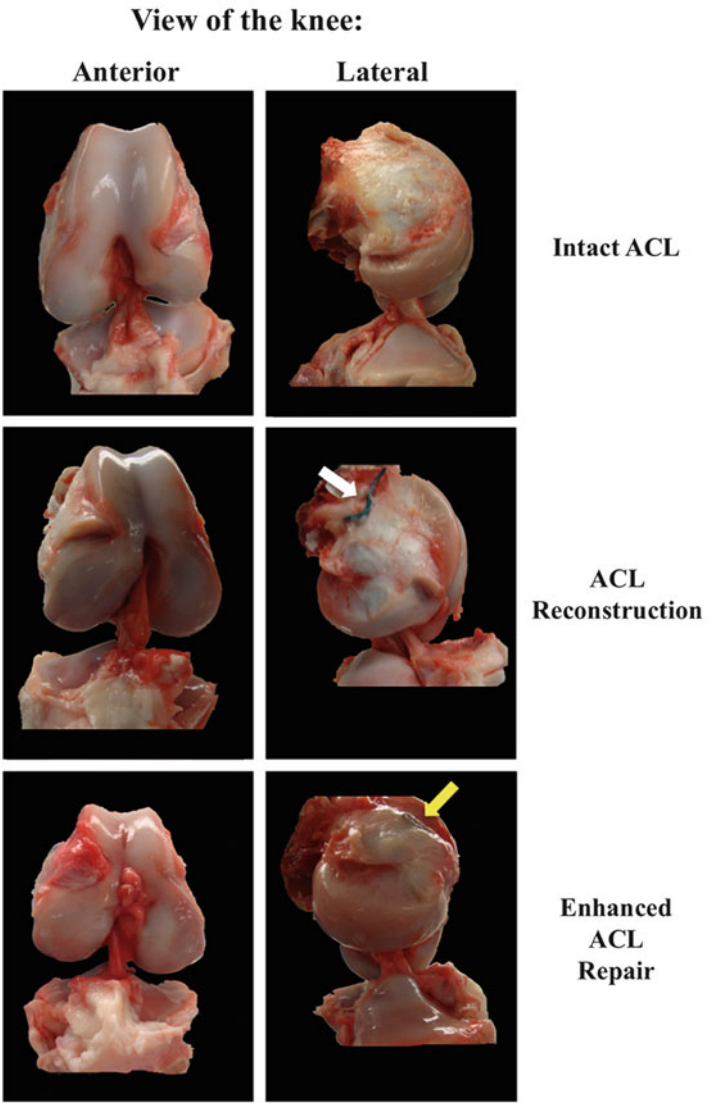

Fig. 12.5 *Tissue engineering enhanced ACL repair*. An alternative to ACL replacement, with a tissue graft or an artificial ACL, is tissue engineering-enhanced ACL repair. Briefly, the torn ends are sutured together and a biomaterial-signal composite is used to enhance healing. In the figure, we can see a normal ACL, an ACL reconstruction (15 weeks after the procedure), and an enhanced ACL repair (15 weeks after the procedure), all from a pig model. The *arrows* show the fixation devices for the surgical procedures. Biomechanical comparison at 15 weeks showed no difference in the strength of an ACL reconstruction or ACL repair (Reprinted from *Arthroscopy*, vol 28, Patrick Vavken et al. [32], with permission from Elsevier)

histology [30]. In a more challenging animal model, complete transections of the ACL in pigs were repaired using the same technique, and significant improvement in mechanics was shown [31] (Fig. 12.5).

Conclusions

Tissue engineering uses combinations of cells, scaffolds, and growth factors to form a biomaterial that can be used to replace or regenerate injured tissues. Cell choices include those found in the mature tissue of interest (i.e., fibroblasts for ligament engineering) or cells that are found in the developing tissue (e.g., mesenchymal stem cells) that can be coerced into turning into the desired cell type. Cells can also be implanted with a scaffold or encouraged to migrate into a scaffold from their residence in the local environment of the wound. Scaffolds can be mechanically strong, particularly for replacing load-bearing structures, or they can be purely biologic in function, as in supplementing a suture repair where the sutures will carry the load and the scaffold provides the biology. The desired signaling molecules may be multiple and complex as presented in the prior chapter on wound healing, thus autologous cells capable of releasing these factors over days to weeks might be useful as sustained delivery systems. The possible combinations of these three elements provide enormous flexibility and potential for regenerating musculoskeletal tissues.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 13
Outcome Assessment for ACL Tissue Engineering

Patrick Vavken, Martha M. Murray, and Braden C. Fleming

Where Do Outcome Assessments Come from?

In the current, global discussion of health care and its future, terms such as "outcomes research" or "health technology assessment" are frequently encountered, usually in combination with financial arguments. But while everybody stresses the importance of "outcome assessment," there is relatively little discussion regarding the definition of outcome research or how to conduct it in a way that provides meaningful results.

Florence Nightingale is often credited for one of the earliest reported uses of outcome assessment in improving patient care [1]. During the Crimean War of the 1850s, she was serving as a volunteer nurse in the British barracks at Scutari (modern day Istanbul). As part of her job, she recorded the rates and causes of deaths of the wounded soldiers brought back from the nearby front. She studied the effects of

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_13,

Fig. 13.1 *Ernest A. Codman*, *MD* (*Dec 30*, *1869–Nov 23*, *1940*). Ernest A. Codman was a pioneer in many fields of medicine. During his work as a radiologist and surgeon at the Massachusetts General Hospital, he contributed to the fields of anesthesiology, radiology, general surgery, orthopedic oncology, and shoulder surgery. He is perhaps best remembered among orthopedic surgeons for his work in bone oncology. In this picture we can seen him with his registry of bone sarcoma. The same meticulous character also led him to initiate the first morbidity and mortality conferences, periodical meetings where cases with poor and/or avoidable outcomes are discussed and to initiate a system of "end result cards" reporting patient outcomes (see text) (Courtesy Bill Mallon, MD) [3]

improved sanitation and supplies and used the mortality rates to justify the need for more funding for the military hospitals from the British government. Her efforts resulted in greatly improved conditions for the soldiers and reportedly reduced the death rate from 42 to 4 % [2].

Another pioneer of outcome assessment was Ernest A. Codman, an orthopedic surgeon at the Massachusetts General Hospital (MGH) in Boston. An active surgeon in the early 1900s, he was the first American physician to systematically follow patients through their recovery. Codman used what he called "end result cards" on which he collected demographic data, diagnoses, treatments, and the resulting outcomes for at least one year for all of his patients. He was a firm proponent of expanding this practice to cover all surgeons at MGH and to make this data public. However, in 1914 the hospital refused his proposal and he was asked to leave the hospital. He published his own "end result cards" from 1911 to 1916, including 337 patients with 123 treatment errors in his 1916 book *A Study in Hospital Efficiency* [3] (Fig. 13.1).

So What Is Outcome Assessment?

In simple terms, and as the name implies, outcome assessment describes how an outcome for a given medical intervention change over time [4]. Outcomes can be many things, including measures of improvement in quality of life and relief of pain, as well as complication, reinjury, mortality, and/or readmission rates. Outcomes can be patient-based, where patients fill out questionnaires regarding their perceived outcome. Physical examination parameters also are frequently measured and recorded for outcome studies. Outcome measures can also be practice-based, including measures of appointment waiting times, rates of readmission to a hospital after discharge, costs of care, or safety measures. These outcomes are collectively referred to as endpoints. In technical terms, medical outcome assessment aims at establishing four parameters: Efficacy ("can it work?"), Effectiveness ("does it work?"), Efficiency ("does it produce value?"), and Safety ("are the adverse effects acceptable?") [4]. Thus, outcome assessment should be understood as part of the general trend towards evidence-based medicine, where treatments are chosen and administered based on scientific data, instead of eminence-based medicine, where treatments are selected based on the opinion of clinical practitioners on the basis of their experience.

Outcome Assessment in ACL Tissue Engineering

Outcome assessment is also a crucial part in the development of tissue-engineering-based ACL treatments. In developing a new treatment, different endpoints have to be considered. In this chapter, we will explain which endpoints we think are important to consider, how they are assessed, and what they mean. Many of these endpoints have been and will be mentioned repeatedly throughout this book. For the purpose of this chapter, we will divide the development of new treatment into three broad stages: (1) the in vitro phase of cell-based research ("benchtop" or "test tube" experiments), (2) preclinical testing (i.e., large animal testing), and finally (3) human clinical trials.

Outcome Assessment in the In Vitro Phase

In vitro is Latin for "within glass," referring to the glassware, such as petri dishes, test tubes, and culture flasks. As such, the in vitro phase describes the earliest stages of the development of a new treatment where cells are grown in a lab and tested for the response to the new intervention. Thus, outcome assessment during this phase revolves around how the cells behave in response to some biological and/or mechanical stimulus. Common outcome measures include (1) proliferation, or the

multiplication of cells; (2) biosynthetic activity, that is, the proteins and signals produced by cells; and (3) extracellular matrix deposition and remodeling, that is, what cells actually do with the proteins and macromolecules they produce. The methods and assays for the assessment of cellular behavior are countless, but there are broad categories that have been and will be mentioned throughout this book. For the purpose of this chapter, we will first focus on the biology of cells and tissues, starting at the tissue level with histology, moving to the cell level with biochemistry, and finally to the subcellular level with molecular biology.

Histology

Histology is the study of the microscopic anatomy of tissues and cells. It is performed by cutting very thin, translucent slices of the tissue of interest and then using special dyes and antibodies to stain particular cells, proteins, or other structures (e.g., extracellular matrix) in the tissue. These sections can then be examined using a microscope. Specialized stains exist that will react with specific cells and proteins, thus allowing researchers to identify and quantify cell types or structures. The most frequently used staining technique is H&E, which is short for hematoxylin and eosin [4]. Briefly, hematoxylin is a blue-colored dye that stains cell nuclei and eosin is a red-pink-orange dye that stains other, non-nucleic structures such as the cytoplasm or extracellular structures such as collagen (Fig. 13.2). Not all tissue components react with H&E, thus other dyes and antibodies have been developed to help identify cartilage, nerves, and other structures in tissues more easily.

Immunohistochemistry is a process where antibodies to cell surface markers or proteins are placed in contact with the tissue [5–7]. Antibodies can be designed that bind to a specific cell surface marker or protein and also carry a binding site for a staining dye. The antibody can thus stick to the structure of interest and the attached dye visualized in the microscope. This technique can be used to identify structures of interest with extremely high specificity, allowing one to visualize not only cells but also proteins involved in the cellular life cycle or specific cellular activity.

Once cells, cellular constituents, and extracellular components of tissues have been labeled, this labeling can be used to assess the type and quality of tissues. The most straightforward way to do this is to describe the distribution and quantity of cells, proteins, such as collagen, or more complex structures such as vessels or nerves within the tissue. As good tissue function typically requires the presence and interaction of more than one cell type (e.g., ACL fibroblasts and blood vessel cells), it is helpful to assess tissue quality by a compound measure or scoring system. For example, the Ligament Maturity Index is a histological ACL quality score that includes a quantitative assessment of H&E stained sections of the healing ACL in three different categories: cellularity, collagen, and vascularity, where each section

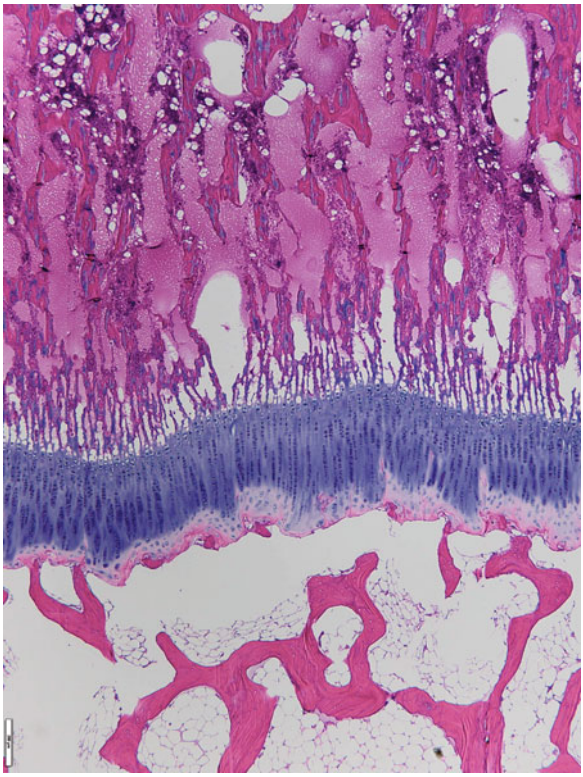

Fig. 13.2 *Histology of the growth plate*. This figure shows a histological section of the growth plate of the tibia, at 40× magnification. The staining technique used is hematoxylin and eosin. The stain is able to show various types of tissues. In the bottom third, we see bone, with dense lamellae and fatty vacuoles of bone marrow. In the center, we see a blue and purple band of cartilage that is the actual growth plate. Long columns of cells can be easily discerned, and the change of the cells from small to large with a dense center can be easily seen even at this low magnification. In the top part, we can see newly formed bone and its change from *blue* to *red* as it mineralizes. In summary, simple hematoxylin and eosin is an extremely versatile technique in the descriptive and semiquantitative assessment of tissues

is given a rating of 0, 1, or 2 by two independent scorers [8]. Using the Ligament Maturity Index, the relationships between the ligament biology and biomechanics have been established following bio-enhanced ACL suture repair [9]. While the Ligament Maturity Index focuses on ligament healing and maturity, other investigators have developed scores focusing on ligament degeneration. For example, Mullaji et al. developed a score that includes five categories of H&E stained tissue: inflammation, mucoid degeneration, chondroid metaplasia, cystic changes, and collagen

orientation [10]. As with most outcome scores, there are no universally accepted standards to guide researchers and clinicians. The best system is typically dependent on the question being asked.

Biochemistry and Molecular Biology

Histology and histological scoring are excellent tools to describe tissues in total, but it has shortcomings. Most importantly, histology is generally a semiquantitative method, that is, it does not produce numerical results directly relating to the many cell and tissue functions, such as the number of cell divisions or the amount of newly formed protein. Also, while histology provides an excellent "road map" of what is occurring in the tissue, sometimes a study searches for more specific answers such as what a particular cell is doing at a given location and time point. A number of methods exist that are able to address these issues.

Biochemistry is the study of the chemical processes or organisms and of living matter. Biochemical tools can be used to quantify DNA, RNA, and proteins. Typically, a chemical reagent is used that binds to the DNA or protein in question resulting in a change in color of that reagent that then can then be measured using photometry. More sensitive methods use a fluorescent reagent, that is, a chemical that emits light of a specific wavelength after being excited by a laser. This light emission can be detected more reliably than a color change. A more versatile and very popular method is the ELISA, or enzyme-linked immunosorbent assay. Similar to the immunohistochemical procedures outlined above, an antibody attached to a tissue culture plate is used to isolate a target substance from a sample. This captured substance is then labeled with a "detecting antibody" that contains a detectable color or that is labeled with another, colored antibody. This color is then measured to quantify the amount of target substance.

Molecular biology is a branch of biology that addresses the molecular basis of cellular behavior. Some of the most important tools in molecular biology are polymerase chain reaction, or PCR, and gel electrophoresis [11, 12]. PCR is an extremely useful method to expand DNA. Briefly, PCR facilitates copying a single DNA sequence millions of times so it can be detected with further methods (Fig. 13.3). New forms of PCR also allow for the amplification of RNA or to search for a specific DNA sequence. The benefit of PCR is that DNA, even from a single cell, can be multiplied to a level at which it can be detected, measured, and described. Gel electrophoresis, in turn, uses the natural charge of DNA, RNA, and proteins to separate them by means of an electric field [13–15]. Based on size and charge of DNA, RNA, and protein molecules, they will travel at different speeds, thus separating from each other. They can then be visualized using dyes and UV light. In 1993, Kary Mullis and Michael Smith won the Nobel Prize in Chemistry for their work on PCR.

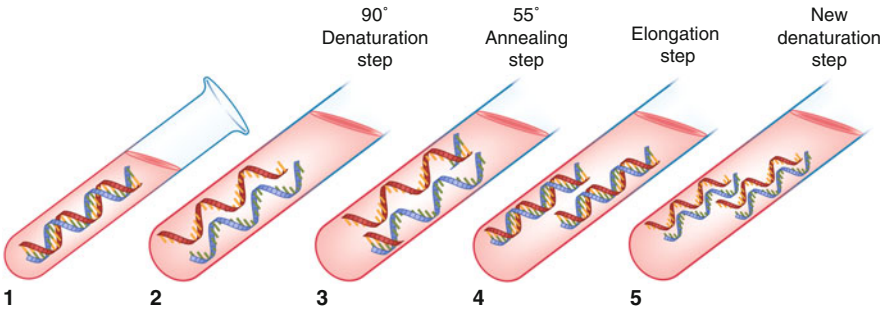

Fig. 13.3 *The principle of polymerase chain reaction* (*PCR*). Briefly, DNA is isolated from a sample of cells, for example, from blood or tissue. Heating the DNA to 90° results in separation of the two strands of the "double-strand DNA" by melting the hydrogen bonds between the two strands (*the denaturation step*). In the next step (*the annealing step*), the temperature is lowered to 55° and primers, that is, markers of the start point of the DNA sequence to be multiplied are added. In the third step (*the elongation step*), the polymerase, after which the process is named, nucleotides (DNA fragments) are added. These fragments bind to the separated strands, starting at the location of the primers, resulting in two new strands of DNA. Typically, these three steps are repeated 20–40 times, resulting in a doubling each time

Outcome Assessment in Preclinical Model Testing

As mentioned above, the development of a new tissue-engineered ACL treatment begins with an idea based on biological and mechanical principles, the feasibility of which is usually first evaluated with in vitro studies. Once the in vitro feasibility is shown to be promising (e.g., ACL fibroblasts are capable of migrating into a scaffold), preclinical models are needed to evaluate the efficacy and safety of the new idea in living systems (in vivo). It is through preclinical models that researchers are able to evaluate, refine, improve, and optimize the treatment without placing any patients at risk. Furthermore, the United States Food and Drug Administration (FDA), which regulates how a medical device or drug is safely translated from the laboratory to the clinic, requires the use of preclinical models to prove that the new treatment (e.g., a tissue-engineered ACL) is likely to be safe and efficacious before it is used on patients [16, 17].

There are many advantages for using preclinical models when designing tissue-engineered constructs. A detailed consensus statement from experts in the field regarding the best models and outcome measures to consider for tissue engineering development has been published [18]. When selecting a preclinical model, researchers attempt to recapitulate the relevant biological and biomechanical mechanical with that of the human. Some of the outcome measures (e.g., functional performance) should parallel those used in humans so that the relationship between the model and human can be established. Preclinical models are also advantageous because they allow researchers to harvest joints or tissues at different time points during healing, something which is not possible in humans. With these tissue-specific biomechanical and biological assessments of ACL or graft healing

(e.g., “Is the biologically enhanced ACL stronger than traditional ACL reconstruction after 1 year of healing?”) or articular cartilage health (e.g., “Does this bioenhancement have a positive or negative effect on the articular cartilage?”) can be performed. Another advantage of preclinical models is the use of control groups to compare the results to normal joints (negative controls, i.e., a comparison of the new treatment to a normal control knee) and/or untreated joints (positive controls, i.e., a comparison of a new treatment alternative to the untreated ACL injured knee) and/or sham-treated joints (sham controls, i.e., a comparison of the new treatment to a knee in which the same incisions are placed in the joint without injuring the ligament itself). All of these controls enable researchers to evaluate the intervention while eliminating the effects of other variables that may confound the outcome and hence the understanding of how the new treatment works. This is a limitation of many clinical trials of ACL treatment in humans, which limits the types of controls that can be used.

Because the ACL primarily serves a mechanical function in that it guides normal motions between the tibia and femur while restraining excessive motion, biomechanical analyses are critical for evaluating ligament or graft healing. The American Society of Biomechanics has defined the term “biomechanics” as “the study of the structure and function of biological systems using the methods of mechanics” (http://www.asbweb.org/html/biomechanics/Biomechanics.html). Preclinical models are optimal for biomechanical testing and are generally performed at the ligament and/or joint levels. Common examples of these include the biomechanical evaluation of the healing ligament or graft itself (e.g., structural properties, material properties, and the viscoelastic properties), passive measurements of joint laxity with the muscles relaxed to assess the ligamentous restraints (e.g., anteroposterior knee laxity), and functional tasks (i.e., activities of daily living) which incorporates the interactions between the muscles and ligaments (e.g., gait analysis). These commonly used outcome measures are briefly described below.

Biomechanical Evaluation of the Healing Ligament

Given that the ACL serves a mechanical function, one of the most common and useful methods to evaluate healing is to measure its mechanical performance. Ligaments support tensile loads (i.e., forces that are applied to the ends of the ligament and are directed along its long axis). Therefore, tensile failure tests, in which forces are applied to the ligament ends and ramped up until the ligament fails (Fig. 13.4a), provide information about how the ligament stretches and how it will fail, parameters that change as an injured ligament or graft replacement heals. The data obtained from a tensile failure test enables the determination of the “structural properties” and the “material properties,” which will be defined and briefly described below. Another feature of soft tissues such as ligament and tendon is that these structures are “viscoelastic.” This means that the biomechanical properties of the tissue are also dependent on the load history, load rate, and the time of the applied loading.

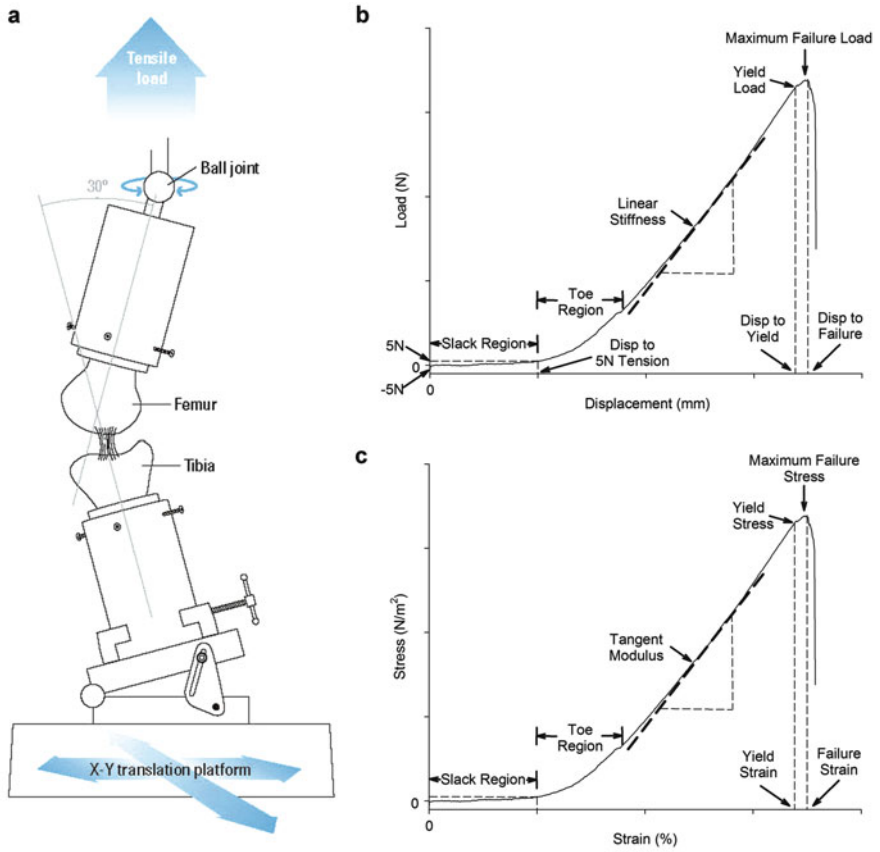

Fig. 13.4 *Tensile failure testing*. (**a**) Failure testing involves isolating the femur-ACL-tibial complex, mounting it on a material testing system and then applying a tensile load to failure. To test the porcine knee, the knee is placed at 30 degrees of flexion, a slight compressive load (−5 N) is placed on the joint so that the two articulating surfaces are touching each other before the tensile load is applied (Braden C. Fleming et al. [19], copyright © 2009 by (Sage Publications), Reprinted by Permission of SAGE Publications). (**b**) During the test, the applied tensile load is plotted as a function of the displacement. This enables the user to determine the length of the slack region and the structural properties of the ligament (Used with permission from Fleming et al. [20]). (**c**) The material properties can be determined by normalizing the tensile load by the cross-sectional area (stress) and the change of length by the initial length of the ligament (strain). The material properties are therefore geometry independent (Used with permission from Fleming et al. [20])

Establishing the viscoelastic properties requires a different set of tests. When tissue engineering a ligament, it is important to consider all of these properties.

The structural properties of a ligament or tendon are derived from the load–displacement relationship measured during the tensile failure test (Fig. 13.4b). Typically the ligament of interest (e.g., the ACL) is left attached to the tibia and femur (i.e., the bone-ligament-bone complex) while all other soft tissue structures are removed. The specimen is then mounted to a material testing system so that the

tensile load can be applied along the length of the ligament (see Fig. 13.4a). Because the structure of the ACL is complex and the planes of the ligament insertions are not parallel to one another, it is important that the tibia and femur are free to move in the other directions during the test so that the applied tensile load is distributed over the entire cross section of the tissue. This approach ensures that the constraints of the testing system will not influence the ligament load–displacement response [21]. As the bone-ligament-bone complex is loaded in tension, elongation of the ligament occurs in both the ligament and its attachments. The three most commonly reported structural properties are yield load, failure load, and linear stiffness (see Fig. 13.4b). The yield load is the point on the curve where the ligament fibers begin to fail (plastic deformation). After passing this point, the displacement will not return to zero if the load were removed. The failure load is the point at which the load drops off and the ligament fails either at the mid-substance of the ligament or the insertion site. It is important to note that failure should occur at the mid-substance if the specimen is properly aligned with the load during the test [21]. The linear stiffness of the ligament or graft is represented by the slope of the load–displacement curve and is typically calculated between the points corresponding to 20 % and 80 % of the yield load. There are other features that are also important, particularly those relating to the length of the toe region (see Fig. 13.4b), the initial portion of the loading curve. The toe region is formed by the greater displacements that occur as the crimped (wavy) collagen fibers begin to straighten. Once straight, the curve becomes linear and remains so until it reaches the yield load. Most structural property analyses focus on the yield load, failure load, and linear stiffness. Unfortunately all of these are related to the upper end of the load–displacement curve. However, the toe region may be more important since most normal activities of daily living are performed at low loads [22]. As one of the principal functions of ligaments is to maintain a specific bone-to-bone distance, measuring the distance between the tibiofemoral contact (−5 N of compressive load) to a "low" tensile preload (+5 N) would be one way to quantify this (see Fig. 13.4b) [20]. For example, a repaired ligament that allows the articulating surfaces to move apart by 10 mm before restraining that motion (i.e., very long toe region) is likely to be nonfunctional even if it could support high yield and failure loads.

The "material" properties are derived from the tensile stress–strain relationship. In simple terms, stress is defined as the applied load divided by the cross-sectional area of the ligament. Strain is the resulting change in length divided by a reference length (the initial length of the ligament just prior to bearing load). The stress–strain relationship (Fig. 13.4c) can be determined from the load–displacement data used to calculate the material properties of the tissue. The force and displacement are normalized by the cross-sectional area and initial length of the ligament to derive the stress and strain, respectively. Material properties of interest include the yield stress, failure stress, and tangent modulus which are related to the yield load, failure load, and linear stiffness. The normalization eliminates the dependence of the structural properties on the size of the ligament. What this means is that there are two mechanisms by which ligament function can be restored: (1) replace with a bigger ligament of lower quality (e.g., the scar in a healed MCL is usually bigger than the

original ligament at the wound site to partially compensate for the poorer quality of the scar tissue) or (2) replace it with a smaller size ligament of higher quality. Thus, the material properties enable researchers to evaluate the quality of the replacement tissue, while the structural properties evaluate the global response of the treatment to the joint. An example of this would be a comparison of the structural and material properties of a graft following bio-enhanced ACL reconstruction with that following traditional reconstruction in the juvenile porcine model [19]. When comparing the average linear stiffness values (a structural property representing the slope of the load–displacement curve) between the two procedures, there was no significant difference between the two. However, the tangent modulus (a material property representing the slope of the stress–strain curve) of the bio-enhanced ACL graft was much higher than that of the traditional graft. Typically the structural properties are reported in the preclinical models of ACL or graft healing. This may be due to the fact that clinicians are interested in the healing response of the entire construct. There are also challenges when trying to measure the material properties of a ligament since the material properties vary depending where you look in the ligament. Nonetheless, the material properties provide important insight which may be valuable for the development of new ACL treatments.

The determination of the structural and material properties from tensile failure testing does not consider the viscoelastic nature of the soft tissue. These properties are dependent on the load history, load rate, and the time of the applied load and are due to the interaction of the collagen fibers, proteoglycans, and water content of the tissue. For example, with repetitive "sub-failure" loading of the ACL, the peak ligament load at a prescribed sub-failure displacement will decrease during initial cycling. In addition, the ligament stiffness is reduced. Once the loading ceases, the ligament returns to its initial state. The viscoelastic properties of a ligament or tendon protect them during repetitive loading to lessen the impact of the loads and thus prevent fatigue damage. This is one reason why it is important to stretch before engaging in strenuous exercise. The temporal loading history of a ligament is described by the phenomenon of creep and stress relaxation. Creep is defined as an increase in ligament length (strain) with time in response to an applied load (stress). Stress relaxation occurs if a ligament is stretched to a predetermined length and fixed at that length. This causes the stress in the ligament to decrease over time. Although most studies evaluating tissue engineering constructs have focused on the material and structural properties of the ligament or graft, the viscoelastic properties are also important and will eventually need to be assessed as well.

As described above, determining the structural properties of the native ligament or its replacement, *ex vivo*, is commonly performed to evaluating the biomechanical properties of the tissue [23]. However, longitudinal assessments of healing within subjects would be possible if an accurate noninvasive method were available. If successful the number of animals needed to run a preclinical study could be reduced, and the technique were validated, it could potentially be applicable to monitoring healing in clinical studies as well. Magnetic resonance imaging (MRI) is showing considerable promise for this purpose [24, 25]. Signal intensity (i.e., the grayscale level of the image) is a MR parameter that is affected by tissue type and water

content, both of which change during healing. The use of MR grayscale as an outcome measure was founded on research showing that the graft grayscale values decrease with time in humans postoperatively [26] and that they negatively correlate with the structural properties of the graft in an ovine model [25]. Although not yet validated in the clinical setting, MR measures of signal intensity have been used to establish graft integrity and maturation following ACL reconstruction surgery in humans [26–29]. Research is currently under way to combine, refine, and validate these methods [24].

Biomechanical Evaluations of Joint Function

A variety of measures have been developed to measure the biomechanical function of the joint. The most common includes passive laxity measurements and gait analysis. These are techniques that are commonly employed in both preclinical and clinical trials so that the link between the two can be established.

Measurements of passive knee laxity, a variable that quantifies how loose the knee joint may be, are commonly performed. Knee laxity evaluations are commonly used to diagnose ACL injuries and to determine how well ACL treatment methods restore overall joint function in ACL injured patients [30]. To evaluate passive knee laxity, loads and/or torques are applied to the relaxed knee and the resulting translations and/or rotations are determined. The clinical Lachman test provides a common example for this. The clinician or researcher applies anterior (forward) and posterior (backward) directed forces to the tibia with respect to the femur, while he or she subjectively evaluates the amount of anteroposterior translation occurs. The resulting displacement is subjectively graded on a scale ranging from 0 (tight) to 3 (very loose). Instrumented systems, such as the KT-1000 Knee Arthrometer [30], were subsequently developed to objectively quantify the amount of anteroposterior translation (AP laxity) of the tibia that occurs when specific forces are applied to the knee. This test is particularly useful because the ACL restrains the anteriorly directed motion of the tibia relative to the femur. Thus, it provides an overall assessment of ACL integrity [31] and has been shown to be proportional to the strength and stiffness of the healing graft [32, 33].

Preclinical models frequently include a measurement of AP knee laxity at different time points after joint harvest. This is done in part because it provides a direct link to measures commonly used in clinical trials. However, in a preclinical model, it is possible to perform these measurements more accurately because the motion can be measured directly between the tibia and femur as these bones can be clamped directly to a material testing system after limb harvest (Fig. 13.5). Knee laxity measurements should also be performed at different knee flexion angles since the amount of laxity is dependent on the knee flexion angle in the normal ACL. A treatment method should therefore restore laxity over a range of knee flexion angles. Although restoring normal AP knee laxity at the time of surgery is possible in preclinical models [35], it is interesting to note that knee laxity following ACL

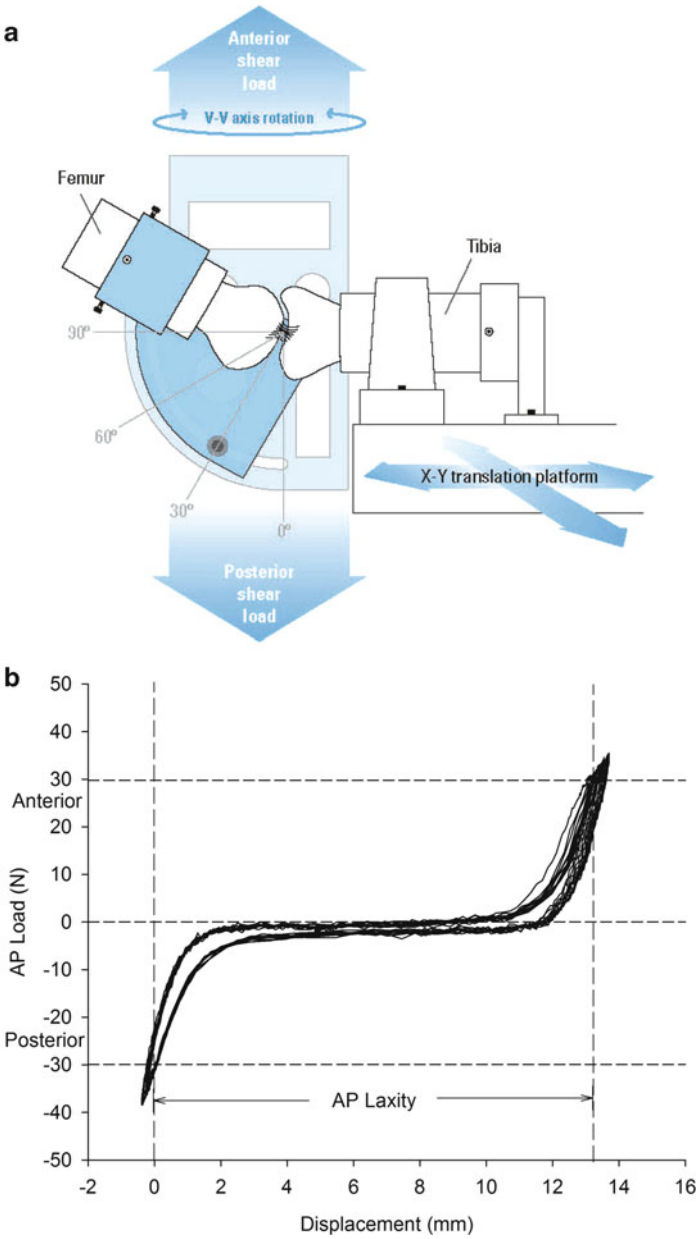

Fig. 13.5 *Anteroposterior laxity testing*. Knee joint laxity can be directly measured after joint harvest. (**a**) The system shown here measures the translation of the tibial relative to the femur in the sagittal plane. The joint is mounted to a fixture to position the joint, while a material testing system applies the shear loads (Used with permission from Murray et al. [34]). (**b**) The load versus displacement response for the anteroposterior (*AP*) laxity test is shown here. Laxity is defined as the total distance of travel that occurs between specified AP shear loads. The magnitudes of the shear loads typically used are both model and study dependent (Used with permission from Murray et al. [34])

reconstruction becomes much looser with time in the commonly used preclinical models [36]. For example, Cummings et al. reported an increase in laxity of approximately 8 mm in the ACL-reconstructed knee when compared to the contralateral control after 16 weeks of healing with most of the increase occurring within the first 2 weeks of surgery [36]. This average increase is much greater than that generally seen in humans following ACL reconstruction (~2 mm). This emphasizes the need for novel approaches to improve healing and joint function after ACL injury. It is thought that the increases in laxity seen in most animal models as compared to humans may be due to our inability to control rehabilitation in the animal models immediately following surgery. Although the laxity increases are greater in animal models, it has recently been shown that a bio-enhanced ACL reconstruction reduces the magnitude of the increase as compared to traditional ACL reconstruction in the porcine model [19].

Functional assessments of the treated joint are also important for preclinical models. These assessments include activity level monitoring (i.e., does the animal become less active?), the detection of a limp (i.e., does the animal protect the treatment leg?), and in vivo joint motion (i.e., even if the animal appears not to limp, are the kinematics of the knee different that normal?). Gait analysis techniques are becoming increasingly popular. One of the challenges with doing gait analysis, particularly for human studies, is in the accuracy of these measurements since the goal is to measure bone-to-bone motion through the soft tissue envelope (skin, muscles, and fat) surrounding the joint. However, with preclinical models, it is possible to place pins directly into the bone so that accurate measurements can be obtained [37]. It is now possible to make accurate 3D bone-to-bone measurements using stereo videoradiography to assess gait following injury and treatment in preclinical models and human studies [38, 39]. As the availability of these technologies is expanding, they should become important assessment tools for the evaluation of different tissue-engineered constructs to treat the ACL injury. With an accurate representation of the joint kinematics, it is then possible to look at the changes in the articular surface interactions which may shed light on cartilage health [38, 40].

Outcome Assessment in Human Trials

Throughout the years, clinical outcomes research has grown immensely. Generally, there are three categories of outcome measurements, including general health status questionnaires, pain measurement tools, and ACL-specific outcome measurements.

One of the best known general health instruments is the Short Form-36 (SF-36), a 36-item questionnaire that quantitatively measures physical and mental outcomes (Fig. 13.6) [42]. The SF-36 has been extensively investigated to confirm its validity and reliability, and it has been translated into more than 40 languages, as part of the International Quality of Life Assessment Initiative. To further improve the efficacy associated with the SF-36 form and to decrease costs, a shorter version of the questionnaire was constructed in the mid-1990s, aptly named the SF-12.

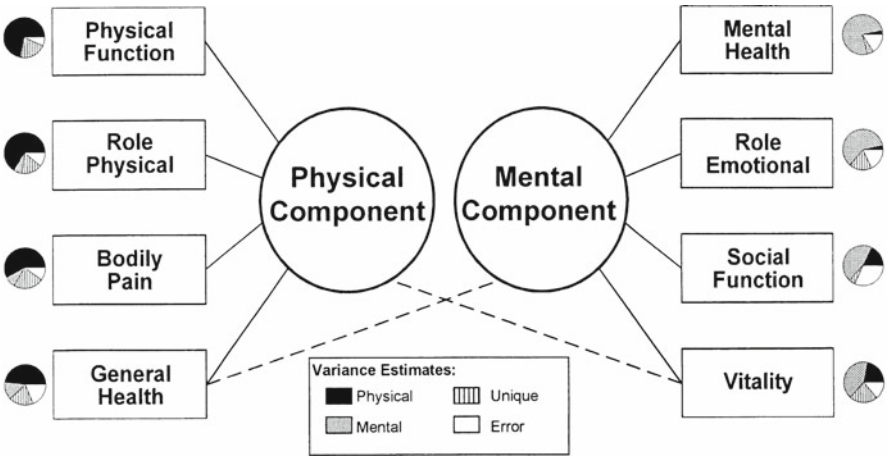

Fig. 13.6 *Components of the SF-36 quality-of-life score*. The Short Form (SF)-36 is an eight-scaled quality-of-life assessment tool that is frequently used in health economics and medical research to establish quality-adjusted life years (QALY). QALY are calculated by multiplying years with the quality-of-life during those years. The eight scales assess parameters of mental and physical health, where each scale is transformed to a 100-point scale, assuming that each area carries equal weight. A commercial form of the SF-36 is available for purchase, but it is important to know that the original SF-36 is available free of charge in the public domain of the RAND corporation (rand.org), the developers of this score (Reprinted from *J Clin Epidemiol*, John E. Ware and Barbara Gandek [41], with permission from Elsevier)

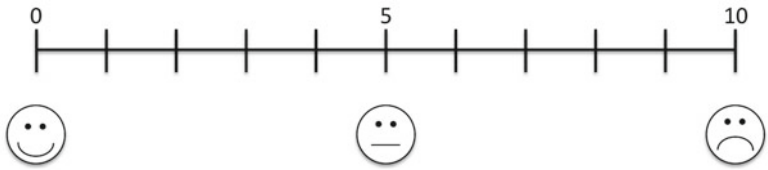

Fig. 13.7 *VAS scale*. Figure shows a Visual Analog Scale, or VAS for short. The image, or a similar variation, is shown to the patient and he is asked to rank a health care outcome – be it pain, knee function, subjective satisfaction, etc. – using the pictograms. The bar is typically normalized to 10 cm, or 100 mm, and the distance in cm or mm of the patient's evaluation from the left side is the single- or double-digit VAS score

A number of pain measurement tools exist, but none is as popular and easily performed as the Visual Analog Scale, or VAS for short [43, 44]. The Visual Analog Scale (VAS) consists of a straight line with the endpoints denoting extreme limits, such as "no pain" and "pain as bad as it could be." Patients are simply asked to show their pain level between the two endpoints of the line (Fig. 13.7). The line is generally approximately 10–15 cm in length, because studies have shown this length is the easiest for patient use, and it results in the smallest measurement error.

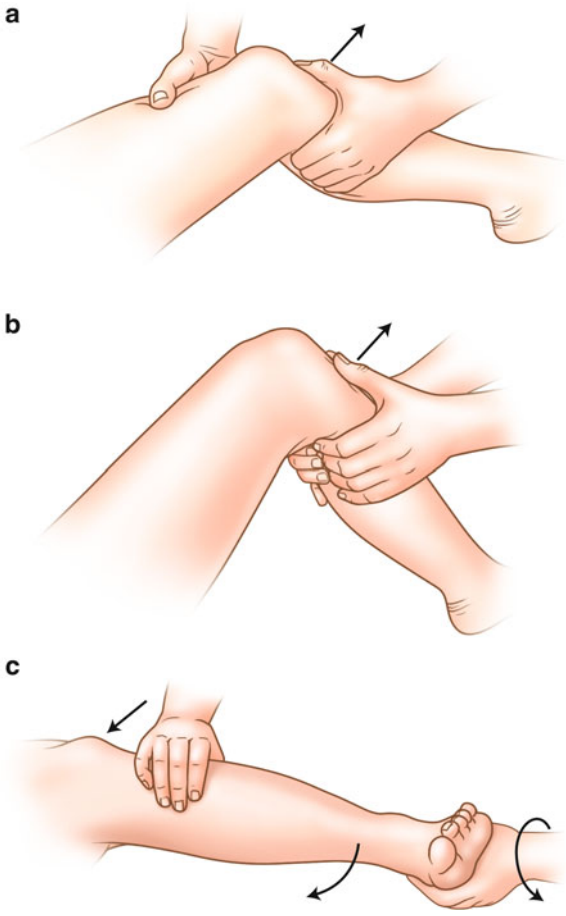

Fig. 13.8 *Lachman test and pivot shift test*. How to perform three common clinical tests for the ACL, the (**a**) Lachman test, (**b**) anterior drawer test, and the (**c**) pivot shift test. (**a**) The Lachman test assesses ACL-dependent AP stability of the knee. The patient is supine and the examiner is on the side of the involved leg. The knee is grasped firmly at the distal femur and the proximal tibia and flexed to 30°. An AP force, that is, pull on the tibia, is applied as the femur is stabilized. The amount of AP translation (in mm) as well as the stop (hard versus soft) is recorded. The absolute AP translation as well as side-to-side differences is noted. Alternatively, the femur of the patient rests on the knee of the examiner and is stabilized with one hand while the other hand applies an anterior force. This modified Lachman test is particularly helpful for examiners with small hands or in athletes with well-developed, muscular legs. (**b**) The anterior drawer test is a second test of anteroposterior knee stability. With the patient lying on his back, the knee is flexed to 90° and the foot is flat on the stretcher. The examiner sits on the foot to stabilize it and holds the tibia just below the joint line with both hands. The index fingers should palpate the hamstrings in the back of the knee to make sure they are relaxed; the thumbs palpate the tibia and femur in the front to assess relative translation between these bones. The anterior drawer test has a potential for bias and can be false negative. For example, both the hamstrings (by holding back the tibia) and the menisci (by acting as "door stoppers") can affect the anterior drawer test. (**c**) The pivot shift test assesses ACL-dependent rotational stability of the knee. The patient is supine and the examiner is on the side of the involved leg. The knee is held at the heel and flexed to 30° and rotated internally. The second hand of the examiner is placed just below the head of the fibula to create a valgus stress. As the knee is flexed, the lateral tibial subluxes if the ACL is incompetent. At approximately 30 degrees of flexion, the tension or the iliotibial band increase enough to reduce the subluxed tibia with a palpable clunk

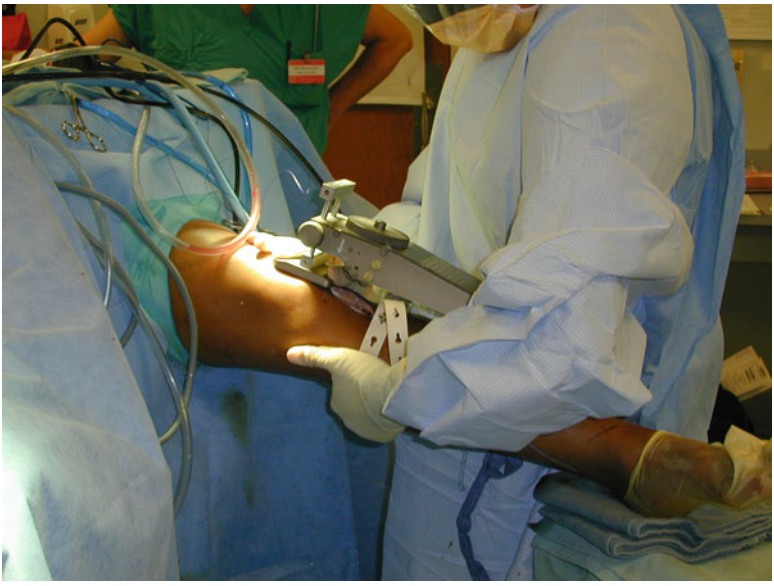

Fig. 13.9 *KT-1000*. The KT-1000 is an instrumented test of anteroposterior stability of the knee. The apparatus, as shown in the figure, is placed over the knee joint and strapped to the tibia. This device allows one to measure the anteroposterior translation with a specified or maximum force. Different variations exist, including one that can be sterilized for use in the operating room

The distance from the "no pain" endpoint represents the patient's pain score. To simplify the process, a mechanical VAS is available; it has a sliding tool that patients move corresponding to their pain. Overall, the VAS is a sensitive, reliable, and easy assessment tool to use for evaluation of pain in patients.

ACL-specific outcome assessment consists of clinical exams and clinical scores. Simple tests of anteroposterior and rotational stability are the Lachman test, the anterior drawer test, and the pivot shift test (Fig. 13.8) [45]. The Lachman test assesses anteroposterior stability *near full extension* and is the most reliable ACL test and probably the oldest one, too. Georges Noulis (1849–1919), a Greek surgeon living in practicing in Paris and Athens in the mid-nineteenth century, described the principle of the Lachman test as early as 1875. The anterior drawer is a test of anteroposterior stability *at 90 degrees of flexion* but can be biased by the menisci acting as wedges, or "door stoppers" that affect translation.

In order to standardize laxity testing, instrumented devices have been developed. Instruments, such as the KT-1000, are displacement measuring instruments that are strapped to the leg and are able to measure knee anteroposterior translation in mm at specific loads (usually 15, 20, and 30 lb) [30]. A sterilizable version of the KT-1000 has been developed for use in the operating room (Fig. 13.9). Another similar mechanical instrument is the rolimeter, which gives translation in mm without measuring load and can also be used in a sterile setting [46] (Fig. 13.10).

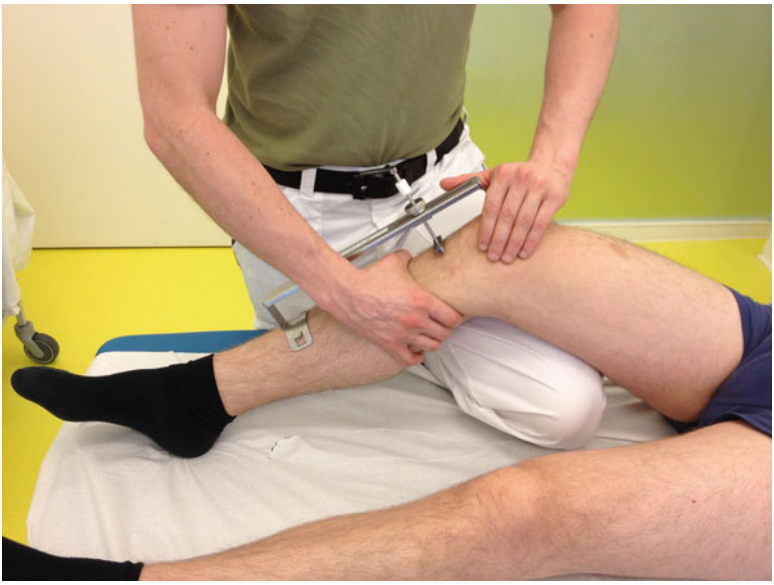

Fig. 13.10 *Rolimeter*. The rolimeter is a simplified version of the KT-1000. It is a purely mechanical instrument that measures anteroposterior translation at maximum stress. It can also be sterilized for use in the operating room and is cheaper than the KT (Courtesy of Matt Wimmer, MD)

The pivot shift assesses rotational stability, by causing a subluxation in the knee through internal rotation of the tibia and valgus stress at full extension. The knee is then bent and the iliotibial tract reduces the subluxation, during flexion, with a palpable clunk. Given this crucial role of the iliotibial tract, its integrity is crucial for test to work.

Lastly, ACL-specific scores exit that combine assessment of pain and function. Currently more than 50 such tools have been published. The best known and most commonly used ones are the Lysholm scores, the Tegner score, the Noyes/Cincinnati rating, the Knee Osteoarthritis Outcome Score, and the IKDC form. These are summarized in Table 13.1.

Summary

Outcome assessment measures are used to assess how well a medical treatment functions. For clinical treatments, this can include measures of safety (i.e., the rate of complications) and effectiveness (i.e., resolution of pain or improvement in function). For basic science, outcome assessment often refers to objectively quantifying

Table 13.1 *Knee scores*. An overview of some of the more commonly used scoring systems for the knee. These scores are time-tested and scientifically validated. Numerous other scores, some validated some not, exist

Score name	Abbrev.	Area	Maximum points (best result)	Description	Reference
Knee society score	KSS	OA	200	Two subgroups (knee exam and function) 100 points each	Insall JN. *CORR*. 1989;248:12
Oxford knee score		OA	48	12 multiple choice questions	Dawson J. *J Bone Joint Surg Br*. 1998;80:63–9
Knee injury and osteoarthritis outcome score	KOOS	OA/ACL	100 points each on five subscales	Five subgroups (symptoms and stiffness, pain, function – ADL, function – sports, QoL)	Roos EM. *J Orthop Sports Phys Ther*. 1998;28:88–96
Western Ontario and McMaster Universities Arthritis Index	WOMAC	OA	96	24 items in 3 subgroups (pain/ stiffness/physical function)	Roos EM. *J Orthop Sports Phys Ther*. 1998;28:88–96
International knee documentation committee	IKDC	OA	100	Ten items in three subgroups (symptoms, sports activity, function, and ADL)	
Modified Cincinnati rating system		ACL	100	Eight multiple choice questions	Noyes FR. *CORR*. 1989;246:238–49
Tegner Lysholm knee scoring scale		ACL	100	Eight multiple choice questions	Tegner Y. *CORR*. 1985;198:43
Knee outcome survey – activities of daily living scale	KOS – ADLS	OA/ACL	70 (=100 %)	14-item scale (5 points each)	Irrgang JJ. *J Bone Joint Surg Am*. 1998; 80:1132–45
Knee outcome survey – sports activity scale	KOS – SAS	OA/ACL	55 (=100 %)	11-item scale (5 points each)	Irrgang JJ. *J Bone Joint Surg Am*. 1998; 80:1132–45
Hospital for special surgery score	HSS	OA	100	Six questions and one category for subtraction of points	Insall JN. *JBJS*. 1976;58A:754

cellular behavior and characteristics and other laboratory measurements. When developing tissue engineering treatments for improving outcomes following ACL injury, evaluation of the tissue-engineered structure is necessary to optimize the function of the repaired or replaced ligament. These evaluations are important in generating confidence that the method will be safe and efficacious prior to moving to a first-in-human trial.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR047910, RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Part IV
Stimulating Healing of the ACL Using Tissue Engineering

Chapter 14
Scaffolds and Biologic Additives for ACL Surgery

Ryu Yoshida and Martha M. Murray

Why Study Tissue Engineering?

Most ligaments in our bodies have excellent healing capabilities. Ligament ruptures typically do not require any surgery and can be treated with conservative management through rehabilitation. However, the ACL is an exception. ACL injuries are notorious for their poor healing and often require surgical treatment. The poor healing ability of ACL may be due to the intra-articular environment of ACL, as discussed in previous chapters.

If the local environment is limiting ACL healing, then appropriate modifications of the environment may allow ACL to heal better. This is the basic concept behind tissue engineering of the ACL. In this chapter, we will review various tissue engineering scaffolds and biologic additives tactics that have been applied to ACL treatment.

Autografts

Reconstruction surgery using hamstring or patellar tendon graft is the current gold standard of treatment for a torn ACL. In addition to providing initial mechanical strength, these autografts are thought to serve as natural scaffolds for cell and vessel ingrowth and healing. Several studies have looked at the remodeling process that

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_14,

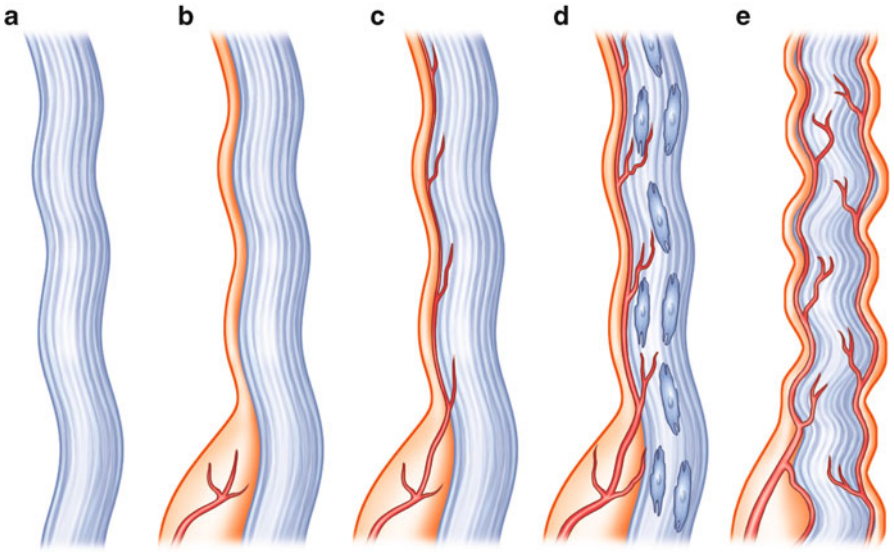

Fig. 14.1 Schematic of the "ligamentization" process. In (**a**), an acellular autograft tendon is seen to have no living cells, vascularity, and a long wavelength of crimp in the collagen fibers. Over time, the tendon is synovialized (*light red*, **c**, and **d**) and blood vessels from the surrounding tissues gain access to the graft (*red*, [**b**]). As the graft revascularizes (**c**), cells also move into the graft and begin to remodel it (**d**). At the completion of ligamentization, the graft fibers have a shorter wavelength, more similar to that seen in the normal ACL (**e**)

autografts go through postoperatively (Fig. 14.1). Janssen et al. studied human hamstring autografts after reconstruction surgery. They found that cell densities, cell morphologies, and alignment of collagen fibers change over time [1].

Rougraff et al. showed that human patellar autografts also go through postoperative changes such as vascular ingrowth, fibroblast proliferation, and collagen realignment. Thus, use of autografts can be considered an example of biological scaffolds [2].

However, while most scientist and surgeons believe there is a process of "ligamentization" that occurs for ACL autografts, it may only occur in the outer portion of the graft. What revascularization of the graft tissue takes place has been investigated extensively in several studies, with early revascularization of the surface of the graft seen at 2–4 weeks, but a persistent avascular zone in the mid-substance of the graft existing even after 6–12 months after graft implantation (see Chap. 20; Fig. 14.2).

A key advantage of autografts over other scaffolds is the absence of immunogenicity. Implanting any foreign material poses some risk of immunogenic reactions, but because autografts are patients' own tissues, immunogenicity is not a concern. While autografts do have some disadvantages, such as donor-site morbidity, their safety and long track records make them the most widely used scaffolds for ACL injuries.

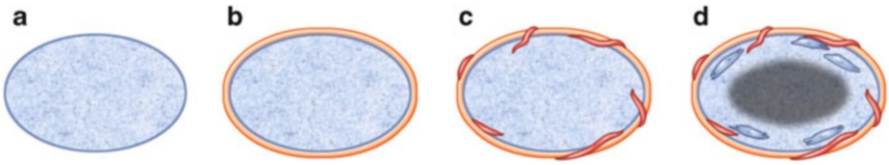

Fig. 14.2 Incomplete "ligamentization." When the graft is implanted (**a**), it has the internal structure of tendon and is not covered with synovium. In addition, it has been removed from its native location and no longer has its own blood supply. While a synovial covering grows around many grafts *in situ* (**b**), bringing vascularity to the outer graft (**c**), the entire graft may not be revascularized, leaving a central, necrotic core (**d**)

Allografts

Allografts are tissues taken from a donor of the same species. For ACL surgeries, patellar, Achilles, or anterior tibialis tendons harvested from cadavers are typically used. Like autografts, allografts also serve as biological scaffolds. After surgery, the graft becomes hypervascular and is infiltrated with fibroblasts, but the cell and vessel densities normalize within 18 months. Collagen bundles also realign during that time [3].

There are several advantages of allografts over autografts. The operative time is shorter because allografts can be prepared prior to surgery. The incision is also smaller with allograft surgery as no graft harvest is required. Because allografts do not have to be harvested from the patient, there is no donor-site morbidity such as pain, paresthesia, discomfort, and decrease in range of knee motion.

However, a major concern when using an allograft is the risk of disease transmission. The cadaver donors are screened, and the tissues are tested carefully, but it is possible to contract serious diseases such as HIV and hepatitis from allografts. The risk of contracting HIV from an allograft is estimated to be one in 1.6 million [4].

Rejection is also a concern with any allograft. Immune reactions from the recipient may slow graft incorporation or prevent ligamentization. In such cases, the graft can fail [5]. Nevertheless, multiple studies have shown that allografts and autografts have similar outcomes in terms of function and symptoms [6], particularly for older, less active patients (see Fig. 1.7). Thus, allograft is an effective scaffold for ACL reconstruction chosen by many surgeons and patients [7].

Synthetic Materials

Many synthetic materials have been evaluated for ACL treatment. Synthetic grafts typically have advantages such as availability, ease of processing, and good initial mechanical strength compared to autographs or allografts. In 1973, a polytetrafluoroethylene (PTFE)-based graft called Proplast by Vitek Inc. was FDA approved as a ligament replacement. However, it yielded satisfactory results in only 52 % of patients and quickly fell out of favor.

Several more products were introduced in the 1980s. W. L. Gore & Associates introduced a PTFE material called Gore-Tex, and Stryker developed a Dacron (PET-polypropylene) graft. Gore-Tex and Dacron were permanent grafts designed to hold mechanical strength without relying on tissue ingrowth. Although these grafts provided good initial stability, they deformed under repeated stress and eventually failed [8].

Also introduced in the 1980s were synthetic materials designed to serve as scaffolds for healing. Leeds-Keio by Neoligaments Ltd and ABC by Surgicraft Ltd were polyethylene terephthalate (PET) devices designed to promote tissue ingrowth, but their success was limited. Many patients experienced knee instability with Leeds-Keio after 2 years [9]. As for ABC, only 41 % of patients had good results [10].

The Kennedy Ligament Augmentation Device (LAD) was another synthetic device manufactured by 3M. This product was designed to be used in combination with autografts to relieve the mechanical load on autografts. The problem with this device was that it triggered inflammatory responses in many patients, leading to effusions and reactive synovitis [11]. Furthermore, addition of this device to a reconstruction with patellar tendon did not show any benefit [12].

The key lesson from these older devices is that native tissue ingrowth is essential for long-term success. More recent studies on synthetic materials have focused on biocompatible, biodegradable polymers. For example, a class of polymers called polyhydroxyesthers such as poly(L-lactic acid) (PLLA) and poly(lactic-co-glycolic acid) (PLGA) has gained much interest. Three-dimensional braiding of these polymers creates micropores in the scaffold that allow fibroblast ingrowth. The PLLA scaffold degrades slowly in vivo, and pre-coating the scaffolds with fibronectin promotes attachment and proliferation of ACL cells [13]. Another biodegradable material that has been studied for ACL reconstruction is poly(urethane urea) (PUUR). PUUR scaffold reportedly allowed ingrowth of connective tissues, and a rabbit study of ACL replacement with PUUR showed good long-term knee function [14] in that model.

While there are no synthetic ACL grafts available for clinical use, synthetic materials have great potential to be an effective graft. Biodegradable, biocompatible scaffolds that allow cell ingrowth seem the most promising at this time.

Collagen Scaffolds/Xenografts

Collagens are a group of fibrous proteins that are abundant in our bodies. Collagen is one of the most widely used biomaterials. Its clinical applications include artificial skin, suture, bandage, and hemostatic agents (materials to help stop bleeding). Because collagen is the main constituent of native ACL, supplementing an injured ACL with a collagen scaffold would seem helpful. In vitro, fibroblasts are able to attach, proliferate, and express collagen on collagen scaffolds [15]. However, an in vivo study in animals showed that addition of collagen scaffold alone to an ACL wound site does not improve the functional outcomes of a suture repair of the ACL [16].

The collagen-based scaffolds in both of these original in vitro and in vivo studies were made from bovine collagen. One of the limitations of these scaffolds is that the collagen fibers are not aligned like the native ACL. To overcome this limitation, some have turned to xenografts. Xenografts are tissues transplanted from one species to another. ACLs from animals have similar structure and composition to the human ACL, so ACL xenografts may be more helpful than a scaffold with disorganized collagen fibers. However, Good et al. found that ACL reconstruction with bovine xenografts led to severe immunologic reactions in humans [17]. Decellularization protocols with detergents can reduce immunogenicity, but the use of xenografts is still an ongoing area of research [18] and these are not currently in use in clinical ACL surgery.

Cell Seeding

One potential way to improve the effectiveness of scaffolds is to seed them with cells. By delivering cells that can participate in ligament healing, such as fibroblasts, the ACL may heal better and faster. Several studies suggest that the combination of collagen scaffolds and fibroblasts may be particularly beneficial. Huang et al. showed that fibroblasts strengthen collagen scaffolds in vitro [19]. Dunn et al. found that fibroblasts synthesized tenfold more collagen when cultured on collagen scaffolds instead of culture plates [15]. Bellincampi et al. implanted fibroblast-seeded collagen scaffolds in rabbit knees. The seeded fibroblasts remained attached to the scaffold and were viable 6 weeks after the implantation (Fig. 14.3) [20].

Others advocate the use of bone marrow stromal cells (BMSCs, also known as mesenchymal stem cells (MSCs)) over fibroblasts. BMSCs are multipotent cells that have the capability to differentiate into several cell types; they can be obtained via a bone marrow aspiration. Van Eijk et al. compared BMSCs and fibroblasts cultured on resorbable suture material. After 12 days, BMSCs had significantly more cell proliferation and collagen expression than the fibroblasts [21]. Applying

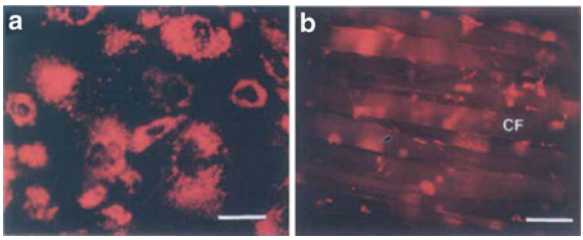

Fig. 14.3 (**a**) Anterior cruciate ligament fibroblasts labeled with PKH26-GL red fluorescent dye attached to a tissue culture plate (bar = 40 um.) (**b**) ACL fibroblast-seeded ligament analog after 3 days of incubation in vitro (bar = 150 um). Cells remained viable and attached to the collagen fiber (*CF*) scaffolds under these cell culture conditions. Similar observations were noted for skin fibroblast-seeded constructs (not shown). *Arrowhead* indicates an anterior cruciate ligament fibroblast (Used with permission from Bellincampi et al. [20])

mechanical forces can further induce expression of collagen and ECM proteins and realign collagen fibers [22].

Other combinations of cells and scaffolds have also been studied. Fan et al. used modified silk scaffolds with BMSCs to reconstruct rabbit ACLs in vivo. Compared to the control scaffolds with no BMSCs, the scaffolds with BMSCs healed better as measured by biomechanical studies and histology [23]. They also used a silk scaffold seeded with BMSCs for pig ACL reconstruction; abundant cells and ECM were seen at 24 weeks postoperatively [24]. Soon et al. reconstructed rabbit ACLs using Achilles tendon allografts with and without BMSCs. Grafts with BMSCs had significantly higher load-to-failure rates and better healing seen on histology [25].

Because ACLs have a poor healing capability on their own, the delivery of cells with high activity such as fibroblasts and BMSCs is an attractive strategy to improve healing. Results of in vitro studies and in vivo animal studies seem promising.

Growth Factors

Growth factors are signaling molecules that regulate activities of cells. Various growth factors are known to be involved in ligament healing, and many studies have been done to determine which growth factors are beneficial to ligament healing. In vitro studies with ligament cells from animals have shown that epidermal growth factor (EGF) [26–28], fibroblast growth factor (FGF) [28], insulin-like growth factor (IGF) [28], platelet-derived growth factor (PDGF) [27, 28], and transforming

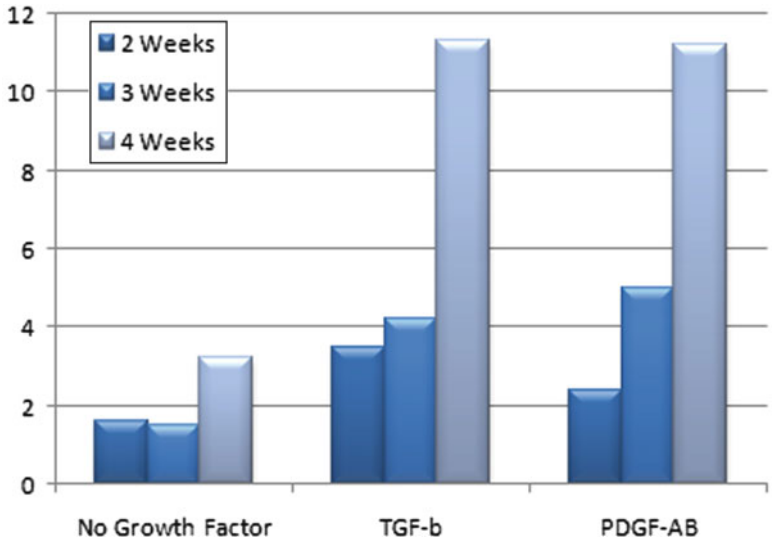

Fig. 14.4 The effect of TGF-b1 and PDGF-AB on collagen synthesis of ACL cells in vitro. Note the strong increase in collagen synthesis, particularly after 4 weeks of culture, when the ACL cells are cultured with either TGF-b1 or PDGF-AB (Data from Murray et al. [29])

growth factor (TGF) [26] improve collagen synthesis and/or cell proliferation. A study by Murray et al. using human ACL cells found that TGF, PDGF, and FGF increased cell proliferation. TGF and PDGF also increased collagen synthesis (Fig. 14.4). However, EGF did not have such effects [29].

A few in vivo studies in animals have also been done. Kobayashi et al. showed that FGF improves the healing and neovascularization of a partially lacerated ACL in canines [30]. Spindler et al. found increased collagen expression and maximum load when recombinant human TGF was added, but they used MCL in rabbits, not an ACL model [31]. Letson et al. showed that rat ligaments increased their stiffness when PDGF was added, but this study was also done using the MCL [32] rather than the ACL.

Platelet-Rich Plasma (PRP)

Supplementation of ACL repair or reconstruction with growth factors may be an effective strategy, but it has a few limitations. Growth factors are difficult to purify and can be very expensive. They often degrade or dissipate quickly, so even with a large loading dose at the time of surgery, the growth factor concentrations near the injury site may quickly become low. Because of these limitations, some researchers have turned to other ways of delivering growth factors.

Platelet-rich plasma (PRP) has been a topic of great interest in orthopedics. It is made from whole blood and contains high concentrations of platelets. Platelets are rich reservoirs of various growth factors such as PDGF, TGF, FGF, IGF, and vascular endothelial growth factor (VEGF), so they seem ideal for delivering growth factors.

While there have been clinical studies on the clinical benefit of PRP in the treatment of ACL injuries, the results are quite variable. For example, Nin et al. conducted a randomized control trial comparing the outcomes of patients undergoing an ACL reconstruction with an allograft supplemented with PRP and those undergoing allograft reconstruction alone. No difference was seen in inflammatory parameters, appearance on MRI, or clinical outcomes between groups with and without PRP [33]. Vogrin et al. also studied if PRP would affect the outcome of ACL reconstruction with a hamstring autograft. They found no difference on MRI in vascularization of the intra-articular part of the graft. However, they did see an increase in early vascularization of graft at the interface of the graft and bone [34].

Orrego et al. reported different results. Six months after surgery, patients who had received PRP supplementation of their graft were significantly more likely to have low-intensity signal on MRI, which is a sign of graft maturation [35]. Silva et al. also found that PRP did not affect healing at the bone-graft interface. They conducted a prospective study of patients having an ACL reconstruction with hamstring tendon autograft. One group had PRP injected into femoral tunnels at the end of surgery. The second group was given PRP at the end of surgery and intra-articularly at 2 and 4 weeks after the surgery. The third group was given PRP

activated with thrombin at the end of surgery. The control group did not receive any PRP. At 3 months after the surgery, there was no difference on MRI among all four groups [36].

One of the reasons the reports on the use of PRP as a biologic adjunct to ACL surgery vary so widely is that the different studies use different PRP formulations, surgical techniques, and methods of evaluation. When there are studies with seemingly conflicting results, a systematic review that compares results from various studies can be helpful. Vavken et al. recently conducted a systemic review of the current evidence for PRP treatment, which suggests some benefit on graft maturation but limited effect on graft-bone interface healing [37]. However, additional studies are needed to determine the true effect of PRP on ACL healing.

Extra-cellular Matrix (ECM) Scaffolds and Platelets

The combination of an extra-cellular matrix (ECM) scaffold and platelets deserves specific attention because beneficial effects have been seen recently in large animal models. Fleming et al. showed that supplementation of an ACL graft with an ECM-platelet composite improves the biomechanical properties of ACL allografts in pigs [38]. Murray et al. showed that supplementation of a primary repair of the ACL in pigs with a collagen-PRP hydrogel improves the biomechanical properties of the healing ACL after 4 weeks in vivo [39]. The PRP-collagen hydrogel also increased healing of a central wound in ACL of canines [40]. Finally, while the ACL is known not to heal after primary repair with suture, primary repair with an ECM-platelet composite healed just as well as ACL reconstruction, the current standard of treatment, when measured using biomechanical outcomes [41].

It is not entirely clear why ECM-platelet composites are so effective, but there seems to be a synergistic effect between ECM molecules, including collagen, and PRP. Neither PRP alone nor collagen alone improved outcomes of primary ACL repairs in pigs [16, 42]. Collagen is known to activate PRP and induce platelets to release growth factors [43], but this probably does not fully explain the synergistic effect. Perhaps plasma proteins play a role. PRP contains not only platelets but also proteins such as fibrinogens and prothrombins. In vitro studies have shown that these proteins can influence interactions between collagen gels and fibroblasts [44, 45]. Cheng et al. have also shown that the combination of plasma proteins and platelets significantly enhance collagen synthesis by fibroblasts cultured in collagen gels (Fig. 14.5) [46].

Summary

In summary, the number of different scaffolds and biologic additives available now for tissue engineering are somewhat endless. Scaffolds can be biologically based (tendons, collagen or extracellular matrix are the most common) or synthetic. Biologic additives can include cells, platelets or growth factors. While the current

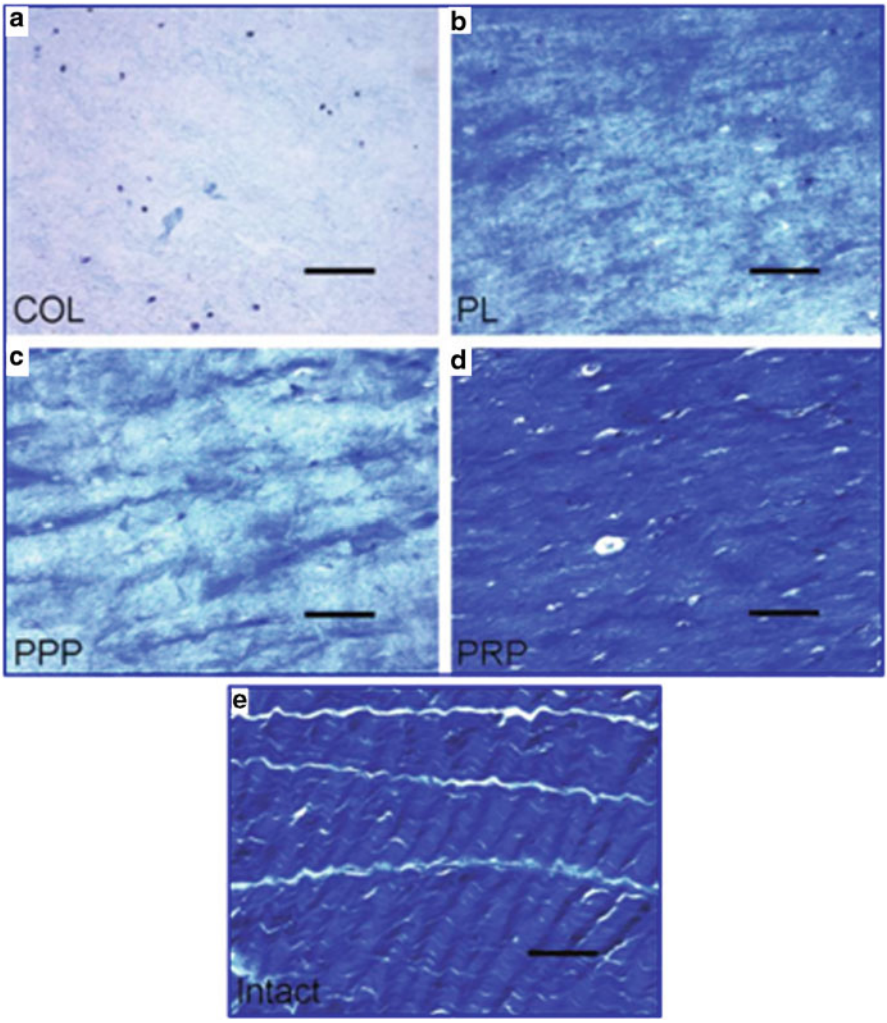

Fig. 14.5 Collagen in constructs of ACL cells and the addition of collagen only (**a**), platelets only (**b**), plasma without platelets (**c**), and platelets and plasma (**d**). All are compared with the intact ACL (**e**), Scale bars: 100 um. Note the similar intensity of staining in the intact and platelet + plasma groups (Used with permission from Cheng et al. [46])

most useful strategy for bio-enhanced ACL repair appears to be a combination of an extra-cellular matrix scaffold and platelets, there are likely to be many more combinations of scaffolds and biologics to come in the future as we continue to design and validate new solutions for repair of tissues inside the joint like the ACL.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 15
Use of Biologics to Treat Partial ACL Tears

Martha M. Murray

It is well known that complete ACL ruptures fail to heal spontaneously. Interestingly enough, even partial ACL tears regain little strength or function after injury. In animal models, partial ACL transections have been shown to regain only 12–14 % of the ACL failure load of the contralateral ACL-intact knee (control) after 6 weeks of healing [1, 2] and only 26 % of that for the control ACL-intact knee after 3 months [1, 3]. As we worked to understand how to get the ACL to heal, we felt that perhaps learning how to get a partial ACL tear to heal would help us make progress toward our eventual goal of getting a complete ACL tear to heal.

Partial tears can be created either by cutting one side or one bundle of the ligament or by making a central cut (leaving tissue on both sides of the cut, Fig. 15.1). A biologic stimulus, like a growth factor or a scaffold, can be placed into the defect and the effects of that treatment then evaluated over time. The ideal treatment would provide an environment for development of a viable, cellular tissue which adheres intimately with the surrounding tissue, increases in strength with time, and is capable of repairing damage as it occurs (a functional scar). To accomplish these goals, the scaffold used for treatment should initially integrate with the host tissue, support the gradual ingrowth of surrounding cells, supply nutritional needs via diffusion or vascular invasion, and facilitate cellular extracellular matrix production and organization to strengthen the repair site (Fig. 15.2). The wound healing process in extra-articular tissues that accomplishes these goals is vastly complex as we've seen in Chap. 6. To review in brief, the process starts with primary hemostasis and formation of a platelet plug. The platelets release over twenty known growth factors in a sequential fashion. These growth factors stimulate the surrounding cells, encouraging cells from the blood and surrounding tissues to migrate into the wound site (chemotaxis) and release of other growth factors and matrix proteins.

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_15,

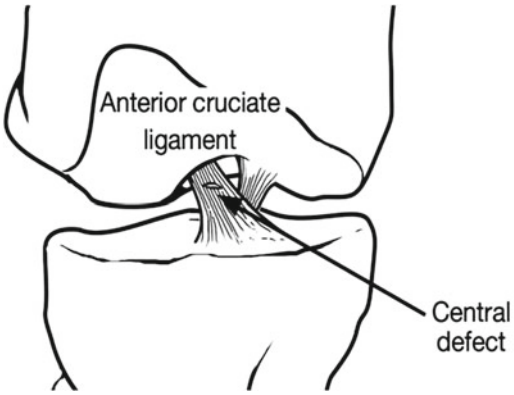

Fig. 15.1 Schematic of the central ACL defect model. A modified Beaver Blade is used to transect the central 40 % of the ACL fibers from anterior to posterior, leaving the peripheral fascicles intact for wound site stability (Used with permission from Murray et al. [4])

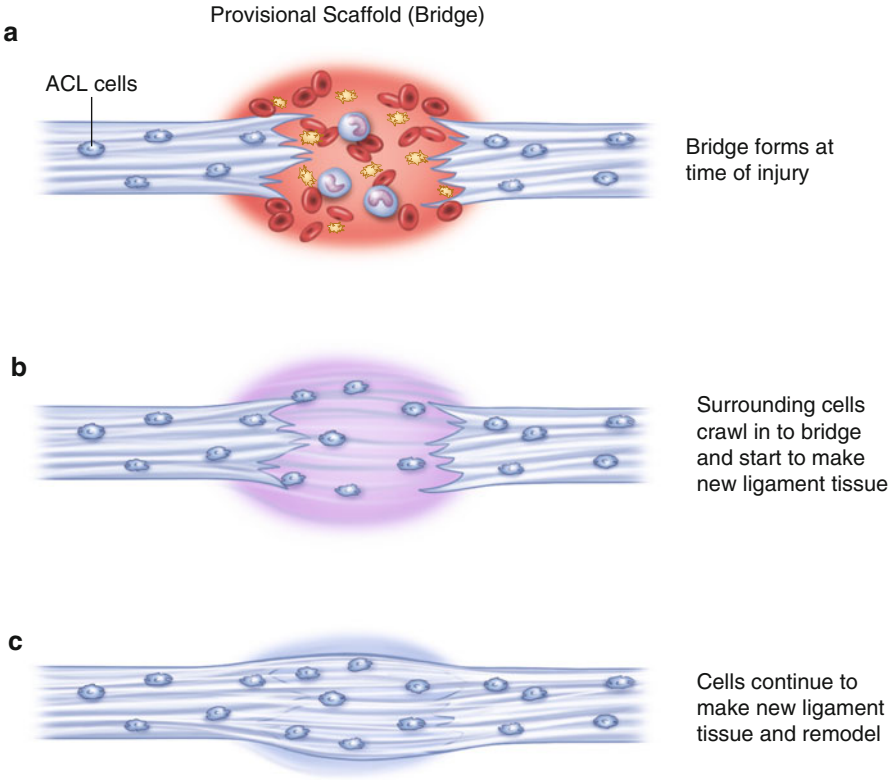

Fig. 15.2 Schematic of provisional scaffold formation and remodeling into a functional scar. Initially, a blood clot forms between the two torn ends of the ligament (**a**). Surrounding cells crawl into the provisional scaffold and begin to create new ligament tissue within this "bridge" (**b**). The provisional scaffold is gradually replaced by collagen and other extracellular matrix proteins commonly found in the normal ligament, and the ligament is considered healed (**c**) (From Murray et al. [5])

As adding one or two growth factors has only met with limited success in stimulating ligament healing [6–8], we have worked on adding both a scaffold material and a biologic stimulus to try to get a central ACL defect to heal, and those results are presented here.

Use of Individual Growth Factors to Treat Partial ACL Tears

In connective tissues which heal, such as the medial collateral ligament (MCL), the initial step in healing is the formation of a blood clot in the site of tissue injury, which serves as a provisional scaffold for healing. The clot contains platelets that release growth factors, including isoforms of PDGF and TGF-β, thought to be important in tissue healing [27]. An inflammatory reaction follows, during which time the provisional scaffold is invaded by neutrophils and then macrophages. Next, the scaffold is gradually invaded by fibroblastic cells that proliferate and produce extracellular matrix proteins [22] and form a vascular [23] scar. The early repair tissue is subsequently remodeled and increases in the ratio of type I: type III collagen are seen, as well as increases in the degree of covalent, intermolecular collagen cross-linking and collagen fibril diameter [27]. This remodeling results in scar tissue that becomes increasingly similar to the normal ligament with time [24].

FGF-2, PDGF, and EGF are present in elevated concentrations in healing tendons [28]. TGF-β1 has been found in elevated levels for up to 8 weeks after wounding of the patellar ligament in rats [29] and of the flexor tendon in rabbits [30]. Initially the TGF-β1 is extracellular, suggesting platelet degranulation as its source, but at later time points, in situ studies have found it to be cell-associated, suggesting that cells are being stimulated to actively produce this growth factor [29]. In the medial collateral ligament of the rabbit, mRNA for TGF-β1, IGF-1, IGF-II, and endothelin-1 remain elevated at 3 weeks after injury [31], while FGF-2 was inhibited initially, then rose back to normal levels [31]. The receptors for IGF-II [31], FGF-2 [32], and EGF [33] have all been found in injured ligaments.

Thus, one strategy for encouraging partial tears of the ACL to heal would be to add one or more of these growth factors found in successful tendon and ligament healing to see if that would stimulate the ACL to also heal successfully.

Others have tried this approach for a partial tear of the ACL. FGF-2, or basic fibroblast growth factor, has been used to try to stimulate healing of a central defect of the ACL [9]. In that study, the investigators created central defects in the ACL using a biopsy punch. In one group, a pellet containing FGF-2 was placed into the defect, and in the second group, the pellet without any FGF-2 was placed as a control. The ligaments were allowed to heal for time up to 24 weeks and then examined under the microscope to see if the treatment with FGF-2 made any difference. The researchers found that the use of FGF-2 resulted in improved orientation and organization of the healing ligament, as well as improved blood supply to the injury site with use of FGF-2 (Fig. 15.3).

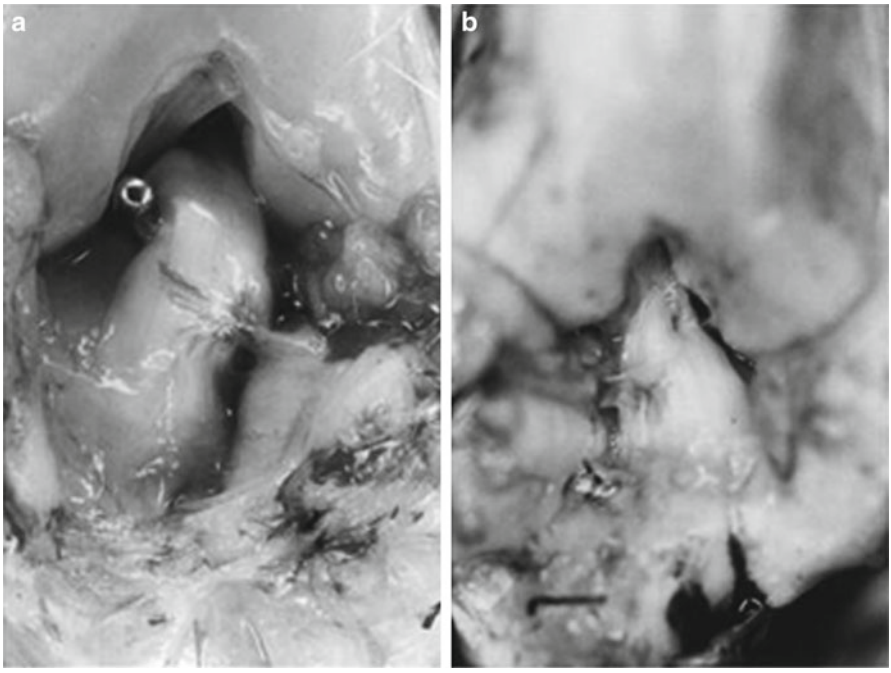

Fig. 15.3 (**a**) Macroscopic observation of the basic fibroblast growth factor (FGF-2) group specimen (the left knee) at 6 weeks; the defect was covered with reparative fibrous tissue. (**b**) Macroscopic observation of the control group specimen (the right knee) at 6 weeks; the cylindrical defect created with a 1.8-mm diameter biopsy punch remained unfilled (From Kobayashi et al. [9])

In our lab, our initial studies focused on the use of only a collagen-glycosaminoglycan sponge to facilitate ACL cell migration [10, 11]. However, migration rates using this substrate were slow [10, 11]. We attempted to stimulate additional cell migration and collagen production using individual growth factors, as other investigators had done, including TGF-β, FGF-2, PDGF-AB, and EGF. However, we only had modest success, with moderate increases in cell proliferation and collagen production rates in the scaffold [12, 13], and we felt the ingrowth was still inadequate to fix the problem of an ACL defect.

We were still left with the problem of which growth factors to add to the provisional scaffold to stimulate wound healing. In examining the wound healing process in other tissues, it quickly became clear that this was a very complex process that starts with platelet activation. The platelets then begin to sequentially release a variety of growth factors and proteins, which summon additional repair cells to the wound site (including neutrophils and macrophages). Rather than trying to design a de novo implant which contained all of the factors required for the wound healing process at the right concentrations, we elected to work toward stabilizing the platelet-fibrin plug for the intra-articular environment and allowing the wound healing cascade to occur as it does in tissues which successfully heal.

We found that mixing the platelets and plasma proteins with a soluble collagen gel resulted in a composite provisional scaffold that was resistant to plasmin

degradation in vitro. This is likely due to the fact that collagen requires an MMP cofactor to be degraded and cannot be degraded by plasmin alone when placed into a fibrin matrix [14]. An additional benefit of the collagen was that it also activates platelets, thus triggering the initial phase of the wound healing cascade. Cell migration assays and cell proliferation assays proved that this combination was fibroinductive for ACL cells and supported cell proliferation and collagen production [15]. Using these assays, we were able to optimize the concentration of platelets that optimally stimulated cell migration, cell proliferation, and collagen production, and this provisional scaffold used for our in vivo pilot study to treat a partial ACL tear.

Development of a Model for Partial ACL Tears

While we had what appeared to be promising results for encouraging ACL cell growth and collagen production in the petri dish, the key question still remained: could use of a substitute scaffold in the wound site of a living ACL promote wound healing in the synovial environment? To answer this question required the development of appropriate animal model for ACL injury and determining the effect of treatment using clinically relevant outcome measures including histologic and biomechanical measures [4, 16–20].

To approach this question, we developed a model of simulating primary repair of an intra-articular tissue injury which failed to heal, even after 6 weeks in vivo. The model was a central ACL defect in a large animal [17] (see Fig. 15.1). The canine model was selected given the similar histologic cell and vessel distribution in the canine and young human ACL [20]. The defect was stabilized mechanically on either side by intact ligament fascicles. However, even though there was no gross motion between the re-approximated ligament ends, the untreated defect did not fill with any type of healing tissue at any time point (Fig. 15.4). This model represented the clinical situation of failure of primary suture repair (where sutures stabilize the ends of the ligament but healing still does not occur). As the model eliminated the mechanical variability inherent with different suture techniques, it was an ideal model for evaluating any change in healing due solely to improved biologic stimulation.

Functional Histologic Response in the Animal Model

As we had seen in our published studies of ACL wound healing in the canine model, there was a substantial lack of provisional scaffold, therefore poor wound site filling in the ACL defects. This was similar to what had been observed clinically in the human condition. Therefore, we had validated a biologically relevant model to test the hypothesis that placement of a substitute scaffold into the wound site of the ACL would stimulate healing.

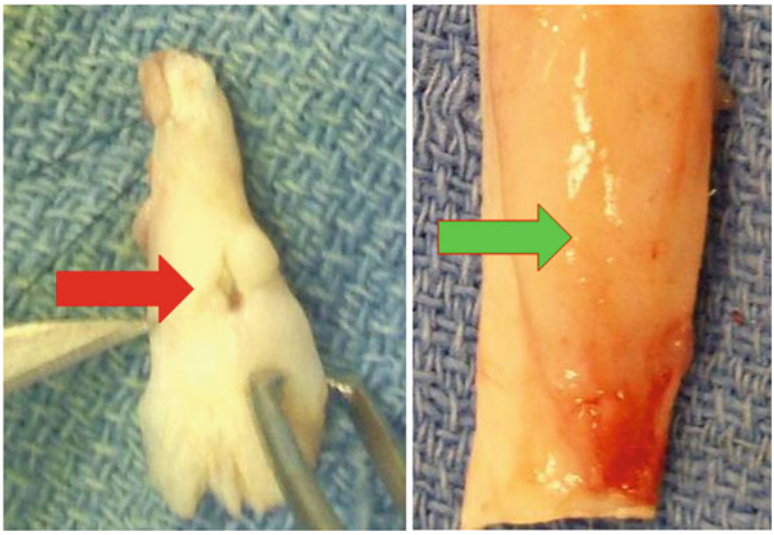

Fig. 15.4 Comparison of wound sites for the intra-articular ACL (*left*) and extra-articular patellar ligament (*right*) after 6 weeks in vivo. Note the abundant granulation tissue covering the area of the defect in the patellar ligament wound on the right and the lack of defect filling or surrounding vascularized tissue for the ACL wound on the left

We developed and validated a histologic scoring system to grade the ligament healing response [19]. In the defects in the extra-articular ligament, wound healing progressed in an orderly fashion with filling of the defect with a fibrin-platelet plug seen at 3 days, infiltration with inflammatory cells at 3 days, gradual fibroblast and capillary invasion at 10 and 14 days, collagen production at 21 days, and almost complete remodeling of the defect at 42 days (Fig. 15.5, first row). However, in the untreated ACL defect no healing was observed (Fig. 15.5, second row). Minimal, if any, filling of the defect was observed. There was often a hypertrophic synovial layer response near the defect which did not extend to fill the defect.

The Use of Extracellular Matrix (ECM)-Platelet Composites as a Substitute Provisional Scaffold

To test the hypothesis that placement of a substitute scaffold could stimulate functional healing of the ACL, pairs of knees had a central defects placed bilaterally. One ACL defect was left untreated, while the contralateral ACL defect was treated with a ECM-PRP hydrogel. In comparison to the untreated defects, the healing response in the ACL wound changed to a sequence that looked markedly similar to the successful histologic healing response seen in the MCL and patellar ligament (see Fig. 15.5, third row). Instead of an empty cavity, the wound site was filled with the hydrogel initially and then quickly invaded by inflammatory cells. Subsequent

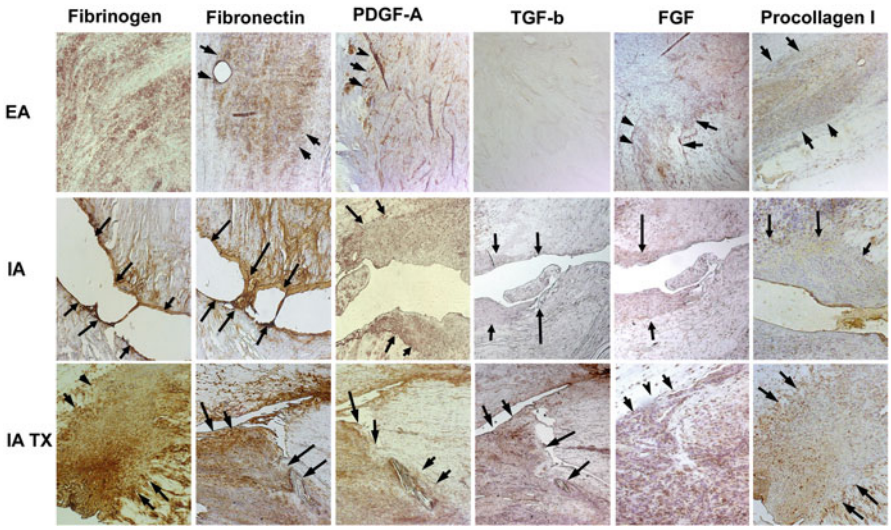

Fig. 15.5 Representative photomicrographs of the patellar ligament wounds (extra-articular (*EA*); *first row*), untreated ACL wounds (intra-articular (*IA*); *second row*), and ACL wounds treated with ECM-PRP scaffold (intra-articular (*IA*) *TX*; *third row*) 21 days after wounding (10×) (Used with permission from Murray et al. [19]). Similar distributions of protein presence were noted in the treated ACL wounds and the healing patellar ligament wounds (*short arrows*). The untreated ACL wounds remain relatively empty of any substratum (*long arrows*)

invasion by capillaries and fibroblasts was seen as early as 21 days, and by 42 days, fibrovascular scar complete with collagen alignment and crimp visualization was seen. Expression of TGF-β, FGF-2, and PDGF-AB was all observed within the wound site for up to 3 weeks. Fibrinogen and fibronectin persisted in the provisional scaffold for the entire 6 weeks [19]. Thus, the use of a ECM-platelet composite placed in the central wound of the ACL stimulated healing via pathways seen in tissues which naturally heal successfully, like the MCL.

Biomechanical Strength of the Healing ACL

We also hypothesized that use of a substitute provisional scaffold would enhance the biomechanical strength of the healing ligament. While histologic improvement in healing was encouraging, clinically relevant outcome measures would be the restoration of mechanical strength and stiffness to the tissue. To test whether the histologic response observed at 6 weeks corresponded with a return in mechanical strength, an additional experiment was conducted where treated and untreated ligament tensile strengths were measured after 6 weeks in vivo [4]. A significant 40 % increase in strength was measured in the treated group, while there was no significant restoration of ligament strength in the untreated knees for the same time period

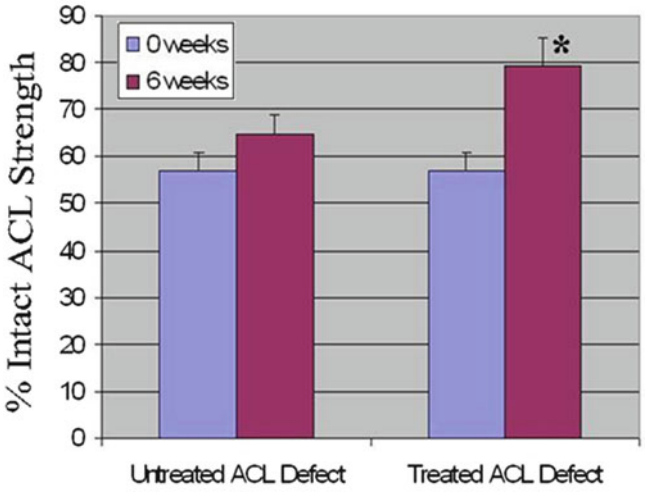

Fig. 15.6 Influence of treatment on restoration of ligament strength after injury. In the untreated group, no significant return of strength was observed. In the treated group, a significant increase in strength of 40 % was seen in the 6-week period (denoted by *) (Used with permission from Murray et al. [4])

(Fig. 15.6). The maximum tangent modulus also increased in the treated knees from 56 % of the intact ligament to 79 % of the intact ligament value at 6 weeks. Thus, the hypothesis was proven, and the increase in strength in the ligaments treated with the ECM-PRP hydrogel was similar to that previously reported in the successfully healing MCL at the 6-week time point [21].

Partial ACL Tear Conclusions

While use of single growth factors may not have enough of a stimulatory effect on healing, placement of a ECM-platelet scaffold into a wound site of an intra-articular ligament (the ACL) resulted in the transformation of a non-healing persistent defect into a biologically active healing wound (see Fig. 15.5). The healing process resulted in partial return of wound strength at 6 weeks to a level expected in a ligament that goes on to heal successfully. These findings suggest that a partial tear of the ACL can be successfully stimulated to heal if an adequate provisional scaffold substitute is provided. In the next chapter, we will discuss the results of such a technique for complete ACL tears.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 16
Can We Get a Complete ACL Tear to Heal?

Carla Maria Haslauer and Martha M. Murray

As previously discussed in Chap. 2, reconstruction of the ACL is the current gold standard of treatment, as the failure rate with suture repair alone has been described as greater than 90 % [1]. ACL reconstruction relies on the removal of the torn ligament and replacement with patellar tendon or hamstring autografts or with a cadaver allograft, and while it is an excellent operation for restoring gross stability of the knee, one wonders if a solution that preserves the ACL and repairs it might be better. If repair of the ACL could be performed successfully, this might provide several advantages over reconstruction, including preserving the natural anatomy of the ligament, such as the insertion sites and multiple bundle morphology. It is also believed that repair of the ACL would preserve the native physiology, such as the nerves and intrinsic cell populations, as well as some of the complex biomechanical properties of the ligament.

The medial collateral ligament, another commonly ruptured ligament of the knee, has been shown to heal uneventfully with nonoperative treatment. Thus, many comparisons of the ACL to MCL have been made in an attempt to determine differences which may prevent the ACL from healing in a similar manner. Studies have shown that cells in both the MCL and ACL are capable of proliferation. Cells from both tissues have also been shown to produce extracellular matrix molecules, which are essential for forming the framework and structure of the tissues. Studies have also verified that cells from both tissue types are capable of migrating, which is a necessary component of the healing process as fibroblasts and immune cells all play an active role. There were, however, two main differences observed between MCL

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_16,

and ACL healing. First, the MCL is not located within the articular capsule, while the ACL is found within this capsule and is continuously bathed by synovial fluid. Second, it was found that the MCL is capable of forming a bridge across the wound site. The ACL on the other hand is incapable of forming such a bridge, or provisional scaffold. It is believed that in order to develop a method by which one could repair the ruptured ligament, one must overcome the loss of the bridge across the wound site, preserving the tissue clot and allowing healing to commence.

Selection and Validation of an In Vivo Model of Complete ACL Tear

The porcine model has become an important part of our task of understanding the mechanisms of ACL healing because of its large size, anatomic similarity to the human knee (Fig. 16.1), and similar blood characteristics to humans. This animal model has been used for both histologic and biomechanical evaluation of functional ACL healing. The porcine model is chosen because of its similarity in gait

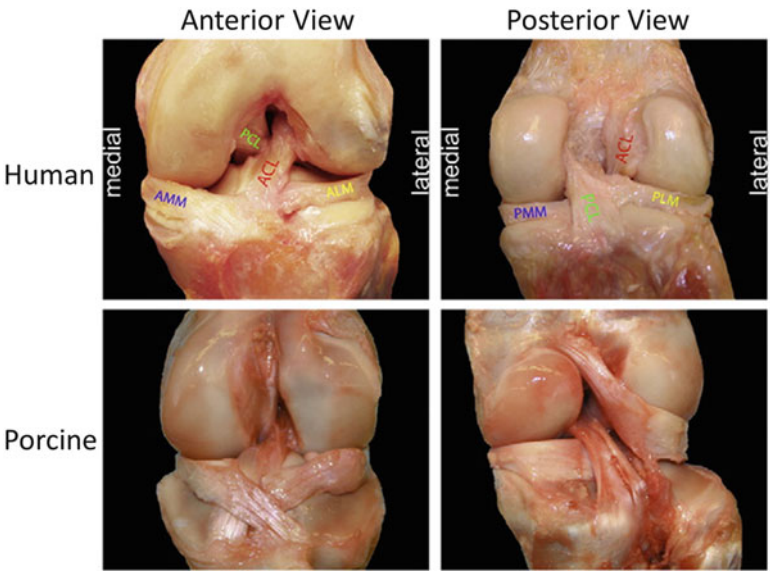

Fig. 16.1 Different aspects of the human and porcine knee. The *left column* shows the anterior aspect of the knees with the medial side being on the left and the lateral side on the right. The *right column* represents the posterior aspects of the knees. In both knees the medial meniscus passes behind the PCL. In the human knees, the posterior meniscofemoral ligament inserts more inferiorly on the medial femoral condyle (*ACL* anterior cruciate ligament, *ALM* anterior lateral meniscus, *AMM* anterior medial meniscus, *PCL* posterior cruciate ligament, *PLM* posterior lateral meniscus, *PMM* posterior medial meniscus) (Reprinted from *Knee*, Benedikt Proffen et al. [2], with permission from Elsevier)

biomechanics to the human. It is necessary to employ an in vivo model to examine the changes which occur after ACL injury as conditions within the culture dish do not replicate those in the joint environment, where synovial fluid, hypoxia, and lower tissue temperatures are only three of the factors making the in vivo ACL environment unique. As discussed in Chap. 11, the porcine animal model was chosen because the ACL in smaller animals—specifically mice and rats—is very small and difficult to access surgically. In addition, the small size of the ACL in these animals makes nutritional supply to the healing wound less dependent on blood vessel ingrowth—a key biologic component of healing in ligaments like the human ACL. Thus, most investigators choose large animals models, such as the dog, sheep, or goat, to study the injured ligament. However, the canine model is biomechanically different from the human knee, due in part to its steep angle of the top of the tibia, which makes procedures that work in humans unsuccessful in this model. Sheep and goats are also good models, but to study blood-based therapies can be problematic, due to their differences in blood characteristics from humans. The biomechanical and hematological similarities of human and pig knees combined with practical reasons of housing availability and familiarity of the veterinary staff with the pig physiology have led us to use this animal model in the subsequently described studies.

Our first studies examined the porcine knee and whether it would spontaneously heal an ACL injury caused by transecting (cutting) the ligament in the midsubstance. We evaluated a primary suture repair of the ligament after transection and found only a 12 % return of strength at 4 weeks after injury (81N was the average strength for suture repair and intact knees averaged 700N) [3, 4]. Thus, suture repair alone was relatively ineffective at stimulating functional ACL healing, and the model was thought to be representative of the human condition.

The Use of Platelet-Rich Plasma to Stimulate Healing of a Complete ACL Tear

As we know that platelets are one of the key biologic activators of successful wound healing (see Chap. 6), our first attempt to get a complete ACL tear to heal used platelets to try to biologically stimulate healing of a sutured ACL tear. One way to isolate platelets from blood is to centrifuge the blood to remove the red blood cells and then concentrate the platelets, so a greater number of platelets can be delivered to the wound site (Fig. 16.2). This solution of concentrated platelets is called platelet-rich plasma, or PRP. Once we were able to make PRP from porcine blood, we performed several studies using this model to examine the effects on the healing ligament through the addition of PRP to a ligament treated with suture repair.

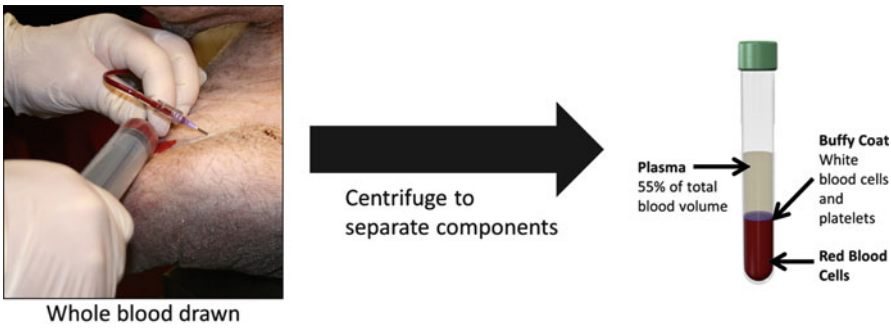

Fig. 16.2 Diagram depicting platelet-rich plasma (PRP) preparation. Whole blood is drawn from the patient and centrifuged to separate the different blood components. The layer of platelets can then be diluted to the desired concentration

In a study involving six skeletally immature, 4-month-old Yorkshire pigs, we tried to ascertain the answer to a relatively simple question: Is the addition of platelet-rich plasma enough to improve the mechanical properties of a repaired ACL? The pigs were subjected to bilateral ACL transection, with one knee treated with suture repair alone (control) and the other treated with the same suture repair plus the addition of 3 cc of a 2X concentration of PRP [5]. The ligaments were allowed to heal for 14 weeks. Several parameters were examined, including white blood cell and platelet count in the blood and in the synovial fluid, knee flexion and extension, anteroposterior (AP) knee laxity, and linear stiffness, maximum load, displacement at failure, and energy to failure of the repaired ligament. No significant differences were noted between groups for any of these parameters. Therefore, it was determined that the addition of PRP to a suture repair of the ACL was not sufficient to enhance any of the measured biomechanical results.

Why did this approach fail? Platelets provide the biologic magic to stimulate wound healing, and we added them in high concentrations. Why was there no functional effect?

To understand why the use of PRP alone had no effect on functional ACL healing, we have to go back to what we found in Chap. 8, where we looked at the mechanism for impaired healing of tissues within joints (like the ACL). In that chapter, we learned that for the ruptured ACL tissue, there was a complete lack of bridging between the two torn ends of the ligament. We also noted that prior work by Harrold and his group [6] had demonstrated that the fibrin the clot does not form in the intra-articular milieu, likely due to enzymes that break down the clot in the synovial fluid [7]. One of the major proteins found in synovial fluid after injury is plasmin—an enzyme that works to quickly break down fibrin or dissolve clot. The major protein that causes platelet-rich plasma to clot in a wound site is the same fibrin that makes a blood sample clot. Thus, the fibrin-based PRP that was injected into the wound site of the ACL was bathed in the same solution of plasmin and likely was dissolved before it had a chance to effectively act as a provisional scaffold for the ACL.

Is There a Way to Deliver the Platelets in a Scaffold That Would Resist This Premature Dissolution by the Synovial Fluid?

Could we deliver the platelets in a scaffold that would hold them in the wound site long enough? What could we use as a carrier for the platelets? We thought the carrier should meet several major requirements. First, it would need to be resistant to dissolution by plasmin and the other enzymes present in synovial fluid. Second, it should activate the platelets. Third, it should be a material that won't cause an excessive inflammatory response within the joint. Finally, it should be a material that encourages the ingrowth of ACL cells and the migration of important blood cells into the ACL wound site.

Collagen is a molecule that can potentially meet all of these requirements. When collagen is combined with fibrin, it forms a copolymer that is resistant to degradation by plasmin. To verify this for ourselves, we conducted an experiment where we subjected collagen-fibrin composites to enzymatic degradation by plasmin, elastase, and matrix metallopeptidase-1 (MMP-1) solutions at physiologic concentrations and measured the degradation of each scaffold at varying time points. The collagen scaffolds had a significantly greater resistance to degradation by MMP-1, elastase, and plasmin over the fibrin-based scaffolds. The results suggest that atelocollagen-based scaffolds may provide some protection against premature degradation by synovial fluid enzymes over fibrin-based matrices [8].

Secondly, collagen is a known activator of platelets. The first step in wound healing is platelet activation by the exposed collagen of the tissue ends in the wound. In addition, we ran in vitro studies to verify the platelet activation by collagen. In these studies, we compared the release of growth factors by platelets using both collagen and thrombin as platelet activators. In that study, we found that the use of thrombin as an activator resulted in immediate release of TGF-β1 and PDGF-AB, while the collagen-activated PRP clots released the growth factors more slowly over a period of days, in a pattern more consistent with that observed in normal wound healing [9–11].

As for the third requirement, collagen is known for its very low reactivity, even when animal collagen is used in human patients (e.g., for collagen injections in dermatology). As we wanted to minimize the chances that the scaffold would not cause a significant inflammatory reaction in the joint, we elected to modify the collagen in a way that would make it less immunogenic. The basic core of the collagen molecule is very similar among species, with the order of amino acids that makes up the collagen a G-X-Y amino acid sequence that differs little even among different animal species. The slight amount of antigenicity that is seen in collagen is thought to be due to the telopeptides attached to each end of the collagen molecule, which do not contain the G-X-Y sequence. Since the telopeptides are not present in atelocollagen, the antigenicity of atelocollagen is even lower than that of collagen (Fig. 16.3).

In addition, we also carefully observed the reaction within the joint and synovium when we placed our collagen-based scaffold within the knee. We did not find any

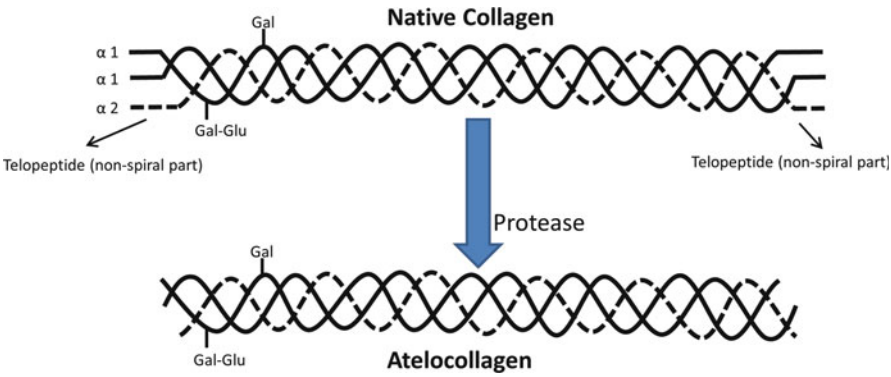

Fig. 16.3 The use of pepsin to digest the bovine collagen cleaves off the telopeptides at each end of the collagen molecule. This removes the majority of the part of collagen that differs among species

evidence of a significant inflammatory reaction, and the specific findings for these studies are detailed in Chap. 19.

To meet the fourth requirement, we knew that other extracellular matrix molecules, proteins such as fibrinogen, decorin, and other glycosaminoglycans were also likely important in encouraging cellular attachment to the scaffold and encouraging their migration through the scaffold. We proceeded to conduct a series of experiments to look at the characteristics of scaffolds that encouraged ACL cell migration, proliferation, and collagen production [9, 12–17], as well as the scaffold materials that would encourage participation in the wound healing process by blood cells [9–11, 18, 19]. The resulting scaffold is made up predominantly of type I collagen but also has other proteins and glycosaminoglycans within it (MIACH, Children's Hospital Boston, Boston, MA).

Will Use of an Appropriate Carrier Allow the Platelets to Stimulate ACL Healing?

In our first attempt to see if the combination of the extracellular matrix-based scaffold and platelets would be effective at stimulating ACL healing, we performed a study where we completely transected both ACLs in the porcine model. On one side, we treated the ACL injury with a suture repair, and, on the other side, we augmented the suture repair with the extracellular matrix scaffold that had been soaked in a platelet solution with a platelet concentration twice that of the circulating blood (2X PRP) [3]. Four weeks after surgery, the knees were retrieved and evaluated. The scar mass was determined to be qualitatively more intense in knees where the transected ligament was treated with the extracellular matrix-platelet scaffold than in

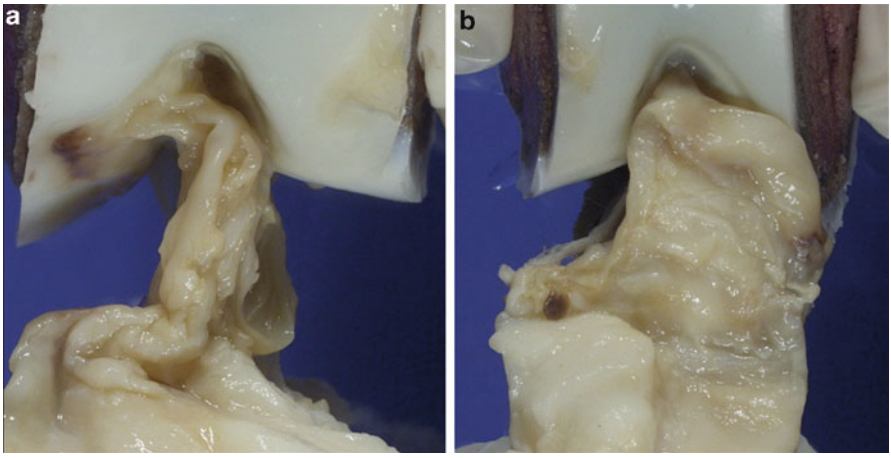

Fig. 16.4 Scar mass between treatment groups at 4 weeks after surgery: (**a**) Suture repair-only group. (**b**) ECM-platelet composite group. At 4 weeks, ligaments treated with ECM-platelet composite scaffolds had larger scar masses (From Joshi et al. [4], copyright © 2009 by (Sage Publications), Reprinted by Permission of SAGE Publications)

knees treated with suture repair alone (Fig. 16.4). In addition, the maximum tensile stress was also twice as high in the group repaired with the ECM-platelet scaffold, though this difference was not statistically significant. These results supported the hypothesis that the use of an appropriate carrier to deliver the platelets to a wound site could stimulate functional healing of the ACL [3].

In a second study, the stimulating effects of an ECM-platelet composite on ACL healing were characterized over 3 months of healing following complete ACL transection [4]. At 3 months, knees that were repaired using the ECM-platelet composite sustained 76 % greater load, 320 % increase linear stiffness, and 47 % decrease in displacement load at yield when compared with knees treated with suture repair alone [4].

Do We Need the Platelets, or Is the Carrier Alone Sufficient?

The results of the study using both the extracellular matrix-based scaffold and platelets were encouraging, but they also raised the question as to whether adding platelets to the carrier was necessary. Perhaps simply adding the carrier would allow for in situ blood to come into the porous carrier at the time of surgery, and the platelets in the blood would be equally effective to those placed in the wound site at the time of surgery.

To answer this question, we performed a study comparing the use of the extracellular matrix collagen scaffold alone versus a suture repair to see if the scaffold alone

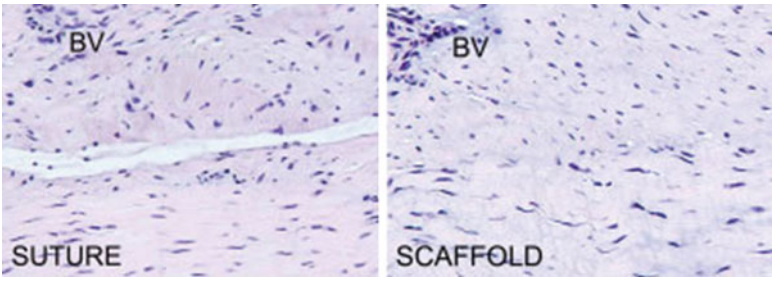

Fig. 16.5 Representative histological images of the healing ligament from the SUTURE and SPONGE groups stained with hematoxylin and eosin. Note that the healing ligaments had areas of parallel collagen fascicles (*bottom* of both micrographs) as well as more irregular areas (*top* of both micrographs) (*BV* blood vessel) (Used with permission from Fleming et al. [20])

would produce the desired effect. Eight Yucatan minipigs were subjected to bilateral ACL transection, and all were treated with suture repair alone in one knee and suture repair augmented with a extracellular matrix (ECM) scaffold in the other [20]. Following 13 weeks of healing, biomechanical and histologic assessments were performed on the ligaments. No significant differences were observed in the yield load, maximum failure load, linear stiffness, displacement to yield, AP laxity, or displacement to failure between the two groups. Histology revealed that ligament tissue in both groups was highly cellular and vascular at 13 weeks (Fig. 16.5). Cells possessed the characteristic shape of mature fibroblasts, and no residual ECM scaffold was visualized. The results of this study indicated that the use of a ECM scaffold alone to enhance primary suture repair of the ACL was not effective. No significant improvement was found in any of the biomechanical parameters evaluated [20].

Are There Ways We Can Improve the Surgical Technique for Repair?

As it appeared that the use of the extracellular matrix-based scaffold and platelet combination was a biologically effective treatment for ACL injury, we next turned to ask whether the surgical technique for the suture repair itself could be improved. Variables such as suture placement location and suture material selection could play a critical role in the repair of the ACL, as they do for repair of the majority of connective tissues. Thus, our next set of experiments worked to define the importance of several of these variables.

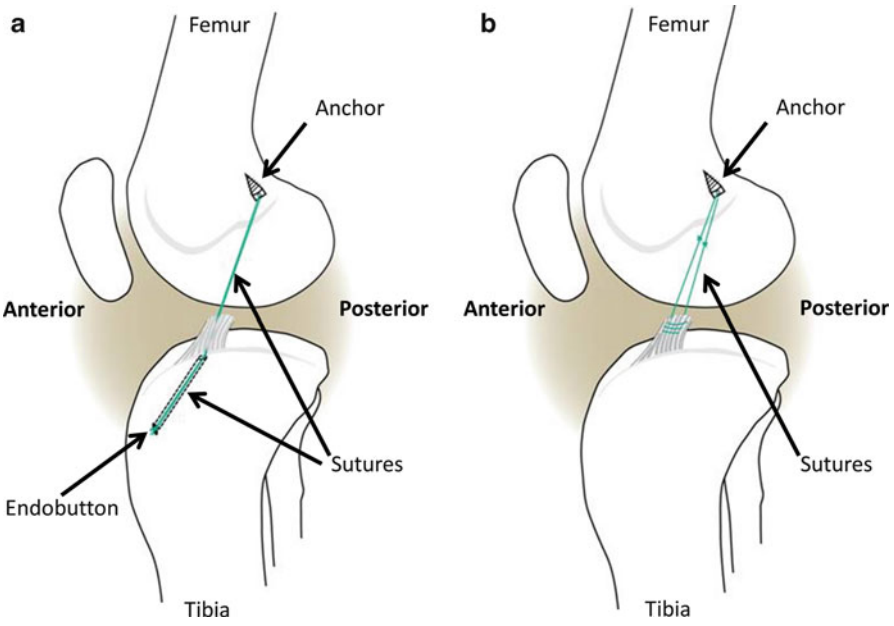

Fig. 16.6 Schematic of two suture repair techniques. (**a**) A suture anchor was placed into the bony femoral ACL insertion site, and the sutures were passed through the tibial tunnel and tied over a Delrin button at the anteromedial tibial cortex. (**b**) Two #1 Vicryl sutures (*green*) were secured in the distal ACL stump, and the femoral anchor was secured in the same manner, with a suture anchor placed into the bony femoral ACL insertion site. The sutures from the anchor were then tied to the Vicryl sutures in the distal ACL stump in maximum tension

Suture Technique

Several suture techniques have been previously reported for ACL surgery. Sutures can be placed from the ACL remnants to each other, from the tibial ACL remnant to a femoral bone bridge, from a femoral remnant to a tibial bone bridge, from femoral bone to tibial bone, or any combination of these fixation techniques.

The objective of our first study was to determine whether one of five different suture repair constructs when performed at two different joint positions would restore normal AP knee laxity. We looked at five different suture techniques ex vivo (using cadaver knees and only checking the knee laxity on the day of repair, not after any time in the body). We used porcine knees and first tested the knees with the ACL intact to determine the amount of anteroposterior translation for the normal knees. We then cut the ACL and placed an anchor with sutures attached to it in the center of the femoral ACL footprint. We took the sutures from the anchor and either passed them through a bone tunnel in the tibia (Fig. 16.6a) or tied it to a suture placed in the tibial ACL stump (Fig. 16.6b). For each technique, we then retested the AP knee laxity to see how close we came to the normal condition. We found that when we connected the femoral sutures to a tibial bone tunnel located in the front

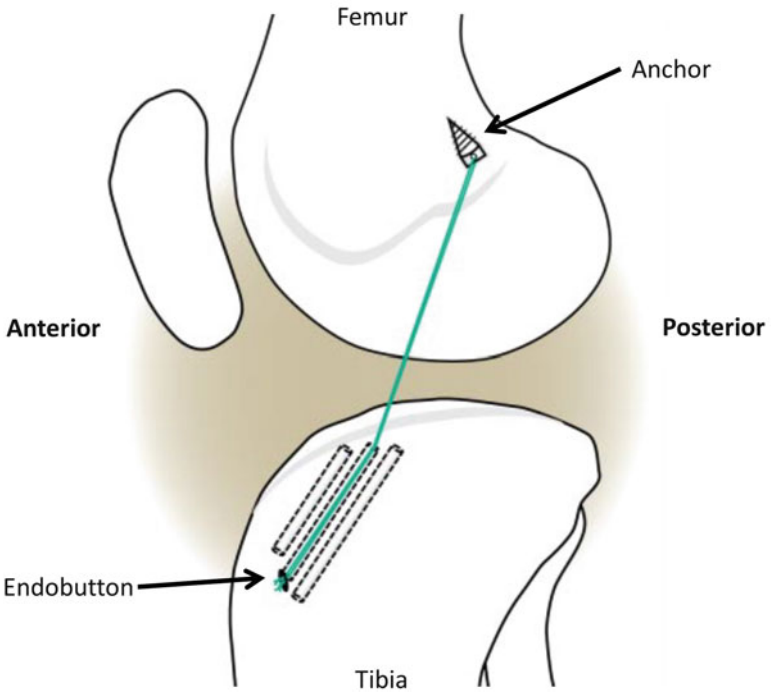

Fig. 16.7 Diagram illustrating the simple suture technique, with suture passing from an anchor located at the femoral insertion site of the ACL through the central tibial tunnel and tied over an EndoButton at the tibial cortex (Used with permission from Fleming et al. [21])

half of the normal tibial ACL footprint, we were able to restore the normal AP knee laxity with this suture bridge alone, as long as we tied the sutures with the knee bent 60°. Suture repair to the tibial stump, or with the knee only bent 30°, did not restore normal AP knee laxity. But this was a start—at least we knew how to get reasonable stability of the knee at the initial time of the repair using a simple suture "stent" technique (Fig. 16.7) [21].

But what would happen over time in a living knee? To answer this question, we then compared the suture technique where we had bone-to-bone fixation with the suture technique where we had fixation from the femoral attachment to the tibial ACL remnant only [22]. In this study, we cut the ACL and repaired it using one of the two suture techniques above supplemented with our ECM-based scaffold and platelets. After 3 months of healing, we found that the yield load and maximum load were significantly higher in the group with bone-to-bone fixation compared with the bone to ligament fixation technique [22]. This suggests that providing some protection to the healing ligament with a suture stent connecting the femur to the tibia may be of some benefit during the initial healing stage.

Absorbable Versus Nonabsorbable Sutures

In a study designed to evaluate the functional outcomes of bio-enhanced repaired ACLs, the effects of absorbable and nonabsorbable sutures were compared. It has been suggested that suture selection may affect the overall strength of the healing ACL, providing initial stability and mechanical protection during the remodeling process. One area of concern, however, is that nonabsorbable sutures may cause stress shielding, which can have a detrimental effect on tissue remodeling. On the other hand, absorbable sutures may reduce stress shielding but may also expose the healing ACL wound to excessive mechanical stress if they break down too early. In addition, the use of a nonabsorbable suture across the growth plate may result in ongoing damage to the growth plate.

To begin to address some of these issues, we conducted another study in skeletally immature Yorkshire pigs. The pigs underwent unilateral ACL transection and were repaired using bio-enhanced ACL repair with either absorbable or nonabsorbable sutures and our extracellular matrix-based scaffold (MIACH, Children's Hospital Boston) soaked with 3 cc of autologous whole blood. Following 15 weeks of healing, the yield and failure loads were both higher in the nonabsorbable suture group. No statistically significant difference was noted in the linear stiffness of the repairs between the two groups. However, significantly larger tunnel diameters were noted in the nonabsorbable suture group, and this group had a larger zone of physeal injury. Thus, surgeons may elect to use absorbable sutures for young patients who still have a great deal of growth remaining to minimize growth plate changes, but may wish to select nonabsorbable sutures for skeletally mature patients who may need a stronger repair.

Is the Combination of the Extracellular Matrix-Based Scaffold and Platelets Enough to Functionally Heal the ACL?

With the optimization of carrier, platelets, and suture technique, are we even close to achieving the mechanical properties of an ACL reconstruction with bio-enhanced ACL repair? To answer this question, we performed another study in pigs comparing the treatment of a complete ACL tear using a bio-enhanced ACL repair (suture repair in combination with placement of the ECM-based scaffold and platelets and using a suture stent between the femur and tibia (Fig. 16.8)) and ACL reconstruction using a bone-patellar tendon-bone allograft [23] and a group which had no treatment (ACL transection only). After 15 weeks of healing, we found that the yield and maximum loads between the bio-enhanced ACL repair and reconstruction were similar after 15 weeks. There were also no significant differences in yield displacement, maximum displacement, or linear stiffness between the two treatment groups [23]. Both the bio-enhanced repair group and the reconstruction group were significantly better than the group which received an ACL transection but no treatment (Fig. 16.9).

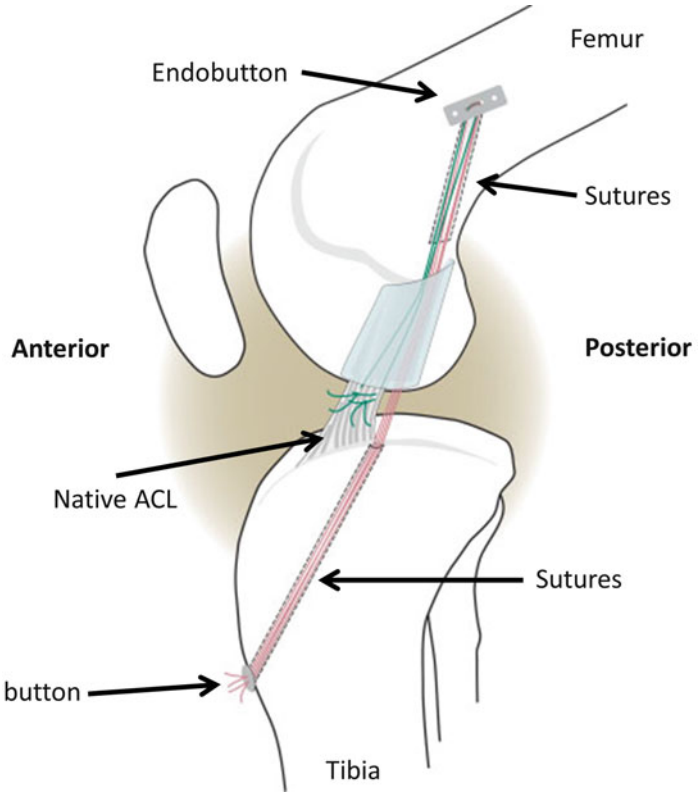

Fig. 16.8 Diagram depicting the primary suture repair of the ACL with the ECM scaffold in place. Sutures were fixed proximally with an EndoButton. The sponge was threaded onto four of the trailing suture ends (*red*) which were then passed through the tibial tunnel and tied over a button to provide initial knee stability. The remaining two suture ends (*green*) were tied to the sutures in the tibial stump of the ACL (Used with permission from Fleming et al. [20])

Summary

Many factors are likely to significantly affect the outcome of a bio-enhanced primary repair of the ACL. These factors include the choice of implanted scaffold, bioactive agent, and suture technique as only a few variables. In this chapter, we have noted that the use of platelet-rich plasma alone is ineffective in stimulating ACL healing, likely due to the fact that it is enzymatically dissolved in the post-surgery joint. In addition, we have noted that use of an extracellular matrix-based scaffold without any additional source of growth factors is also insufficient for stimulating functional healing of the ACL. However, the combination of scaffold and platelet can be quite effective. The strength of the healing ACL can also be enhanced by using a suture technique that restores normal AP stability at the time of surgery. Using all of these findings, the current technique of bio-enhanced ACL repair using

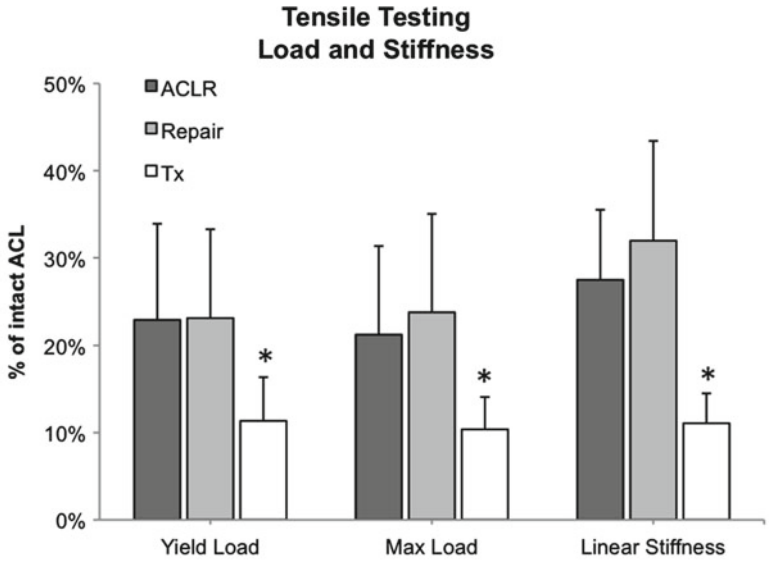

Fig. 16.9 Results of tensile testing in head-to-head comparison of ACL reconstruction (*ACLR*) and bio-enhanced ACL repair (*repair*). Outcomes of both procedures were similar, and both were significantly better than ACL transection alone (*Tx* group) (Reprinted from *Arthroscopy*, Vavken et al. [23], with permission from Elsevier)

whole blood, an extracellular matrix-based scaffold (MIACH, Children's Hospital Boston), and a suture stent protecting the early repair leads to a healing ACL with equivalent properties to that of an ACL reconstruction after 3 months of healing. These findings provide hope that one day, bio-enhanced ACL repair will be a viable option for patients with ACL injuries.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 17
The Effects of Platelets and Their Concentration on ACL Healing

Patrick Vavken

Platelet Biology

Platelets, or thrombocytes in medical terminology, are one type of cell in the peripheral blood. Their main function in normal physiology is the formation of blood clots and release of growth factors during hemostasis. Platelets are fragments of larger cells called megakaryocytes, which are formed in the bone marrow as a part of the white blood cell group. These megakaryocytes produce between 2,000 and 5,000 platelets by a process that is not fully understood yet. The platelets themselves are round discs of approx. 2–4 μm (micrometer) in diameter (Fig. 17.1), and thus the smallest cells in the blood. They contain no nucleus, but do have organelles such as mitochondria (for energy production), microtubuli (for motility), and granules (for growth factor release). The most important granules for platelet-rich plasma (PRP) production are the alpha granules. There are roughly 50–80 per platelet at 200–500 nm in size containing some 30 bioactive proteins, such as growth factors, that play a crucial role in blood clotting and wound healing [1].

Under physiological conditions, platelets are usually found within the confines of a vessel, that is, encircled by endothelial cells, which line the inside of arteries and veins. This endothelial layer keeps the platelets resting, passively by covering trigger molecules, such as collagen, and actively by secreting prostacyclin (prostaglandin I2), which is a chemokine that maintains the resting state of platelets. After injury, this lining is interrupted and the platelets are activated by exposure to surfaces other than endothelial cells, such as collagen. Platelets may also be activated by chemicals released from the injured endothelial cells or other activated platelets, such as thromboxane, ADP, or thrombin.

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_17,

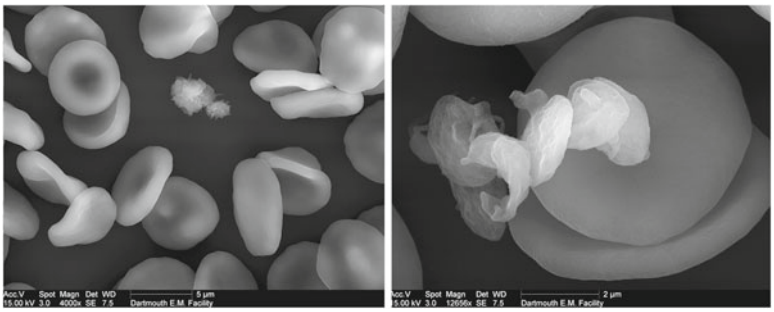

Fig. 17.1 Platelets. Illustrates human platelets together with red blood cells under scanning electron microscopy. The higher magnification on the right shows them in their typical resting state, as smaller cells seen against the background of the red cells (big round discs). (Used with permission from Louisa Howard, Dartmouth College Electron Microscope Facility)

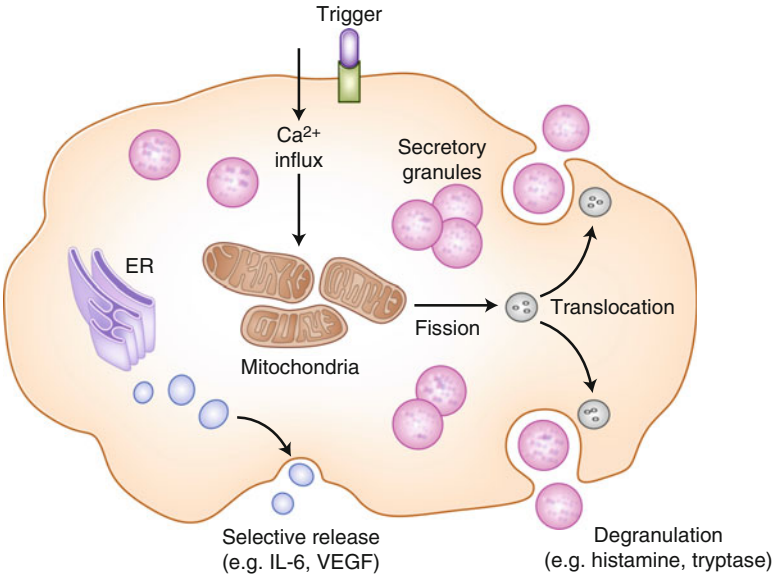

Fig. 17.2 Growth factor release. Platelets contain between 50 and 80 granules that carry a number of bioactive proteins such as growth factors. Once activated these granules move to the cell surface and release the growth factors. Ninety-five percent are released within 1 h

Activation of platelets results in two major processes. After activation, the granules are brought to the cell surface, and their contents are released immediately into the surrounding blood and tissue (Fig. 17.2). A wide range of growth factors is contained in these granules, which stimulate inflammation and subsequent wound healing on multiple levels. These growth factors include, but are not limited to,

platelet-derived growth factor (PDGF), transforming growth factor (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), and also platelet factor 4 (PF4), interleukin-1 (IL-1), platelet-derived angiogenesis factor (PDAF), epidermal growth factor (EGF), platelet-derived endothelial growth factor (PDEGF), epithelial cell growth factor (ECGF), and other proteins including osteocalcin, osteonectin, fibrinogen, vitronectin, fibronectin, and thrombospondin-1. All of the functions and effects of these growth factors have not been elucidated yet, but some have been studied and described in much detail.

Platelet-derived growth factor (PDGF) was the first growth factor to be associated with platelets [2]. It is also found in other cells and has three different appearances, or isomers, and different affinities to receptors and cell types have been described. However, the exact reasons for this variety and functional difference still remain to be elucidated. Currently, we think that the most important function of PDGF is its ability to stimulate proliferation of fibroblasts and collagen production by the fibroblasts. In addition, PDGF is known to induce fibroblast chemotaxis and macrophage activation. PDGF also attracts neutrophils into a wound site, thus further enhancing the removal of debris and pathogens [3]. All of these are cellular events important in the healing cascade. PDGF release is independent of age and gender [4].

Transforming growth factor beta (TGF-β) is probably the most widely studied growth factor. It enhances the production of extracellular matrix, the synthesis of collagen, the proliferation of fibroblasts, and the differentiation of mesenchymal cells. It has also been found to inhibit the degradation and resorption of bones and help build skeletal mass [3]. Similar effects are attributed to insulin-like growth factor (IGF), which enhances fibroblast proliferation and biosynthetic activity, as well as bone formation and osteoblast differentiation [4]. IGF has also been shown to support muscle growth and recovery.

The role of vascular endothelial growth factor (VEGF) is a bit more elusive. As the name implies, VEGF has been traditionally associated with vasculature, more specifically new vessel formation. However, recent data has shown that fibroblasts express VEGF receptors [5]. More importantly, it was shown that the level of VEGF receptor expression is associated with functional, biomechanical outcomes in ACL repair [6].

A full description of all platelet-associated growth factors and their function is beyond the scope of this chapter, but Table 17.1 gives an overview of some of the more important ones.

The second function of platelet activation, in addition to growth factor release, is hemostasis, or blood clotting. The activated platelets change their shape from round to stellate with numerous fingers reaching out to find other platelets (Fig. 17.3). The platelets are able to bind to each other (aggregation) and to the exposed collagen in the endothelial defect (adhesion). Thus, they form a hemostatic plug that seals the defect in the injured blood vessel. At the same time, they release proteins and growth factors that start the blood-clotting cascade. This blood clot has a physical function as a defect filler and cover but also serves as a scaffold for cell movement and activity required for wound healing. However, in some diseases such as atherosclerosis, the

Table 17.1 Growth factors associated with PRP

Name	Effect[a]	Source[b]
Platelet-derived growth factor (*PDGF*)	Proliferation, migration, angiogenesis, collagen production	Platelets
Platelet-derived angiogenesis factor (*PDAF*)	Stimulation of proliferation of endothelial cells and angiogenesis	Platelets
Platelet-derived endothelial growth factor (*PDEGF*)	Stimulate wound healing via proliferation of fibroblasts and keratinocytes	Platelets
Platelet factor 4 (*PF-4*)	Stimulates migration of neutrophils, acts as chemoattractor for fibroblasts, heparin antagonist	Platelets
Vascular endothelial growth factor (*VEGF*)	Angiogenesis, unclear effect on fibroblasts	Platelets
Transforming growth factor-β1 (*TGF-β1*)	Proliferation, differentiation, collagen production, fibronectin production	Platelets
Transforming growth factor-β2 (*TGF-β2*)	Embryonic development, wound healing	Platelets
Fibroblast growth factor (*FGF*)	Fibroblast and myoblast stimulation	Platelets
Epidermal growth factor (*EGF*)	Cell proliferation (mesenchymal and epithelial). Complex interaction with other growth factors	Platelets
Hepatocyte growth factor (*HGF*)	Migration, angiogenesis, antifibrotic effect	Plasma[c]
Insulin-like growth factor-1 (*IGF*)	Fibroblast and myoblast stimulation, muscle growth and regeneration	Plasma[c]

[a]As known [b]some growth factors are released from cells other than platelets. [c]Note that not all growth factors are platelet released

platelets are pathologically activated inside a vessel, for example, within the coronary arteries that feed the musculature of the heart. This type of aggregation can lead to the occlusion of the blood vessel and blockage of the flow of blood to where it is needed.

The physiology of platelets is more detailed and intricate as described above, with various factors including diet affecting these processes [7–11], but even this short summary of platelet action makes their role in wound healing fairly obvious. Thus, it is not surprising that regenerative medicine tries to harness their power, usually by employing platelet concentrates. The best-known platelet concentrate is platelet-rich plasma.

Platelet-Rich Plasma

Physicians have been trying to isolate and employ the stimulatory capacities of platelets described above to improve wound healing. Out of innumerable approaches to solve this problem, one stands out among the others: platelet-rich plasma, or PRP for short. PRP is an autologous platelet concentrate that is made from the patient's own blood. Briefly, blood is drawn and clotting and platelet activation is artificially stopped with a chemical. Subsequently, the blood is spun to separate the different blood cell types based on cell size and weight. Thus, platelets can be isolated, concentrated, and injected into a defect or wounded tissue.

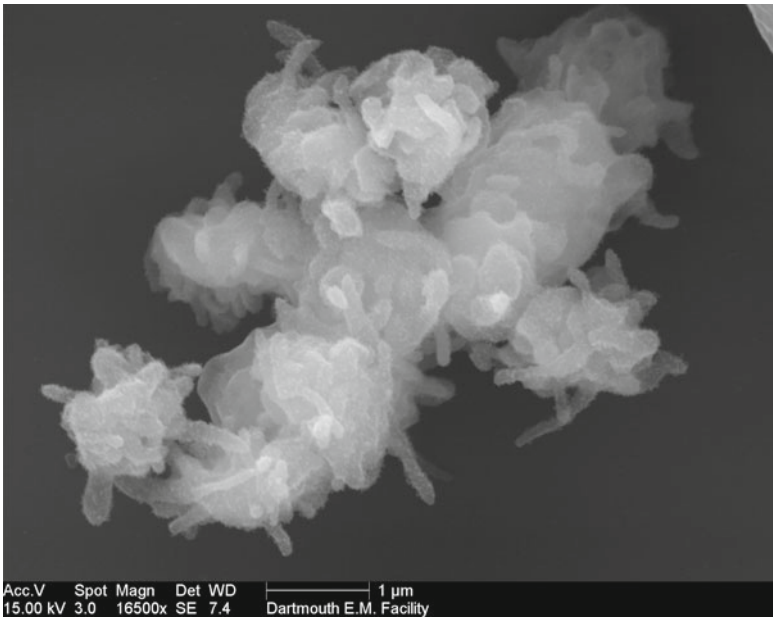

Fig. 17.3 Aggregating platelets. Once they are activated platelets release growth factors and aggregate. The initial step in aggregation is the change of shape from *round disc* (cf. Fig. 17.1) to stellate, spiky cells. The newly formed fingers (pseudopods) allow attachment to each other or to a substrate (Used with permission from Louisa Howard, Dartmouth College Electron Microscope Facility)

Studies have shown that the platelets in PRP can be activated by contact with collagen [12]. Collagen is the most abundant protein of all in all mammals and is ubiquitously present in practically all tissues; thus, PRP can be injected into most tissues without a need for addition of an activating agent. Such applications have shown excellent results in bone healing and tennis elbow. In cases where there may not be enough *in situ* collagen around to activate the PRP, PRP can be combined with a biomaterial that contains collagen and platelets be activated in this way. When PRP is made using an anti-coagulant, the anti-coagulant may need to be reversed to facilitate platelet activation.

Platelet-Rich Plasma and ACL Healing

Other chapters in this book report on the use of PRP in ACL reconstruction and ACL repair. At this juncture we want to discuss the response of the ACL tissue to PRP. The ACL consists of one cell type, primarily, the ligament fibroblast. These cells have surface receptors for the various growth factors released from PRP (see Table 17.1). As mentioned above, PDGF, or platelet-derived growth factor, stimulates fibroblast growth, migration, and biosynthetic activity. Similar effects are

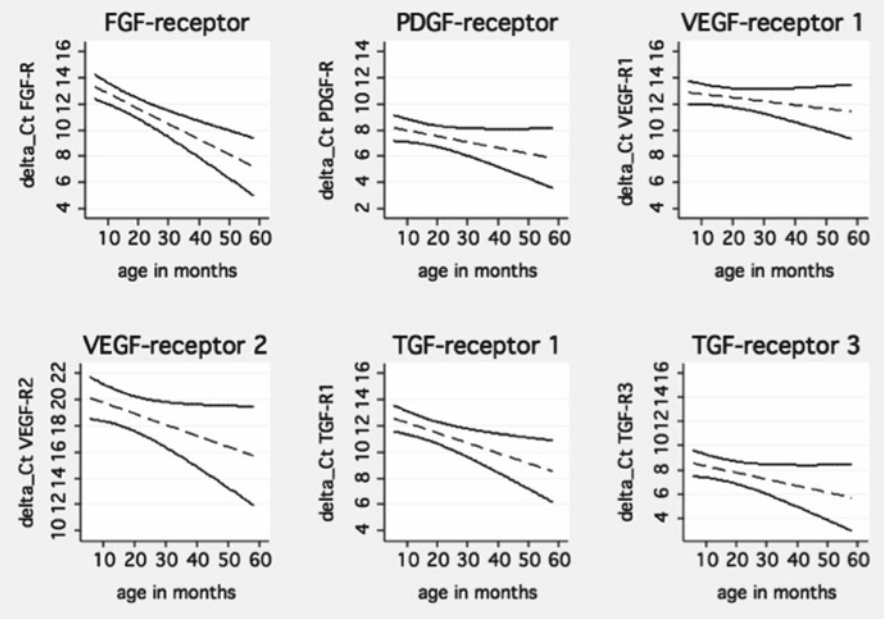

Fig. 17.4 Growth factor receptor expression is age dependent. The release of growth factors from platelets is mostly independent from age and gender, but with age the responder cells express less and less receptors to receive the growth factor message. This fact might be one key to answering why younger individuals heal faster (and better) than older ones (Used with permission by Vavken et al. [5])

seen with TGF-β, or transforming growth factor β, and FGF, or fibroblast growth factor. Recent data has shown that receptor expression is age dependent [5, 6]. These growth factor–surface receptor interactions might also hold the key to the age-related differences in PRP-based healing. Prior research has shown that PRP effects diminish with rising age [13]. This finding is not surprising given the common, empirical knowledge that fractures and injuries heal faster in children than in adults. We were able to show that, with age, fibroblasts express fewer and fewer growth factor receptors on their surface [5] (Fig. 17.4). In combination with the fact described above – the association of receptor expression with functional outcome – it seems only logical that this age-related reduction of receptor expression is causal for the age-related reduction in ACL healing response.

Platelet Concentration

The use of a platelet concentrate begs the question which concentration will produce the best results [14]. Trying to tackle this question, the first thing one realizes is that there isn't even a definition of "PRP platelet concentration." Many studies give the PRP concentration as platelets per volume, usually in millions per microliter, but

this gives no information on how concentrated the PRP is. Another way to describe PRP concentration is to give the relation to the systemic platelet count in the peripheral blood of a patient. Twofold or 2× means twice as many platelets in the PRP as are in the patient's circulating blood, threefold or three times as many, etc.

Many biologic agents have a direct dose-response relationship, that is, a stronger response with higher doses. Following this logic, most commercially available PRP systems try to maximize the platelet concentration in their product and may produce up to an 11-fold concentration. A recent study assessed the differences between PRP made with different, commercially available machines, but found none in mean cell counts or bioactive protein concentration [15–17].

However, when talking about PRP concentration and dose–response relationships, a number of thoughts should be included: First, biological relationships are not always linear but are often confined to a window of concentration. In other words, the cells may act predictably only above or below certain concentration thresholds of the biologic stimulus. Second, even if PRP effects had a linear dose–response relationship, PRP is not an active agent in itself, but a stimulator of other cells. As such, its effects are finally dependent on the presence of responsive cells [6]. If no such cells are available, then the highest concentration of PRP may not be able to produce a result better than a lower concentration. Third, the exact effects of PRP are still elusive, but while there is evidence for many, many positive effects, there is also evidence for negative effects [18–21]. At higher concentrations, the negative effects might match or even outweigh the positive effects of PRP [18, 20, 21]. Fourth, it should not be forgotten that with changing PRP concentration, the relative concentration of other blood cells in the PRP such as red blood cells or white blood cells changes as well. Both cell types have a profound effect on platelets and fibroblasts, which is the subject of the next chapter [22]. And last but not least, all these processes are relevant on the cell level, but we don't know if they are translated to the tissue and organ levels.

Given all these facts, we wanted to assess the effect of different platelet concentrations on ACL healing experimentally. We designed an experiment in which we used a threefold and a fivefold concentrated PRP to stimulate ACL healing in an animal model [23]. After 13 weeks of follow-up, we tested the strength and tissue composition of the healed ACL tissue. We did observe a lower cell number in the threefold concentrated group compared to the fivefold group (Fig. 17.5), but how this finding relates to the function of the ACL is less clear. Generally, high cellularity is agreeable, since the cells fill the defect and repair the tissue. However, high cellularity can also be a panic reaction, exacerbated by overstimulation by PRP, leading to a chaotic, uncontrolled remodeling attempt rather than a balanced and directed repair process. Such overstimulation and undirected repair has been shown to result in poor repair strength. In our study we found no significant increase in the ligament strength with higher platelet concentration. Actually, the mechanical strength of the healing ligament was slightly stronger in the threefold PRP concentration group, corroborating the overstimulation theory.

Others have asked similar questions outside the field of ACL healing. Concerning bone healing it has been shown that there is a dose-dependent, beneficial effect of

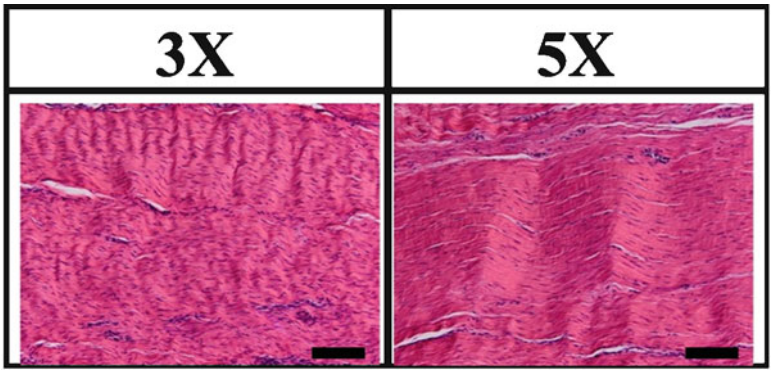

Fig. 17.5 3× and 5× PRP produce equal ACL repair strength. In direct comparison, bio-enhanced ACL repair with 3× and 5× PRP did not show a difference in biomechanical strength of histological ligament maturity index (Used with permission by Mastrangelo et al. [23])

PRP up to a certain threshold [21, 24, 25]. Beyond this threshold PRP inhibits those cells that remodel the bone by removing old tissue and depositing new along stress lines. Weibrich et al. tested such bone remodeling around a surgical implant using different concentrations of PRP [14, 17, 26]. Interestingly, they found that the dose–response relationship of PRP with bone healing is limited to a narrow window of concentrations. In their study in rabbits, the best effects were seen with about one million cells per microliter, which corresponds to a roughly twofold concentration of platelets compared to normal blood [27]. Below this concentration, they did not see any effects; at higher concentrations however (>2 Mio/μL), they found a paradoxically inhibitory effect.

Such findings were seen not only in bone but also in injured soft tissues. A recent study by Rappl et al. used PRP to treat chronic wounds in 285 patients with spinal cord injury [28]. The problem these patients suffer from is the lack of neurological feedback to these wounds, which leads to problems with the perfusion and control of moisture leading to poorly healing, chronic defects. Using a 1.3-fold concentrated PRP, 90 % of their patients responded positively with a 54 % reduction in wound area and a 67 % reduction in wound volume. Yamaguchi et al. used PRP at different concentrations to improve intestinal healing after abdominal surgery [29]. When comparing plasma only with a low concentration of PRP (2 Mio/mL) and a high concentration of PRP (5 Mio/mL), they found the best mechanical strength and the most adequate tissue composition in the low PRP group. Interestingly, the plasma-only group had better outcome than the high-PRP group.

In summary, these findings underpin the need to approach the question of PRP concentration sensitively. We have found that increasing the concentration from three- to five-fold did show some differences in cell behavior, but no effect on the healing tissue strength. Others have reported using a 1.3- to 2-fold concentration to obtain the best results [28]. Currently, we are much in favor of a one-fold concentration of platelets with specific additions of other blood cells such as red and white

blood cells. Our preliminary data has shown that these cells, at controlled concentrations, enhance the effect of PRP on ACL healing. The following chapter gives detailed information on these findings.

Conclusion

Platelets hold much of the regenerative power of natural healing. Platelets can be isolated from the peripheral blood and with them their regenerative power. Recent studies have documented the effectiveness of using platelets to stimulate healing for various injuries, including the ACL. When using PRP, it is important to consider platelet concentrations. The current literature commends the use of low PRP concentrations, in the range of one- to two-fold the concentration of the peripheral blood, to optimize PRP effects and avoid adverse reactions.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 18
The Effects of WBCs and RBCs on Ligament Healing

Linda H. Chao and Martha M. Murray

Introduction

Ligament healing progresses in a manner similar to healing in other soft tissues, with well-described histologic stages: the inflammatory, proliferative, and remodeling phases. These integrated processes result in formation of a provisional matrix, cellular invasion, neurovascular invasion, and remodeling of the matrix into a functional ligament. Many different blood cell types are involved in this process, including platelets, white blood cells, and red blood cells (Fig. 18.1). Each of these cell types contributes in a unique way, and each delivers multiple cytokines and molecules during their residence within the ligament wound. As growth factor delivery vehicles, each of these cell types has a great deal of potential power, and they can be obtained from the patient for use in healing their own tissues at relatively low cost. Thus, these cells are of great interest for use in tissue engineering of healing ligaments and other tissues. However, to begin to understand the orchestration of these cell types during ligament healing, we first need to outline the effects of each of these cell types on the ligament wound environment. What we know of the function of these cell types will be summarized in this chapter as a foundation for future studies that involve delivering these cells to a wound site to heal ligaments in vivo.

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_18,

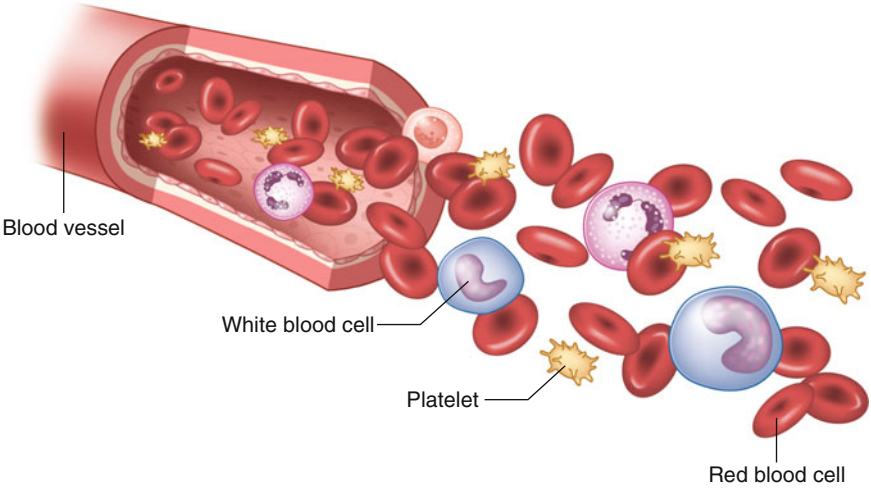

Fig. 18.1 Schematic showing the multiple cell types circulating in the blood that participate in the wound healing process after injury. Platelets are the small cell fragments seen in *gray*. White blood cells, or leukocytes, come in many different forms and are noted here as clear cytoplasm with purple nuclei. Red blood cells do not have a nucleus and are the red biconcave cells seen in high numbers within the blood

Erythrocytes

Erythrocytes, or red blood cells (RBCs), are the primary transporters for oxygen to the tissues in the body. Until recently, their role in wound healing was less extensively studied than the role of platelets or white blood cells. However, recent studies have suggested that red blood cells play a key role in wound repair (blood vessel size changes) [1] in part because of their ability to interact with fibroblasts and extracellular matrix (ECM) during wound healing, to bind inflammatory mediators, and also to scavenge nitric oxide (NO), which is a regulator of vasodilation (blood vessel size changes) [1]. For example, both erythrocytes and erythrocyte-conditioned medium help fibroblasts make IL-8, a growth factor responsible for neutrophils coming into a wound site early in the wound healing response [2].

In addition to their role in inflammation, erythrocytes have been shown to control fibroblast proliferation and programmed cell death, or apoptosis, which are important to the proliferative and remodeling phases of wound healing. A key feature of the transition from the proliferative phase of wound healing to the remodeling phase is the decrease in fibroblast proliferation and increase in collagen production [3]. When the collagen content in the wound reaches a certain threshold, fibroblast proliferation and collagen synthesis are suppressed [4] and the remodeling phase begins.

Erythrocytes have been shown to inhibit fibroblast proliferation and upregulate fibroblast collagen production in several in vitro studies [1, 5, 6]. Two-dimensional tissue culture of human lung fibroblasts with erythrocyte concentrations above 5×10^5 and 5×10^8 erythrocytes/mL[5] (concentrations which were approximately one to four orders of magnitude lower than the physiologic concentration of erythrocytes in whole blood ($4.5–6.0 \times 10^9$ erythrocytes/mL) [7]) showed that including even this small amount the erythrocytes in the culture caused significantly decreased fibroblast proliferation and significantly increased fibroblast apoptosis compared to fibroblasts cultured without erythrocytes [1]. In three-dimensional (3D) cultures, human ACL fibroblast proliferation was significantly inhibited in a collagen-platelet hydrogel containing 1.5×10^9 erythrocytes/mL, as compared to a collagen-platelet hydrogel containing no erythrocytes [5]. Furthermore, 3D culture of fibroblasts in collagen hydrogels with physiologic or higher concentrations of erythrocytes resulted in decreased collagen gel contraction as compared to gels containing lower concentrations of erythrocytes [6].

Despite the inhibition of fibroblast proliferation in both 2D and 3D culture models, erythrocytes stimulate the production of procollagen by fibroblasts within a simulated wound when the erythrocytes are added at a supraphysiologic concentration to the collagen gel [6]. This upregulation of collagen expression may be due to the effects of high levels of hemoglobin released by erythrocytes when they die and burst. Hemoglobin is able to bind and then subsequently release nitric oxide [8, 9]. As nitric oxide has been shown to stimulate collagen synthesis by fibroblast in vitro [10, 11], a high concentration of hemoglobin, and subsequently nitric oxide, in the cell culture media of fibroblasts cocultured with erythrocytes may be responsible for the increase in procollagen expression by the fibroblasts.

At the same time, heme, the functional group of hemoglobin, is highly toxic when released into the extracellular milieu, whether during physiological or pathological cell damage, and is quickly taken up by high capacity scavengers, including macrophages [12]. When macrophages first come into a wound site, they are CD163-negative (they do not express the CD163 receptor) [13]. A day or two later, CD163+ macrophages appear in the wound. The emergence of cells with CD163 on the surface is important, as these cells play a crucial role in the control of inflammatory processes by induction of anti-inflammatory pathways [14]. CD163-positive macrophages are the predominant macrophage population found in the resolution phase of inflammatory processes, including wound healing [13]. Furthermore, CD163 expression is strongly induced by anti-inflammatory mediators such as IL-10 and glucocorticoids [13]. Thus, it has been posited that CD163-positive macrophages play a role in the resolution of inflammation by the scavenger receptor's two related functions: clearance of free hemoglobin and a potential anti-inflammatory function [14].

The effect of erythrocyte lysis on wound healing may be to contribute to the resolution of inflammation – the dampening of the immune response – via signaling through the macrophage hemoglobin scavenger receptor.

Leukocytes

Leukocytes are the white blood cells that circulate in the body and regulate inflammation and response to injury and infection. They come from two major precursor cells – lymphoid and myeloid (Fig. 18.2). Cells of both lineages are critical in wound healing and will be discussed in this next section.

Neutrophils

The role of inflammatory cells, such as neutrophils and macrophages, in the healing wound, has been widely investigated leading to evidence for both positive and negative influences of neutrophils upon the healing wound [15]. It has been recognized that functional neutrophils are required for successful wound healing, as both

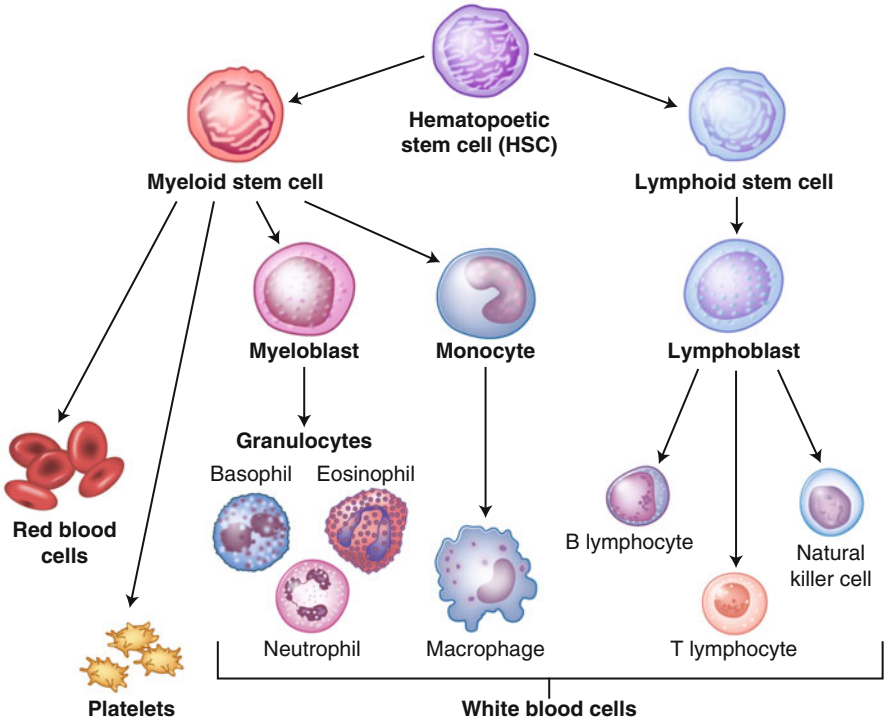

Fig. 18.2 Schematic of blood cell origins. The blood stem cells differentiate into two major cell types – lymphoid and myeloid. The lymphoid cells are the precursors for the lymphocyte cells, while the myeloid stem cells are the precursors for other white blood cell types (including neutrophils, basophils, and eosinophils), red blood cells, and platelets

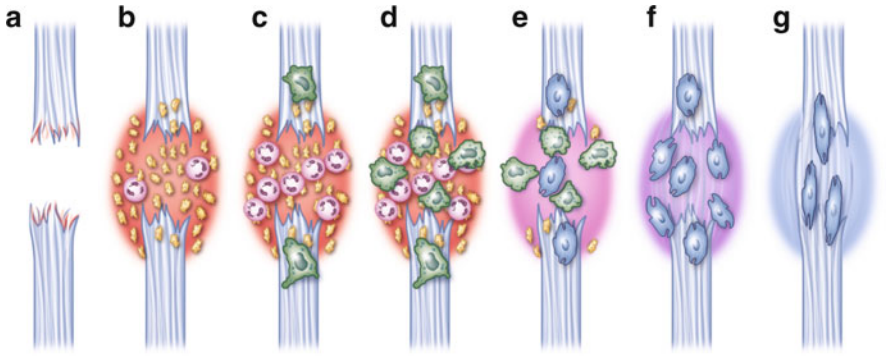

Fig. 18.3 Schematic of ligament wound healing and the role of various blood components. (**a**) Tissue injury, (**b**) immediately after injury: the injury site is filled with a blood clot containing platelets (*yellow*), a few white blood cells (*pink/purple*), and red blood cells in a fibrin network (*red*). (**c**) Four hours after injury: additional neutrophils migrate into the wound site (*pink/purple*), and inflammatory macrophages begin to migrate into the wound site in response to stimuli there. (**d**) One day after injury: The macrophages (*green cells*) begin to engulf (phagocytose) the dying neutrophils. Platelets continue to degranulate and release growth factors in the wound site. (**e**) Two to 4 days after injury: after ingesting the dying neutrophils, the macrophages switch to a reparative phenotype and fibroblasts begin to migrate into the wound (*dark blue cells*). (**f**) One week after injury: the fibroblasts are present in the wound site and actively synthesizing collagen, gradually replacing the provisional fibrin-erythrocyte-platelet scaffold with a collagenous matrix. (**g**) Weeks after injury: the wound site is predominantly composed of new collagen produced by the resident fibroblasts

neutropenic patients and those who have dysfunctional neutrophils exhibit impaired wound healing [16]. On the other hand, robust inflammation can also be detrimental to healing wounds [15]. Functional wound healing takes place in between these two extremes on the spectrum of neutrophil infiltration in acute inflammation. At present, neutrophils have been well studied in the healing of dermal wounds, but their role in the healing of ligament wounds has not yet been well studied. More work needs to be done to elucidate the effects of neutrophils and the chemicals they release on the healing ligament.

Like platelets, neutrophils may be critical in establishing a functional healing response in that they are crucial "first responders" in the acute inflammatory response to injury (Fig. 18.3). Neutrophils remove damaged tissue and dead cells from sites of injury or infection via phagocytosis and release reactive oxygen species (ROS) and reactive nitrogen species (RNS) to kill pathogens. Neutrophils also participate in the resolution of inflammation by promoting the switch of arachidonic acid-derived prostaglandins and leukotrienes to lipoxins, which inhibit the entry of new neutrophils to sites of inflammation, reduce vascular permeability (which is important for entry of white blood cells from the bloodstream into the tissues), and promote the entry of monocytes/macrophages to enter the wound site to ingest and clear apoptotic neutrophils [17]. The short lifespan of neutrophils in tissues

(1–3 days) as well as their release of proinflammatory ("catabolic") molecules contributed to their being thought of as only minimal contributors to wound healing [18], but more recent awareness of the multiple cytokines (IL-4, IL-8, TNF-α) these cells release in the first day of wound healing has led to a greater respect for their role in that process [19]. Furthermore, patients who have conditions that lead to neutrophil deficits or dysfunction exhibit impaired wound healing [18]. For instance, patients with leukocyte-adhesion deficiency-1 (LAD-1) have large nonhealing ulcers [20] and nonhealing wounds [21] despite high neutrophil counts in peripheral blood (based upon CBC analysis), due to an impaired ability of neutrophils to migrate into tissues [20]. Furthermore, patients with chronic neutropenias also exhibit impaired wound healing, which further supports the importance of the role of neutrophils in functional wound healing [18].

It has recently been appreciated that the immune system is finely tuned such that the acute inflammatory response to injury is self-limiting and contributes to the resolution of the inflammatory process that platelets and neutrophils initiate. Thus, while excessive inflammation can indeed be detrimental to wound healing, in functional healing wounds, the immune system takes care of "applying the brakes" to wind down acute inflammation such that healing progresses to the proliferative phase [22]. How does this happen? Once neutrophils have cleaned up the wound site via phagocytosis, they undergo a process of programmed cell death, or apoptosis [23]: The apoptotic process prevents the release of cytotoxic and proteolytic contents of the neutrophils and also engages macrophages to phagocytose (or eat) the apoptotic neutrophils [24, 25]. The phagocytosis of apoptotic neutrophils by macrophages stimulates the macrophages to secrete TGF-β_1 [26], the cytokine crucial for promoting collagen production in functional wound healing. Furthermore, phagocytosis of apoptotic cells inhibits damage to tissue cells by activated macrophages [27] and triggers the secretion of vascular endothelial growth factor (VEGF), which is critical for repair of endothelial and epithelial injury [28]. Finally, the ingestion of dying neutrophils by the macrophages causes the macrophages to switch from a catabolic, scavenging role, to a productive, healing role and this step has been recognized as a key step in the resolution of the inflammatory phase [18, 26]. These observations point to the intimate linkage of mechanisms that promote tissue repair with cell death and clearance.

Some authors have suggested that including neutrophils in platelet-rich plasma (PRP) for tissue engineering applications may have a detrimental effect on wound healing, owing to their release of "catabolic signaling molecules," such as reactive oxygen species (ROS), upon degranulation [29]. These authors claim that the "catabolic signaling molecules" released by leukocytes, such as ROS, can damage tissue and lead to impaired wound healing, but the in vivo effects of high concentrations of neutrophils and other leukocytes in PRP have not yet been well characterized.

A question that remains is, what concentration of neutrophils should we apply to an avascular wound site in order to improve healing, as is the strategy in the delivery of PRP to the wound site in enhanced ACL repair? It is well-known that neutrophils are recruited to sites of injury and infection to debride tissue and phagocytose pathogens, in the acute inflammatory reaction to injury and infection, and are later

phagocytosed by macrophages, themselves, in the resolution of the acute inflammatory phase of wound healing. However, the in vivo effects of placing neutrophils and other leukocytes into spaces where blood cells normally do not infiltrate, as is the strategy employed for bio-enhanced ACL primary repair, have not yet been well characterized.

Macrophages

Monocytes and macrophages have been known for a long time to play important roles in wound healing, but the specifics of those roles are not yet completely known [30]. In the wound site, macrophages are known to both promote and resolve inflammation, remove cellular debris and dead or dying cells, and to promote the proliferation of fibroblasts which create the new tissue within the wound site (Fig. 18.4). In injured tissue, the early macrophage response is necessary for (1) debridement of damaged tissue in the wound and for (2) growth factor release to mediate normal repair processes. Upon tissue injury, monocytes circulating in the peripheral blood are recruited to the wound site within 8–12 h. Once they are within the wound site, they differentiate into macrophages. In the tissue, macrophages engulf or phagocytose any pathogens which may be present and activate the adaptive immune response by presenting pieces of the engulfed pathogens (or processed antigens) to lymphocytes, which in turn activates the lymphocytes and gets them started generating antibodies against the pathogens. Macrophages also work to clear the wound site of debris and dead or dying cells. The cytokines and growth factors secreted by macrophages, including IL-1, IL-6, FGF, EGF, TGF-β, and PDGF [31], regulate and coordinate the cells involved in wound healing. In addition, macrophages release FGF, TGF- β, and PDGF, which promote fibroblast infiltration of the wound site, as well as the synthesis of new collagen and breakdown of old collagen [31].

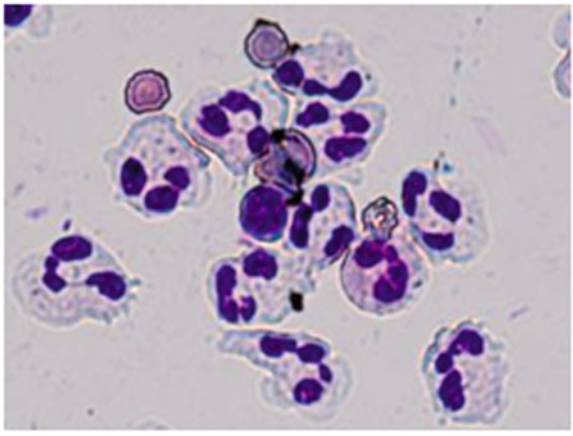

Fig. 18.4 Monocytes/macrophages are characterized by their kidney-shaped nuclei and neutral staining with hematoxylin and eosin (H&E, human peripheral blood prepped via Ficoll density gradient centrifugation, 400X)

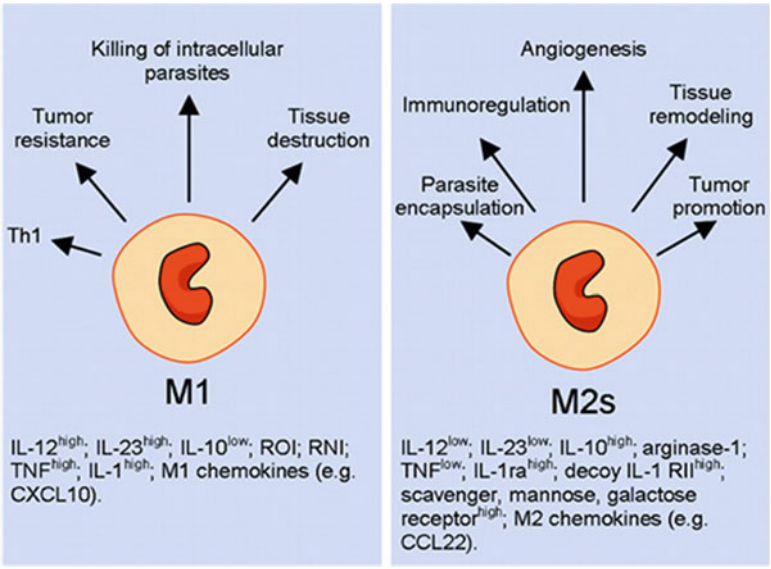

Fig. 18.5 Key properties and functions of the M1 and M2 macrophage phenotype. As is listed here, the same cell, the macrophage, can function very differently in the various stages of wound healing, depending on its activated state (Used with permission by Alberto Mantovani [32])

Recently, it has been recognized that there is more than one "type" of macrophage, that is, macrophages perform differently in different phases of wound healing, and indeed that they change their whole profile of gene expression and function during the wound healing process (Fig. 18.5). These different states of activation have been referred to as "M1" or "classically activated" to "M2" or "alternatively activated" types of macrophages – a classification which likely simplifies the range of activities of the cells but make it easier to talk about them [33]. These two macrophage types promote different parts of the wound healing process. Macrophages are crucial to functional wound healing, and dysregulation of macrophages has been found to lead to excessive or chronic inflammation and/or fibrosis. This cell lineage has to be further studied in the context of wound healing, but a few recent studies have begun to shed light on the role of macrophages in ligament healing, in particular.

"Classically activated" or "M1" macrophages have an enhanced ability to engulf (or phagocytose) dead pieces of tissue, cells, and pathogens and internally produce nitric oxide (NO) and reactive oxygen species (ROS), which the macrophage can use to kill the phagocytosed pathogens [34]. In addition, the M1 macrophages can produce high levels of the cytokines IL-12 and IL-23, proinflammatory cytokines. In contrast, the "alternatively activated" or "M2" macrophages start to slow and turn off the inflammatory response and start the tissue repair process [34]. When M1-type macrophages engulf dying neutrophils, this results in a shift of the macrophage

from the M1 to M2 phenotype, and this step has been recognized as a key step in the resolution of inflammation [17, 25].

Macrophages are not the only inflammatory cell that can switch back and forth between "proinflammatory" and "wound healing" functions – monocytes have recently been found to have similar multiple personalities. The "inflammatory" population of monocytes (characterized as Ly-6C^{high}CCR2^{high}CX3CR1^{low}) migrates into sites of inflammation, including wounds, during the early phase of the injury response, and predominates in the wound site in the first 3 days following injury. In contrast, the "wound healing" population (characterized as Ly-6C^{low}CCR2^{low}CX3CR1^{high}) migrates into inflammatory sites after the "proinflammatory" monocytes do [35–37] and produces VEGF [36]; these "wound healing" monocytes also increase the rate of production of collagen in the wound site. Both monocyte subtypes are likely to be important for optimal wound healing, as it was shown that the depletion of either subtype of monocyte resulted in reduced collagen accumulation in the wound site [36].

How does this relate to the healing of ligaments like the ACL? Studies regarding the effects of macrophages and of macrophage depletion on ligament healing have only recently been published, and most focus on the medial collateral ligament (MCL), an extracapsular ligament, rather than the ACL. Nevertheless, the results are illuminating. For instance, a recent study has also shown that nonspecific inhibition of macrophages early in the wound healing process can control excessive granulation tissue formation (a problem in certain disease processes involving excessive scarring, like liver cirrhosis) but that this inhibition of macrophage function is detrimental to early matrix formation and ultimately ligament strength [38].

Chamberlain et al. (2011) investigated the effects of macrophage depletion on healing of the medial collateral ligament (MCL) in a rat model. Skeletally mature rats were given a drug (clodronate) to knock down the number of macrophages in their bloodstream. The drug was given two days before their medial collateral ligaments (MCLs) were surgically cut in half. The ligaments were allowed to heal and the healing compared to healing in rats with normal circulating levels of macrophages at various times after injury. The animals given the drug had far fewer macrophages in the wound for the first 10 days after injury. They also had fewer myofibroblasts and blood vessel cells (endothelial cells). More new type I procollagen was found in the MCLs of these animals in the first few days; however, these wounds in the treated rats had far less Type III collagen production than normal rats and this was associated with a significant decrease in the strength of the healing tissue. These results suggest that although depletion of macrophages may stimulate early type I procollagen gene expression, inhibition of the normal production of type III collagen leads to inferior healing of a midsubstance ligament injury.

In contrast, Hays et al. (2008) found that depletion of macrophages using clodronate-treated rats in an ACL reconstruction model led to accelerated healing of the tendon-bone interface with more marked interface remodeling, reestablishment of collagen fiber continuity, and direct bone ingrowth into tendon in the macrophage-depleted animals as compared to control [39]. Chamberlain posited that the differences in the responses to systemic macrophage depletion in the MCL

transection model as compared to the ACL reconstruction model were due to differences between the anatomy and physiology of the wound sites in the two models [38]. While the Hays study analysis of the avascular tendon graft healing to the bone, the MCL studies looks at healing of vascularized, living ligament ends. Involves tendon-bone interface healing, the MCL transection wound repair model entails ligament-ligament healing. Kawamura et al. (2005) studied the spatiotemporal profile of cell infiltration into a healing ACL reconstruction wound and found that the process of tendon-to-bone healing occurs by bone ingrowth into the tendon-bone interface and that a macrophage population that invades the tendon-bone interface and differentiates into osteoclasts is likely to play a key role, whereas the intrinsic tenocytes do not contribute directly to healing in this model [39]. Chamberlain et al. (2011) argue that in the ACL reconstruction model, clodronate administration may have ablated the osteoclasts important in bone remodeling and wound repair, thereby stimulating an altered wound healing response in this tendon-to-bone healing model.

In summary, studies of ACL midsubstance tears may shed additional light on the role of macrophages in ACL healing, but it would seem that macrophages play a key role in ligament healing, as they do in other wounds.

Lymphocytes

Lymphocytes are a type of white blood cells. The three major categories of lymphocytes are T cells, B cells and natural killer (NK) cells. Both T cells and B cells work to get rid of foreign cells, bacteria, viruses or materials; however, they do this in different ways. T cells get rid of foreign invaders by producing cytokines that call other cells like macrophages to come and get rid of the invaders (T-helper cells), or by directly releasing toxic granules that kill infected cells or foreign invaders (cytotoxic T cells). There is also a group of T cells which help to prevent the immune system from reacting to cells that are supposed to be in the body (regulatory or suppressor T cells). NK cells recognize infected cells or tumor cells and get rid of them by producting toxic granules that destroy the undesirables. Like neutrophils and macrophages, lymphocytes are also important for wound healing. Patients with impaired lymphocyte function, such as those with AIDS, diabetes, malnutrition, or advanced age, are known to experience impaired wound healing [41, 42]. Recently, it has also been discovered that lymphocytes play an important regulator role during wound healing as well [43]. For example, T lymphocytes, also modulate fibroblast activity during normal wound healing [44]. Furthermore, it has been shown that getting rid of cells that suppress T cell function can result in improved healing in human dermal wounds [45], which also suggests that there is a subpopulation of T lymphocytes that stimulates wound healing. In both humans and animals, the number of T suppressor lymphocytes within the wound increases as healing progresses, findings which led Boyce et al. to hypothesize a role for T suppressor lymphocytes in downregulating healing as wounds close [46]. The role of B lymphocytes in wound healing has been far less well studied than that of the T lymphocytes, and

some authors have claimed that B lymphocytes are unlikely to play a significant role in the regulation of wound healing. However, recent early studies have suggested that the antibodies produced by B lymphocytes may play a more central role in wound healing than previously thought. The interactions between T and B lymphocytes and healing ligaments are a relatively new avenue for research.

T Lymphocytes

T lymphocytes have long been known to be important to wound healing, but their interactions with healing ligament and tendon have not yet been fully elucidated. In a study of the spatiotemporal dynamics of tendon-bone healing, Kawamura et al. (2005) found that T lymphocytes were found sporadically in the tendon-bone interface at each time point (day 0 to day 28) in a rat ACL reconstruction model [47]. Chamberlain et al. (2011) also noted a paucity of T lymphocytes in a rat model of MCL transection [48]. IL-4, a pleiotropic cytokine involved in cell growth, immune system regulation, anti-inflammation, differentiation of T lymphocytes to Th2 lymphocytes, and promotion of macrophages to the M2 phenotype, is produced by macrophages in response to tissue injury, increasing significantly 1 day after injury and peaking at 4 days before decreasing to normal levels by 21 days [48]. Rats that were treated with systemic intravenous interleukin-4 (IL-4) 2 days prior to bilateral MCL transection had reduced wound size, decreased type III collagen, and increased type I procollagen as compared to control animals [48]. In contrast, IL-4 causes decreased fibroblast proliferation when added to these cells alone in vitro. IL-4 is known to mediate immune suppression via its effects on T lymphocytes and macrophages, pushing them toward an "anti-inflammatory" M2/Th2 profile, and it is these cell populations that are likely responsible for the enhanced early healing response in the MCL of rats pretreated with IL-4 [48].

Another part of the wound healing picture that has not yet been explored in orthopedic surgery but has been extensively researched in oral and maxillofacial surgery is that of the tissue resident T cell. Resident gamma/delta T cells have been shown to be important in skin wound healing, as their absence delays healing [49, 50] – this is a different population from the T cells that infiltrate during inflammation. Gamma/delta T cell populations have also been detected in periodontal ligaments [51], but to our knowledge, they have not been studied in other ligaments. Surprisingly, however, it has recently been shown that fracture healing is accelerated in mice lacking a functional adaptive immune system due to the absence of recombination activating gene-1 (RAG-1), which is vital to the development of T lymphocytes [52]. The RAG1(−/−) mice exhibited reduced expression of inflammatory cytokines and strong upregulation of anti-inflammatory interleukin 10 (IL-10) [52]. However, the mechanism for this enhanced fracture healing has not yet been elucidated, and because fracture healing is different from soft tissue wound healing, the results of lymphocyte depletion on soft tissue healing may very well be different.

T cells have also been shown to be important in chronic inflammatory processes in the joint. For example, dogs with inflammatory stifle (knee) arthritis and

degenerative cranial cruciate ligament rupture had an elevated proportion of T helper (CD4+) and cytotoxic T lymphocytes (CD8+) in their stifle synovium and synovial fluid as compared to healthy control animals [53]. T helper lymphocytes have recently been implicated in the progression of osteoarthritis by inducing macrophage inflammatory protein-1γ (MIP-1γ) in a murine ACL transection model [54].

The role of the various T lymphocyte populations in ligament healing likely mirrors that of the monocyte/macrophage cell lineage, in that a balance between the proinflammatory and anti-inflammatory subtypes is involved in successful wound healing. Further study will likely allow us a greater understanding of the complexity of the role of these cells in the wound healing process.

B Lymphocytes

The traditionally recognized role of B lymphocytes is to secrete antibodies to antigens. The role of B lymphocytes in wound healing has not been extensively studied because their main function – secretion of antibodies against antigens in the humoral response – was thought to have little reason to affect the healing of sterile wounds. However, one recent study suggests that B lymphocytes secrete antibodies to wounded tissue that can enhance cutaneous wound healing. The role they play in ligament healing is not yet fully characterized but is likely to mirror their role in healing of other soft tissues, which we are only beginning to understand.

A recent study found that in the wound site, there are detectable levels of immunoglobulins secreted by B cells against both antigens specific for microorganisms as well as tissue antigens and also demonstrated that the restoration of B cells to splenectomized mice can rescue their wound healing capabilities. Although the authors did not detect any B or T lymphocytes in the wound sites in the splenectomized animals by 24 h after they were injected with T lymphocytes, they did detect (via immunohistochemistry) autoreactive IgG1 antibodies, which were secreted by B cells, bound to damaged tissues. Not surprisingly, splenectomy caused a delay in the clearance of neutrophils by macrophages, which stayed in the wound site longer than in normal mice, and subsequently resulted in a delay in the differentiation of fibroblasts into myofibroblasts and in the appearance of endothelial cells, which are important to wound contraction and angiogenesis. However, there was no significant difference in collagen content, as assessed by hydroxyproline assay, between the two groups of animals [55].

Summary

Wound healing is an incredibly complex process involving multiple cell types with multiple roles. The roles of the monocyte/macrophage phenotypes have shown there are a spectrum of behaviors for these cells, depending on the timing and

environment of the wound site. Neutrophils act predominantly early in the wound site, and they die within the first day or two after injury. Their death, however, leads to the switch of the macrophage from a cell which is actively stimulating "clean up" of the wound into a cell type which promotes collagen production and repair of the wound. Other cell types also appear to have the ability to switch from one role to another in this intricately coordinated process. The role of all the inflammatory cells is beginning to become clearer, and future studies of these cells will likely provide critical information about how we can best promote wound healing in all tissues.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 19
Safety of the Bio-enhanced Repair

Elise M. Magarian and Martha M. Murray

In recent years, there has been an increasing interest in using extracellular matrix (ECM) based biomaterials for enhancement of the repair of structures in the knee joint. Applications for collagen and extracellular matrix-based (ECM) biomaterials have included use as a platelet stabilization scaffolds in a central defect model [1, 2], complete ACL transection [3, 4], and bio-enhanced ACL reconstruction [5]. Making scaffolds from human tissues is problematic as sourcing can be limited and expensive. The use of animal tissues may be more reliable and available; however, before xenogenic biomaterials (i.e., material from another species) can be considered for use in surgical procedures, their safety within the body must be established. In this light, it is essential to define the criteria that denote safe use of collagen-based biomaterials in ACL repair. Across species, the amino acid sequence and epitope structure of collagen is remarkably similar, and safe use of bovine or ovine collagen has been documented historically [5].

In past studies of xenogenic collagen safety, only 2–4 % of individuals were found to be clinically reactive to its presence. These reactions were found to occur extra-articularly, and most were inconsequential. Risk of reaction to collagen or other ECM molecules in the intra-articular environment should be even lower due to the immunoprivileged joint space [6, 7] as the avascular and alymphatic environment within the knee further reduces the likelihood of immunologic response to xenogenic collagen. However, the possibility of provoking such a response within the joint space must be considered, especially given that the procedure also introduces a platelet concentrate and thus a higher concentration of cytokines that have the potential to trigger, facilitate, or exacerbate an immunologic response.

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_19,

The biomaterial we examined in these studies was a scaffold comprised predominantly of type I collagen but also contained other extracellular matrix-based proteins, including glycosaminoglycans (MIACH, Children's Hospital Boston, Boston, MA). For clarity in this chapter, we will refer to the scaffold as an "ECM scaffold." This scaffold was created by solubilizing bovine connective tissue with pepsin, treating the resulting digest with several proprietary steps and then lyophilizing the digest to form a spongelike, porous scaffold as previously described [8].

Immunologic response may occur on one of two levels – either systemically, affecting the whole body, or locally, within the enclosed joint space. The former would present as an elevated white blood cell count and an increase in inflammatory markers in the peripheral blood. The latter would typically manifest itself with a joint swelling (an effusion), an increase in the number of white blood cells in the joint fluid and thickening of the synovium (the tissue lining the joint). To determine whether or not an immunologic response occurs following the introduction of our xenogenic ECM scaffold, we examined several systemic and local criteria in 15-week study of a porcine animal model.

Parameters Determining Safety

To determine the safety of the presence of a xenogenic, ECM-based material within the knee joint, we examined the physical characteristics of the knee as well as systemic inflammatory markers indicative of an adverse reaction after its implantation in the knee joint. Flexion and extension of the knee joint, joint effusion as seen on MRI, synovial hypertrophy characterized with histology, intra-articular (synovial fluid) and systemic (whole blood) leukocyte counts, and the level of the systemic inflammatory markers interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) were all considered in the determination of the safety of the extracellular matrix-based implant.

Flexibility of the knee joint, characterized by the differences between passive flexion and extension before and after surgical implantation of the ECM scaffold, is an indicator of overall joint health. Changes in joint mobility, namely, a loss of range of motion, may indicate swelling of the tissues which may be related to an underlying inflammatory process. Similarly, an effusion (or an increase in fluid in the joint space), which can be seen on MRI images (Fig. 19.1), and hypertrophy (or overgrowth) of the synovial tissue, as seen in histological preparations, can also be signs of an inflammatory reaction.

In addition to examining the physical properties of the joint tissues, the synovial fluid in the joint space can also provide valuable information regarding a localized intra-articular reaction. As in systemic body fluids, an elevated white blood cell (leukocyte) count in the synovial fluid of the knee may indicate an inflammatory process occurring in that joint.

Like these indicators of local inflammation, systemic markers in the blood also play a role in determining the body's reaction to the implanted biomaterial. Elevated levels of leukocytes, in addition to an increase in cytokines such as TNF-α and IL-1β, may indicate a systemic inflammatory response.

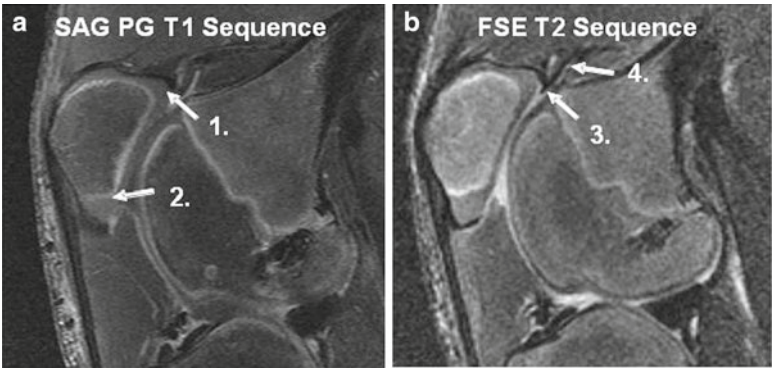

Fig. 19.1 MRI measurement of synovial thickness and effusion. Lateral images of the knee with the largest area of patella visible are represented here. (**a**) SAG PG T1 Sequence (*1*) Suprapatellar synovium. (*2*) Infrapatellar Synovium. (**b**) FSE T2 sequence (*3*) Suprapatellar effusion width. (*4*) Suprapatellar effusion length (Used with permission by Elise Magarian et al. [9])

Leukocytosis, or an elevated white blood cell count in the circulating blood, is indicative of an infectious or inflammatory process somewhere in the body. This elevation may occur due to a variety of processes including bacterial, viral, or fungal infections, or because of an established disease state (such as cancer or tuberculosis). While leukocytosis is not a definitive indicator of the type of infection or inflammation present or its location, it does provide clues that an inflammatory process is occurring somewhere in the body.

Cytokines are molecules secreted by various types of cells that function as intercellular communicators. They can also be referred to as growth factors. Different types of cytokines are produced by specialized cells and indicate different responses to processes occurring in the body. While different cytokines are associated with a variety of different processes, several – including IL-1β and TNF-α – are elevated in the occurrence of an inflammatory response. Because the elevation of each cytokine can be linked to a specific function based upon the receptors they interact with, it is possible to make connections between the occurrence of an elevated cytokine level and the presence of a systemic inflammatory process.

Porcine Model for Intra-articular Collagen Safety

In order to determine the safety of implanting an ECM scaffold into the knee joint, a porcine animal model was utilized in a controlled trial. Eighteen animals were divided into groups based upon the type of surgery applied to one knee, and the results were compared to the contralateral intact (non-operated) knee. Surgical groups included repair with sutures only (SUTURE group), repair with an ECM and sutures (SPONGE group), and repair with sutures and an ECM-based scaffold loaded with platelet-rich plasma (ECM group). Results of physical examination criteria, systemic and synovial, leukocyte counts, inflammatory cytokine levels, joint effusion, and synovial hypertrophy were measured at 15 weeks after surgery. There

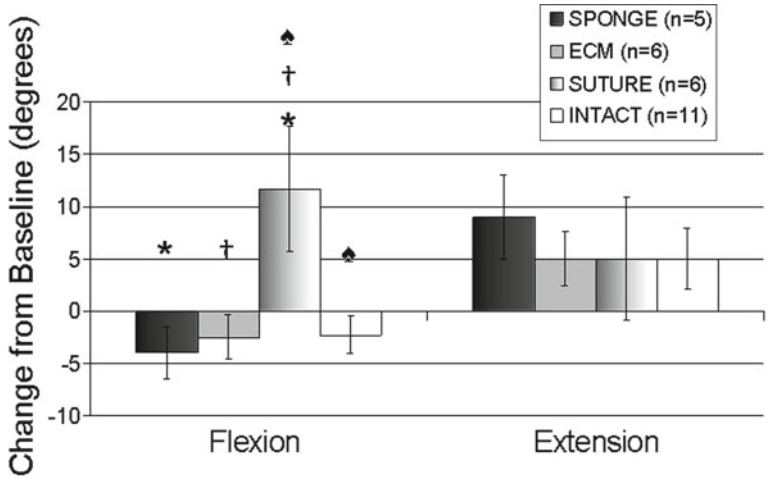

Fig. 19.2 Comparison of suprapatellar effusion dimensions across treatment groups. The addition of atelocollagen in either SPONGE or ECM had no effect on the amount of suprapatellar joint effusion width as measured on the sagittal sections of MRI compared to INTACT knees ($p = 0.201$). Knees treated with suture repair alone had a significantly lower suprapatellar effusion length than either the INTACT or ECM knees ($p < 0.001$). All values represent mean effusion (mm) ± standard error (Used with permission by Elise Magarian et al. [9]). All bars marked with the same symbols are significantly different from each other

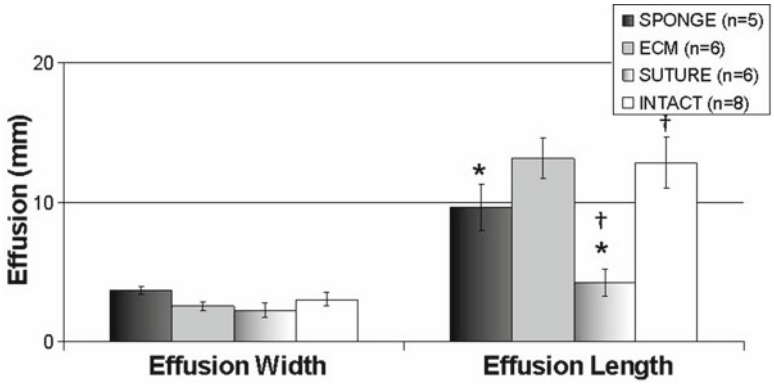

Fig. 19.3 Comparison of suprapatellar effusion dimensions across treatment groups. The addition of atelocollagen in either SPONGE or ECM-platelet form had no effect on the amount of suprapatellar joint effusion width as measured on the sagittal sections of MRI compared to INTACT knees ($p = 0.201$). Knees treated with suture repair alone had a significantly lower suprapatellar effusion length than either the INTACT or ECM-platelet treated knees ($p < 0.001$). All values represent mean effusion (mm) ± standard error (Used with permission by Elise Magarian et al. [9]). All bars marked with the same symbols are significantly different from each other

Table 19.1 Qualitative histological evaluation of ECM and Suture groups

	Cell layers	Lymphocytes	Vascularity	Villi
ECM: ACL-Synovium ($n=7$)	2.88 ± 0.89	0.05 ± 0.08	1.33 ± 0.71	0.02 ± 0.02
ECM: CAPS-Synovium ($n=7$)	3.48 ± 0.96	0.00 ± 0.00	1.24 ± 0.64	0.07 ± 0.07
SUTURE: ACL-Synovium ($n=6$)	3.33 ± 1.054	0.06 ± 0.14	1.42 ± 0.74	0.06 ± 0.09
SUTURE: CAPS-Synovium ($n=6$)	2.94 ± 1.58	0.08 ± 0.14	1.22 ± 0.72	0.11 ± 0.20

The synovial tissue covering the healing ACL (ACL-Synovium) and lining the joint capsule (CAPS-Synovium) at a location remote from the prior incisions was fixed in formalin and sectioned. Three areas of each tissue were analyzed. Within each area, the number of cell layers comprising the synovium was recorded, and vascularity, lymphocytes, and villi were rated on a zero-to-three scale, with zero being none present, one being below normal, two being normal, and three being above normal. All values represent mean ± standard deviation. There were no observed significant differences between the groups treated with suture alone (SUTURE) and those treated with the ECM-platelet composite (ECM) for any of the measures

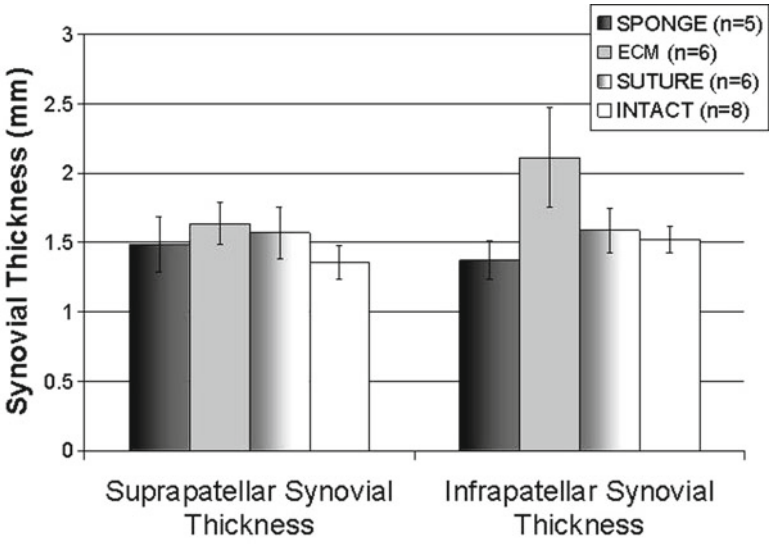

Fig. 19.4 Synovial thickness as measured by MRI at 15 weeks postoperatively for all treatment groups. Infrapatellar synovial thickness was not significantly different when comparing SUTURE and INTACT groups ($p=0.9063$). The ECM-platelet composite (CPC) and SPONGE groups approached significance ($p=0.0665$), and all groups showed no significant differences when compared to INTACT knees ($p>0.1$). All values represent changes in these parameters from the baseline measurements at the time of surgery. Error bars represent standard error (Used with permission by Elise Magarian et al. [9])

was a greater loss of flexion for suture group compared with INTACT ($p=0.0034$), SPONGE ($p=0.0053$), and ECM ($p=0.0077$) groups. There were no significant differences between groups for extension ($p>0.4762$ for all; Fig. 19.2). There was no increase in joint fluid noted between SPONGE or ECM groups and INTACT knees (Fig. 19.3). We found that there were no significant changes in the WBC levels in blood or synovial fluid, which all remained within normal limits. There was no evidence of synovial hypertrophy in any of the animals with the collagen- or ECM-based scaffold by either histological (Table 19.1) or MRI criteria (Fig. 19.4) [9].

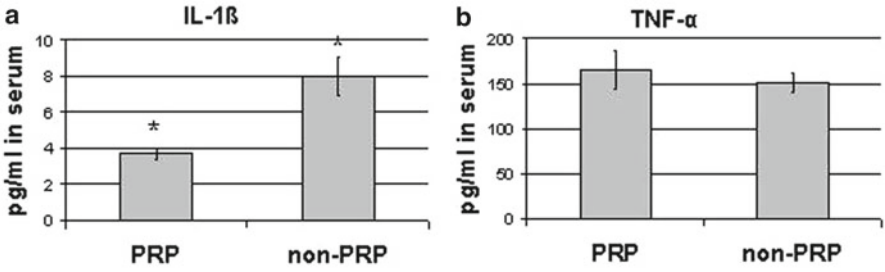

Fig. 19.5 Comparison of inflammatory markers in PRP and non-PRP groups. *Significant difference in the level of IL-1β between the PRP and non-PRP groups ($p=0.002$) (Reprinted with permission from Magarian et al. [9])

In addition to the possible response to collagen alone, we also wondered if the presence of a platelet concentrate in conjunction with the extracellular matrix implant would exacerbate or induce an immunologic reaction. Platelets are known to stimulate cytokine release and thus can be linked to immune response. In our study, however, we found no association between the addition of platelets to the joint and outcome measures for immune response. Levels of TNF-α were not different between the PRP and non-PRP groups, and levels of IL-1β were actually lower in the PRP groups than in the non-PRP groups. These results do not render a definitive conclusion, though it can be suggested that enhanced repair with PRP may result in a reduced release of IL-1β and therefore a subdued inflammatory response (Fig. 19.5) [9].

Overall In Vivo Safety Profile of the ECM Scaffold in the Knee

To date, the ECM scaffold has been implanted in 162 porcine knees of 138 animals in eight studies. In several of these studies, there were surgical and postoperative complications noted in several of the animals (Table 19.2). These included respiratory infection, perioperative respiratory failure, subcutaneous abscess of the jaw, hemolytic anemia, postoperative paralytic ileus, prolonged lameness, as well as several others. The observed complications are listed here along with any interventions performed and resulting deaths.

However, in all of these studies and all of these animals, the operative knees that had the ECM scaffold implanted had no significant complication that could be attributed to the use of this scaffold. Operative knees for both ACL reconstruction and ACL repair with the ECM scaffold did have loss of range of motion at 3 months postoperatively, but the repair and reconstruction groups were not different, suggesting this is a function of surgery and potentially insufficient rehabilitation (the pigs are hard to get to exercise) rather than a reaction to the scaffold [10]. The knees

Table 19.2 Summary of complications observed in animals implanted with the ECM scaffold

Complication	Intervention	Number of occurrences	Number of deaths
Respiratory infection	Euthanasia	1	1
Perioperative respiratory failure	Epinephrine, doxapram, assisted breathing	1	1
Hemolytic anemia	Euthanasia	1	1
Postoperative paralytic ileus	Euthanasia	1	1
Knee infection	Euthanasia	1	1
Prolonged postoperative lameness	Banamine and/or fentanyl for pain management	3	0
Subcutaneous abscess (jaw)	Area cleaned, cephalexin	1	0

treated with an ECM scaffold repair actually had less loss of thigh circumference than their study counterparts undergoing reconstruction. The reconstructions were performed with allograft, so that a pain response to loss of part of the extensor mechanism would not explain this, but perhaps the lesser pain of repair versus reconstruction might.

Summary

Collagen and ECM scaffolds, sourced from bovine animals, have shown significant promise for use as a biomaterial in a variety of intra-articular applications [11–13]. Concerns about the safety of using non-autologous collagen in vivo persist, and little evidence has been produced to counter these claims. However, atelocollagen, like that used in our study, is generally considered a safer option for collagen implants when compared with generic collagen because atelocollagen lacks the immunogenic telopeptide in its molecular structure [5, 14].

Further studies are needed to more fully explore the possible immunologic reaction that may occur in response to the intra-articular use of collagen biomaterials. Additional parameters and longer time points are necessary to validate safety beyond 15 weeks. Although the pig is a validated model for human immunity, [15, 16] additional studies are necessary to confirm that our conclusions are translatable to humans.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 20
Reinnervation and Revascularization in Engineered ACL Healing

Benedikt Lorenz Proffen and Martha M. Murray

Because the ACL plays a crucial role in the stabilization of the knee and its motion, ACL deficiency causes joint instability which will then lead to increased degeneration of tissue and ultimately to early-onset osteoarthritis [1]. Therefore, a sufficient treatment is necessary to prevent the immense socioeconomic burdens created by this long-term complication [2]. The current gold standard of treatment for complete ACL tears is surgical reconstruction of the ligament with an autologous or allogenic tendon graft, which restores anterior-posterior knee stability, but cannot eliminate the risk for osteoarthritis [3]. But why can't ACL reconstruction stop the progression of joint deterioration?

The movement of the legs and especially the knee is not just a simple extension-flexion motion. It is a rather complicated interaction between the central nervous system, the nerve fibers within the ligaments and capsule of the joint, and the muscle groups surrounding the joint. This neurologic system helps maintain knee motion within safe and normal limits by rapidly signaling and coordinating the surrounding knee musculature. The ACL itself is one of the key neurologic regulators of normal knee motion. The ACL has many mechanoreceptors [4–6], and when the tibia starts moving too far forward from the femur, the subsequent stretch on the ACL leads to a reflex contraction of the hamstring muscles which brings the tibia back into line [7–9] (Fig. 20.1). ACL reconstruction typically removes the injured ACL and the nerve receptors within the tissue, which may be the reason that this sensorimotor function seems to be deficient after ACL reconstruction [10, 11].

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_20,

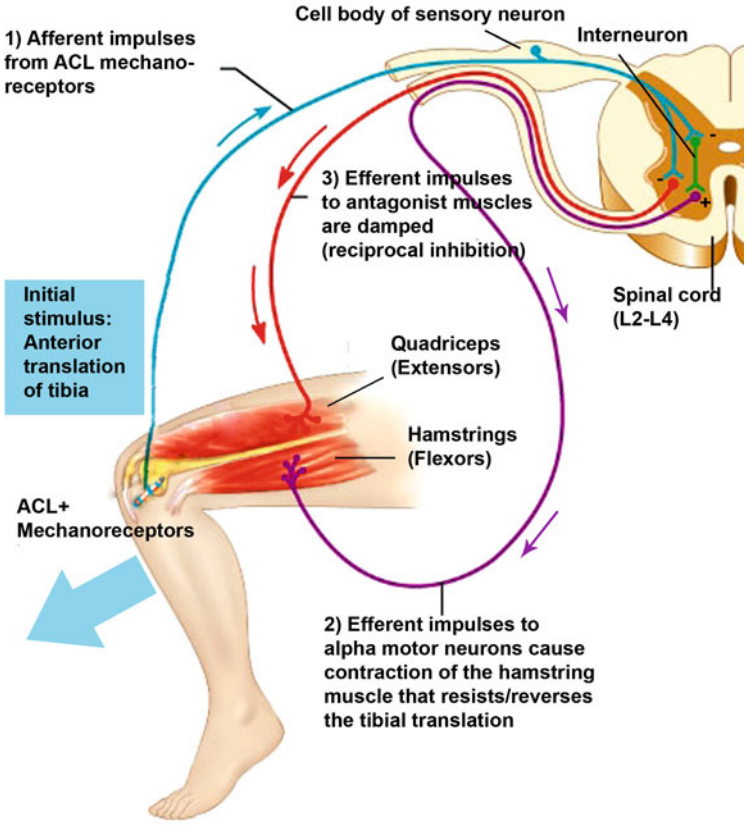

Fig. 20.1 Reflex arc of the knee. The stretch of the ACL mechanoreceptors by anterior translation of the tibia triggers an afferent impulse (*blue line*) which is switched on the level of the spinal cord (L2–L4) to prompt polysynaptic reflex during which the quadriceps muscles are damped and the hamstring muscles are contracted

The blood supply, or vascular system, plays a critical role in the normal function of the ACL and its response to injury. The ACL cells rely upon an adequate blood supply for the delivery and removal of metabolic substrates and cells in normal and injured tissue [12].

After ACL reconstruction, the tendon graft used to replace the injured ligament does not contain a viable blood supply and therefore needs to create one within its substance if it is to function as a biologic graft rather than purely a mechanical one. What revascularization of the graft tissue takes place has been investigated extensively in several studies, with early revascularization of the graft surface seen at 2–4 weeks, but a persistent avascular zone in the mid-substance of the graft exists even after 6–12 months after graft implantation [13]. There is likely also biologic variability in the revascularization process, with some patients having more revascularization of the graft than others.

Bio-enhanced ACL repair, which preserves the native remnants of the ACL (and thus the nerves and blood vessels contained in the tissue), may make it easier for innervation and vascularization to persist and thrive in the ACL after injury. Bio-enhanced ACL repair [14] combines a primary suture repair with a scaffold containing platelets to improve the healing of the ACL. To what extent the nerves and vessels can be preserved and encouraged to regenerate in this bio-enhanced repair process will be reviewed in this chapter.

Innervation and Vascularization of the Healthy ACL

In general, the human anterior cruciate ligament is covered with a layer of synovial tissue. Anteriorly, the synovial covering merges into the connective fibrous capsule tissue via the ligamentum mucosum. Branches from the tibial nerve and branches from the arteria genus media run within the ligamentum mucosum and supply the ACL with nerve fibers and blood vessels [15, 16]. Sensory structures like free nerve endings and Ruffini and Pacini corpuscles are found in the connective tissue between the synovial membrane, the cruciate ligament and the connective tissue septa between the individual fascicles of the ACL [15] (Fig. 20.2). While the blood vessels split into smaller capillaries and build a network which runs between the fascicles of the ACL and supply it with nutrients, the function of the nerve structures is more complex, as evidenced by the presence of a variety of different fibers and sensory structures.

Nerve Fibers in the Synovial Tissue Around the ACL

Three different types of nerve endings have been found in the subsynovial layer encapsulating the ACL in rabbits. These include mechanoreceptors, which send signals to the brain when the ligament is stretched, as well as nerves which can control the dilation and contraction of the blood vessels within the subsynovial layer. In addition, Ruffini corpuscles (a type of specialized sensory nerve endings) were also noted [17] (see Fig. 20.2).

Nerve Structures in the ACL

Within the human ACL, free nerve endings as well as the sensory structures of Ruffini and Vater-Pacini corpuscles have been noted [15]. The free nerve endings are able to act to sense stretch (mechanoreceptors) or pain (nociceptors). The Ruffini

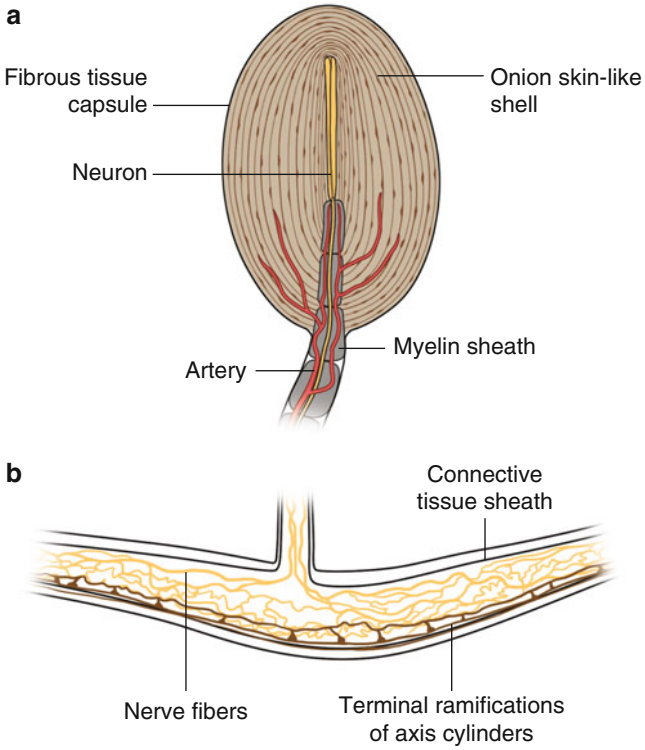

Fig. 20.2 Mechanoreceptors of the ACL. (**a**) Scheme of a Pacinian corpuscle. It is a quickly adapting mechanoreceptor sensitive to vibration. The myelin sheath covered nerve afference in the stem of the corpuscle loses the myelin when advancing to the center of the mechanoreceptor where the naked neuron is surrounded by an onion skin-like shell and a fibrous tissue capsule. (**b**) Ruffini ending. It is a slowly adapting mechanoreceptor sensitive to stretching of collagen fibers which traverse the barrel-like receptor from one end to the other and are surrounded by nerve endings

corpuscles are mechanoreceptors and act as slowly adapting stretch receptors [18], while the Vater-Pacini corpuscles are fast adapting and act as velocity receptors (see Fig. 20.2).

Reflex Arcs of the Knee

In animal models, the mechanoreceptors of the ACL have been shown to provide information about the angle of the joint during motion [19], and to slow the motion of the knee as the motion limits are reached [20]. Stretch of the ACL mechanoreceptors has also been shown to stimulate the hamstring muscles to fire through a polysynaptic reflex arc [21]. This reflex arc has also been noted in human subjects [8] (see Fig. 20.1).

Revascularization and Reinnervation in Functional Wound Healing

The process of wound healing is very complex and involves a multitude of separate processes which are orchestrated in a specific sequence. Briefly, as we have seen in Chap. 6, the wound healing process can be thought of as occurring in three phases: inflammation, proliferation, and remodeling. Angiogenesis or neovascularization begins after injury by the formation of capillaries by vascular endothelial progenitor cells after 4–5 days [22]. These endothelial stem cells originate from parts of uninjured blood vessels, which migrate through the basal membrane of the vessel into the wound site, attracted by angiogenic factors released by macrophages or platelets [23]. Reinnervation is a very important aspect for the wound healing process as well, with the job of regulating vascular and lymphatic flow to and from the wound. Denervated skin exhibits delayed wound healing [24, 25]. All phases of wound healing showed a greater wound surface area and were prolonged when nerve tissue in the wound area was artificially reduced by capsaicin injection [26]. Also, nerve tissue-specific neuropeptides including nerve growth factor (NGF) and substance P (SP) have been shown to increase wound contraction and leukocyte chemotaxis [27, 28].

What Happens to the Nerves and Blood Vessels in the ACL After Injury?

Prior studies have shown that nerve and vascular structures are preserved in the ACL remnant after ACL injury [29–31]. In the human ACL, neovascularization coincides with a hypercellularity in the ligament which occurs between 8 and 20 weeks after injury [29] (Fig. 20.3). Silver and PGP 9.5 immunostaining has been used to look for nerve structures within the ruptured human ACL, and nerves were visualized even at 12 months after injury [30]. The use of immunohistochemical techniques to look specifically for mechanoreceptors (stretch sensors) found these sensors to still be present in the ruptured ACL, but with a reduced number when compared to the intact ACL [31].

What Happens to the Nerves and Blood Vessels After ACL Reconstruction?

Reinnervation After ACL Reconstruction

ACL reconstruction typically involves removing the torn ACL and its nerve supply and replacing it with a new free tendon graft that does not involve any connections

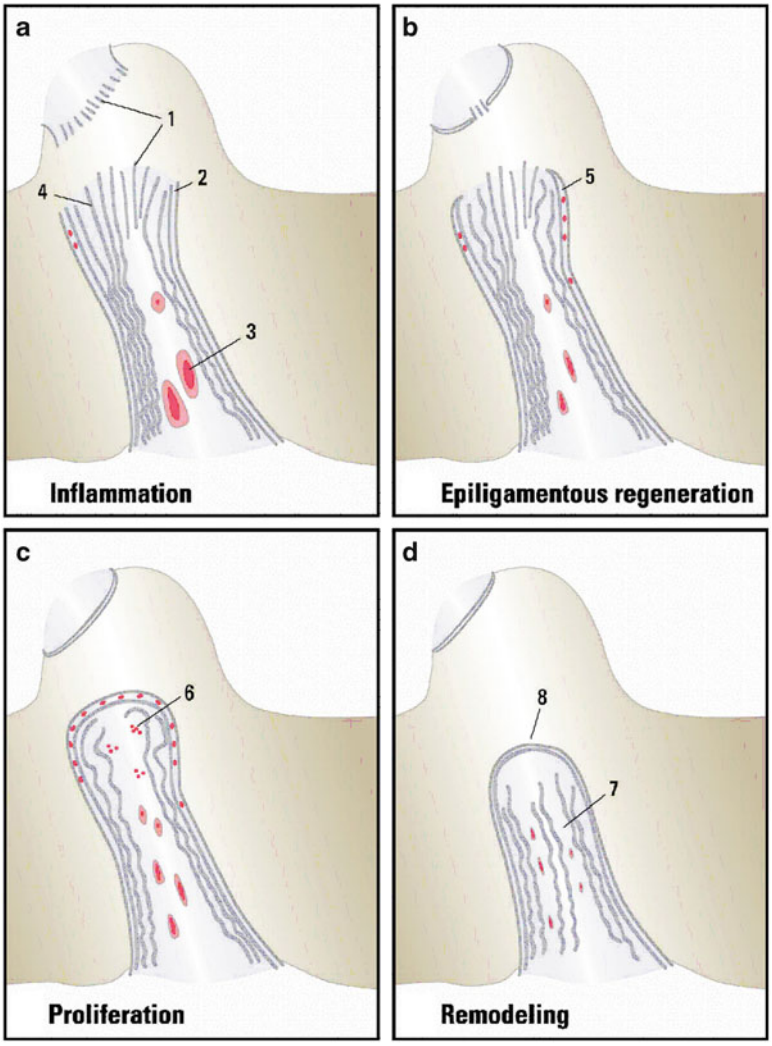

Fig. 20.3 Healing response in the ruptured ACL. (**a**) Inflammation showing mop-ends of the remnants (*1*), disruption of the epiligament and synovial covering of ligament (*2*), intimal hyperplasia of the vessels (*3*), loss of regular crimp structure near the injury site (*4*). (**b**) Epiligamentous regeneration of the ligament remnant by vascularized, epiligamentous tissue and synovial tissue (*5*). (**c**) Proliferation with revascularization of the remnant (*6*). (**d**) Remodeling with decrease of cell number and blood vessel density (*7*), and by retraction of the ligament remnant (*8*) (Reprinted from Sonnery-Cottet et al. [32], with permission from Elsevier)

to the local nerve supply. Thus, with current ACL surgical techniques, any nerve supply to the graft would need to grow in after the graft had been implanted. Aune et al. investigated reinnervation of a patellar tendon autograft in humans but did not find any positive staining for neuropeptides in 5- to 37-month-old specimens [33]. Biedert and Zwick found an increase in EMG activity in the hamstring muscle after a Lachman maneuver but could not initiate the reflex by direct mechanical stimulation of the ACL alone [34]. Iwasa et al. stimulated the ACL electrically and found a decreased hamstring response in patients with ACL grafts [35]. The existence of the inhibitory reflexes found by Dyhre-Poulsen and Krogsgaard [8] was tested on patients after ACL reconstruction by Krogsgaard et al. who had to stimulate the ACL with a 3.5 times higher current than patients with a healthy ACL to trigger the muscle reflex [36]. Combined, these results suggest that the neurologic function of the ACL is not completely restored even months after ACL reconstruction.

Revascularization After ACL Reconstruction

The process of revascularization of the graft starts 2–4 weeks after implantation, with blood vessels from the surrounding synovial tissue and retropatellar fat pad starting to grow onto and into the avascular graft [13, 37, 38]. Arnoczky et al. were one of the first investigators to study revascularization after a patellar tendon graft ACL reconstruction in dogs. Histologically, after 2 weeks, there was no vascularization seen in the implanted graft, and at 4 weeks, only the synovial membrane that had formed around the graft had a blood supply. After 6 weeks, there was still a central area of avascularity, relative acellularity, and fragmentation of collagen bundles in the graft, whereas the synovial covering was hypervascular. Ten weeks after reconstruction, there was an invasion of capillary buds into the avascular graft [39]. In the sheep model, a free tendon graft was found to stimulate a migration of vessels from the synovial envelope toward the center of the graft, with the highest vascular density seen at 6 weeks after grafting [38]. In rhesus monkeys, a microangiographic analysis revealed a relatively hypovascular mid-zone of the graft at 8 weeks after implantation, while after 3 months, the paraligamentous and endoligamentous vascular supply was readily apparent, entering mainly from the posterior synovial fold but also with substantial contributions from the endosteal vessels within the femoral and tibial tunnels [13].

In humans, MRI with gadolinium has been used to evaluate the revascularization process of bone-PT-bone autografts. This study showed a significant increase in blood flow in the intra-articular portion of the graft after 6 months, whereas the bone attachments were slower to revascularize [40]. Shino et al. used laser Doppler flowmetry to measure the surface blood flow of allograft ACL grafts in humans after ACL reconstruction. After 6 months, the grafts had a significantly elevated blood flow compared to the native ACLs and there was a thickening of the synovial layer covering the graft. After 12 months, the elevated blood flow levels in the grafts had normalized, and no difference to the native ACLs was detectable by the Doppler flowmetry [37].

These results show that revascularization of the graft after implantation occurs gradually along its length, the intra-articular site being the first and the faster part to complete this phase, while the intraosseous parts are still progressing throughout the first postoperative year. In addition, a central necrotic core still may remain, even months after ACL reconstruction.

What Happens to the Nerves and Blood Vessels After Bio-enhanced ACL Repair?

During our several studies in animal models of bio-enhanced ACL repair, we described the processes of revascularization and reinnervation of the wound between the ACL stumps [14, 41–44]. Joshi et al. reported a large increase in vascularity in the ACL wound after 4 weeks of healing and an additional 50 % increase in blood vessel density between 4 and 6 weeks. The vascularity then dropped by 40 % between 6 weeks and 3 months as the ligament matures [41] (Fig. 20.4). Mastrangelo et al. described the revascularization after bio-enhanced ACL repair during the first 4 weeks of healing in animals of various ages. After 1 week, thin-walled venules and capillaries were noted in the ACL stumps and the central wound site. Vessel walls were thicker after 2 weeks, and a higher number of venules and capillaries were present with a high density in the epiligamentous region. After 4 weeks, the vessels seemed more mature and were present in the ACL stumps as well as in the central wound site, but the blood vessel density did not change significantly between 2 and 4 weeks [42]. Additional data on these histologic sections demonstrates the ongoing presence of nerve fibers in the healing ACL as well (Fig. 20.5). Although the data are still preliminary, this suggests that maintenance of the neurologic and vascular function of the ACL may be possible with bio-enhanced repair of the ACL.

Conclusion

Both the blood supply and nerve supply play critical roles in the normal functioning of the ACL and the knee. Injury to the ACL can cause loss of these functions, and ACL reconstruction, which has typically involved removing the old ACL tissue prior to placing the new graft, may result in a further loss of these systems. The blood supply appears to be able to reestablish itself, at least on the periphery of the graft, although an avascular core of collagen may remain. Reinnervation of the ACL graft is unpredictable at best and likely does not occur to any useful extent. While the ACL remnants usually are removed during the standard ACL reconstruction procedure, a preservation of these structures might be beneficial to the graft as it may provide a source of vascularity and nerve fibers which could contribute to revascularization and reinnervation of the ACL graft. The new method of biologically enhanced ACL repair may allow for a great number of retained nerve structures and vascularization in the healed ACL.

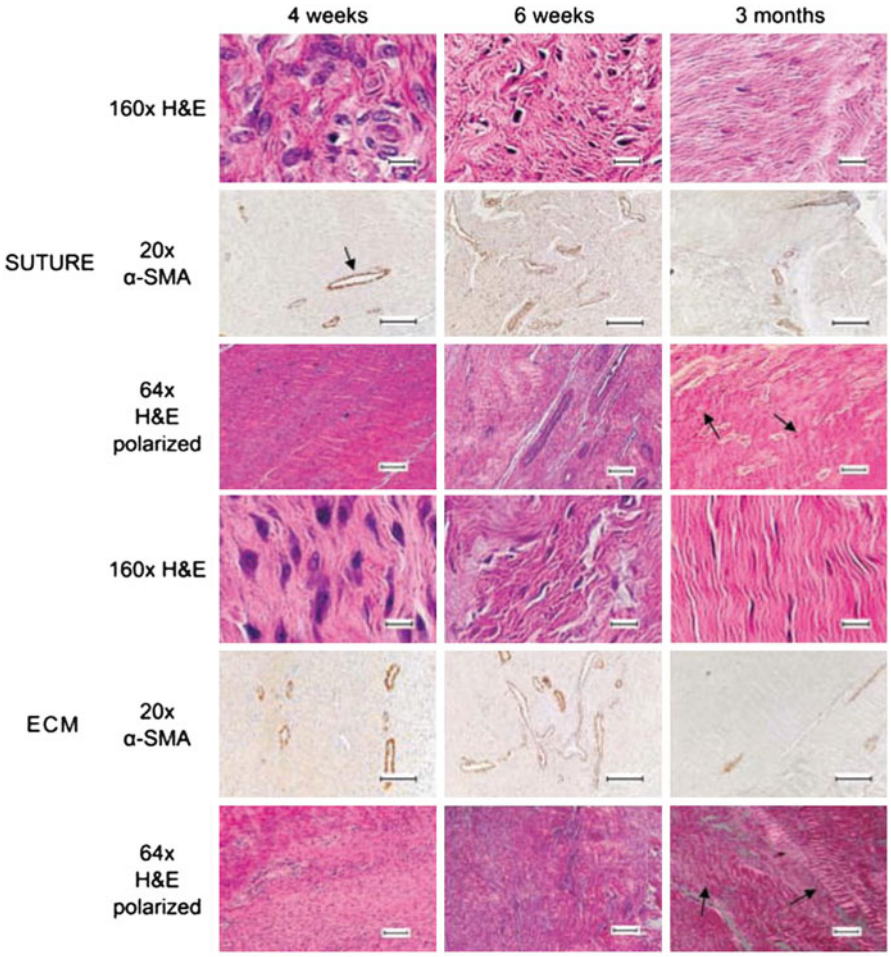

Fig. 20.4 Revascularization in enhanced ACL repair. Changes in suture and ligaments treated with an ECM scaffold containing platelets (CPC) groups over time. Blood vessels are noted as the ringlike structures staining positive for α-SMA (*arrow* points to example). Notice the change in cellularity from ovoid cells in both groups at 4 weeks to spheroid cells in the suture group and fusiform cells in the CPC group at 3 months; note also the increase in blood vessels in both groups at 6 weeks. The arrows in 64× H&E polarized indicate bundles of collagen, which are more consistent in the CPC-treated ligaments at 3 months. The size bar for 160× H&E is 10 μm; for 20× α-SMA, 100 μm; and for 64× H&E polarized, 100 μm. *CPC* extracellular matrix (ECM)-platelet composite, *H&E* hematoxylin and eosin, *α-SMA* α-smooth muscle actin, where *red* is a positive stain (From Joshi et al. [41], copyright © 2009 by (Sage Publications), Reprinted by Permission of SAGE Publications)

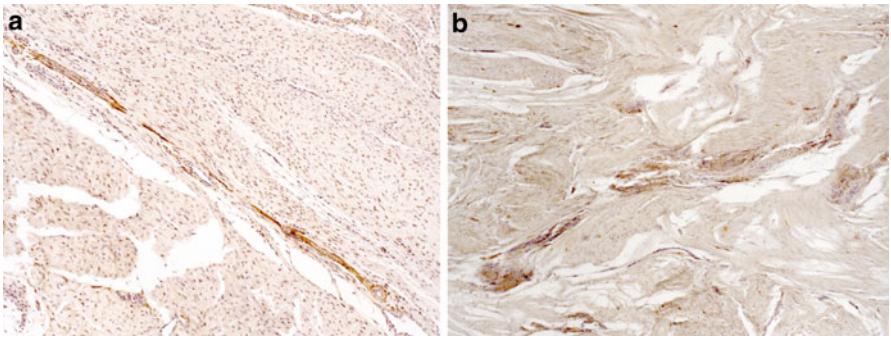

Fig. 20.5 Reinnervation in enhanced ACL repair. (**a**) Low-affinity nerve growth factor (LNGFR) staining at 4 weeks. (**b**) LNGFR staining after 9 weeks

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 21
Bio-enhancement of ACL Graft Healing

Braden C. Fleming

The current "standard of care" for an ACL injury is reconstruction with either an autograft (harvested from another tendon within a person's own body) or allograft (harvested from another donor) tendon. The objective of ACL reconstruction surgery is to provide gross stabilization of the knee, to improve post-injury joint function and patient-oriented outcomes, and to lessen the risk for post-traumatic osteoarthritis. Current surgical treatments of the ACL injury meet some of these goals but not all.

There are many studies that have shown that ACL reconstruction does not completely restore knee joint health. Measurements of passive joint stability (e.g., knee laxity or "looseness") after ACL surgery demonstrate that knee stability is not fully restored despite surgery [1] and that the increase in knee laxity seen after ACL reconstruction typically occurs within the first 12 weeks of healing [2]. Measurements of the three-dimensional motion of the ACL-reconstructed knee during running have shown that the tibia is externally rotated (by approximately 4°) when compared to the uninjured knee and that the sagittal plane translation increases within the first 12 months of surgery [3]. It has been hypothesized that the increased risk of arthritis following knee injury and its treatment are due to these kinematic changes [4].

The hypothesis that ACL-reconstructed patients remain at risk for arthritis is supported by many studies. For example, ACL-injured patients present with arthritis in their injured knee 78% of the time, as compared to 4% in their uninjured knee, whether or not they underwent ACL reconstruction surgery [5]. Studies evaluating patient-oriented outcomes report significant improvements in joint function and patient satisfaction following ACL reconstruction, though these scores do not reach normal even years after surgery [6, 7]. Finally, the risk of graft failure remains a

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_21,

significant factor on the outcome of ACL reconstruction. In a recent prospective cohort study, it was determined that the risk of graft failure is 4.9% for autograft as compared to 10.1% for allograft ACL reconstruction [8]. More concerning is that the risk of graft failure for allograft reconstructions is four times higher than those with autograft reconstruction in teenage patients (Fig. 1.7) [8].

When reviewing the clinical outcome studies of currently used ACL reconstruction techniques, there is no question that there is room for improvement on all fronts. Therefore, new strategies are needed that could improve graft healing to better restore joint motion, function, patient-oriented outcomes, and cartilage health while decreasing the risk of graft failure. One such strategy would be to find a means to enhance the biology of graft healing postoperatively.

Biology of Healing ACL Grafts

Animal models of ACL reconstruction have shown that the histology (i.e., how the tissue looks under the microscope), gross morphometry (e.g., size and shape), and biomechanical properties (e.g., strength and stiffness) of healing ACL autografts and allografts never fully reach those of the normal ACL [9–11]. It is known that an autograft undergoes a period of acellular and avascular necrosis during the first weeks of healing [9], in which the graft essentially becomes a scaffold that is devoid of cells. Cells, and then blood vessels, migrate into the tissue by 3 months, and then, according to some studies, the graft transforms into what appears to be a normal ligament tissue by 9 months [12–14]. It should be noted that this healing response is considerably slower following allograft reconstruction, though the morphometric and histological differences between allografts and autografts become less distinct 1 year after implantation [10]. All of these biological changes that occur during the healing process correspond to distinct biomechanical changes in the graft, a surrogate measure frequently used to determine the extent of graft healing in animal studies, which are also known to influence joint laxity, another clinical outcome measure to assess joint health following ACL injury and its treatment [15, 16].

From the biomechanical perspective, the currently used hamstring and patellar tendon autografts possess similar structural properties (i.e., strength), and both are similar to that of the normal ACL at the time they are put into the knee [17, 18]. However, the tensile properties of the graft rapidly decrease during the first 6 weeks as the graft undergoes avascular necrosis and revascularization [9, 10, 19]. After 6 weeks of healing, the strength of the graft begins to improve, though may reach only a fraction of the values for the intact ACL (reports range from 10 to 40 %) even after a year of healing in animal models [19–22]. Although the histological evaluations suggest that the grafts appear to be normal microscopically at 1 year, the quality of the tissue appears to be inferior. Likewise, knee joint laxity is significantly greater than the opposite normal knee in most animal models, despite graft type [23]. Again, a method by which we could stimulate and/or accelerate the graft healing process may improve the tensile properties of the graft and joint stability.

Bio-enhanced ACL Graft Healing

Given that ACL reconstruction procedures do not restore normal knee function and that graft remodeling within the joint is a slow process, the following question arises: Is there something that can be done to improve or accelerate graft healing? A potential target to achieve this goal is to optimize the environment of the healing graft.

It is known that intra-articular graft healing involves the release of a myriad of growth factors and cytokines that are secreted and activated during different stages of the wound healing process, similar to what is seen for healing of any soft tissue. As discussed in detail in previous chapters, recent studies support the hypothesis that the intra-articular environment of the ACL interferes with the natural wound healing response of ligamentous tissues [24]. Recent evidence suggests that enzymes in synovial fluid prevent the formation of a blood clot in the intra-articular space of the synovial fluid [25], which in turn limits the healing capabilities of the normal ACL (see Chap. 7).

The constituents of a blood clot (i.e., platelets, macrophages) are responsible, at least in part, for initiating and regulating early healing [26–28]. Therefore, if a clot is not permitted to form in the wound site, the healing response of the tissue will be limited. One of the key constituents of blood is platelets, small cell fragments known to initiate and regulate early wound healing. For an injured extra-articular ligament (a ligament that lives outside of the joint such as the medial collateral ligament), natural healing of this structure is initiated with the formation of a fibrin-platelet plug at the wound site. This plug immobilizes the platelets at the site of injury, and the platelets then release high concentrations of growth factors that are crucial to wound healing (Fig. 21.1). Although the exact mechanism of intra-articular healing remains unknown, it is known that bleeding from the injury site within

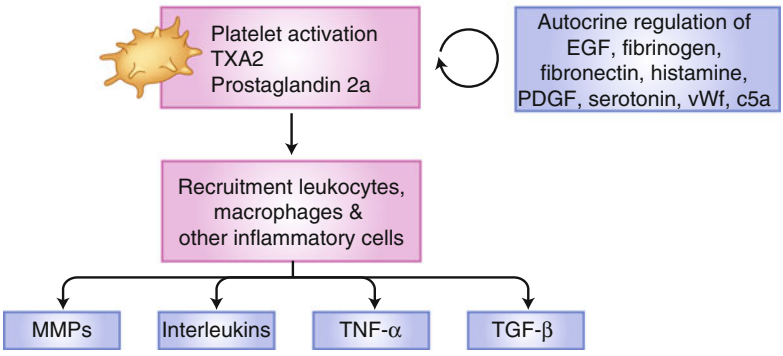

Fig. 21.1 Platelet activation sets off a chain of events that stimulates wound healing through the controlled release of growth factors, anabolic proteins, and cytokines. These growth factors should also be important to stimulate or enhance healing of an autograft or allograft to replace the ACL

the knee is not contained, but rather is dispersed throughout the joint fluid. Thus, the platelets are not localized within the wound site, and their power to influence the local healing environment is substantially diminished. Therefore, it seems reasonable to assume that when an ACL graft is placed in the intra-articular environment, healing would also be compromised because of the same inherent limitation for intra-articular tissue healing. Therefore, methods that involve the direct application of growth factors, cytokines, and/or chemokines involved in wound healing; the placement of a blood clot within a carrier that maintains the clot about the graft at the time of surgery; or possibly the placement of stem cells in and/or around the graft could provide possible solutions that could optimize the graft healing environment.

Growth Factor-Enhanced ACL Reconstruction

Growth factors, including those released by platelets, provide a means by which the intra-articular healing environment could potentially be enhanced and thus may provide a therapeutic alternative to improve outcomes following ACL reconstruction. For example, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and basic fibroblast growth factor (FGF) are important to healing of tendon and ligament soft tissues [29]. PDGF is released from platelets shortly after injury, and it stimulates the production of other growth factors. IGF-1 and TGF-β also stimulate the proliferation and migration of fibroblasts and increase the production of collagen, while VEGF and FGF stimulate angiogenesis and further regulate cell proliferation and migration. Although five promising growth factors for enhancement are listed here, there are many other growth factors and cytokines that participate in the proliferative, revascularization, and remodeling phases of healing, which may also need to be considered. It is possible that the proper introduction and modulation of any of these growth factors could enhance the healing response of the ACL graft. Research studies have been carried out to demonstrate the feasibility of a "growth factor-enhanced" ACL reconstruction technique.

Animal models have been used to evaluate the potential therapeutic benefits of exogenous application of growth factors for the enhancement of ACL graft healing. In 2004, it was determined that the combination of recombinant TGF-β and recombinant epidermal growth factor (EGF), delivered together in a fibrin sealant at the time of surgery, increased the tensile failure properties of the graft after 12 weeks of healing when compared to sham controls (ACL reconstruction with fibrin sealant only) [30]. Likewise, another research team evaluated the effects of applying PDGF, which was locally delivered to the graft for a period of time after implantation via its release from degradable sutures [31]. They also reported a significantly higher linear stiffness value at 24 weeks compared to controls, though there was no difference in the graft tissue histology between treatment groups. Neither study reported

any significant differences in knee laxity as compared to traditional ACL reconstruction [30, 31].

Because the expression of VEGF stimulates graft revascularization [32], exogenous application of VEGF to the healing graft at the time of surgery was evaluated in the sheep model [33]. As expected, it was determined that the VEGF-treated grafts had a greater number of newly formed blood vessels and a higher number of infiltrated fibroblasts in the VEGF-treated grafts compared to controls. Unfortunately, the histological improvements were accompanied by an increase in knee laxity and a decrease in the linear stiffness (a structural property) of the graft after 12 weeks of healing. The study did not characterize the long-term changes that might occur in the later stages of graft healing. It may be that the increased vascularity would have beneficial effects for the long term despite the lower strength of the graft at 12 weeks. The combination of VEGF with TGF-β was also evaluated in the rabbit model using gene therapy [34]. It was determined that the increase in co-expression of VEGF with TGF-β improved the tensile properties of the graft when compared to using either one alone after 24 weeks of healing [34]. This finding emphasizes the role that multiple growth factors contribute to the graft healing response. Given the number of growth factors involved in the healing process, the complex interaction between them, and the variable timing in their release and/or activation, it may be challenging to determine what optimal combination of growth factors would provide the most therapeutic benefit.

Although the preclinical studies evaluating the effects of one or two growth factors on ACL graft healing show potential, there are limitations that must be addressed before these techniques should be translated into clinical practice. Most of the animal studies performed to date have focused on the short-term benefits on the exogenous administration of growth factors. However, their long-term effects on joint health (both beneficial and detrimental) remain unknown. It is possible that the attempts to stimulate healing could damage other joint structures, such as the articular cartilage, particularly if the growth factors are concentrated. Another limitation of the growth factor approach is that the mechanisms of graft healing are not fully known so it is difficult to expect that modulation of one or two growth factors would optimize healing since many are involved in the wound healing response. Even if we knew which is the best growth factor to enhance healing, the optimal dosing, timing, and the interaction between growth factors would need to be established. Finally, the cost of recombinant growth factors such as TGF-β, PDGF, or EGF is extremely expensive. Therefore, a method that could naturally enhance the wound healing may provide a better solution.

Platelet-Enhanced ACL Reconstruction

The use of platelets to stimulate healing of the ACL graft is a plausible means to enhance healing and improve outcomes. Autologous platelets (platelets from a patient's own blood) can be readily obtained, concentrated, and then applied to the

graft at the time of surgery. Several commercially available systems are on the market to process and concentrate platelets from blood aspirates. As mentioned above, platelets release a myriad of growth factors that are essential to wound healing, and their release should be similar to that induced by natural healing given the role that platelets play in healing [35]. Furthermore, many of these growth factors released by platelets have been shown to stimulate the healing response of ligament and tendon tissues [29]. In vitro, ACL cell migration has been stimulated by TGF-β, while PDGF and FGF stimulate cell proliferation in a 3-D extracellular matrix (ECM) scaffold [26]. ECM-platelet composites have been found to release PDGF in relevant quantities, suggesting platelet activation and cytokine release [36]. In vivo, high levels of FGF, PDGF, and TGF-β are found in the area of an intra-articularly implanted ECM-platelet composite for up to 3 weeks after implantation, suggesting sustained presence of these platelet-related growth factors in the wound site after platelet activation [24]. When combined with collagen, platelets have been shown to help heal partial and complete transections of the ACL [24, 37–39]. Thus, it seems reasonable to assume that a platelet-based preparation could also stimulate ACL graft healing.

Two animal studies have recently been performed to determine the efficacy of using ECM-platelet composite to enhance ACL graft healing. In the adult goat model, a ECM-platelet composite was made to augment the healing response of a patellar tendon autograft by adding whole blood to a collagen-based extracellular matrix scaffold [40]. After 6 weeks of healing, a 30% reduction in knee laxity was observed when the autograft was treated with the extracellular matrix-platelet composite as compared to treatment with the collagen-based scaffold only (no platelets). Although no significant differences were found with regard to the tensile properties, there was a significant correlation between the systemic platelet count at the time of surgery with the graft tensile properties after 6 weeks of healing suggesting that increasing the platelet count (i.e., increasing the platelet concentration) could improve the graft tensile properties. Building on the goat study, a subsequent porcine study was designed to evaluate the effects of using an extra-cellular matrix-platelet composite with a platelet concentrate, five times the systemic level placed around an ACL graft. Immature pigs underwent unilateral ACL reconstruction with bone-patellar tendon-bone allograft. Half were treated with a standard patellar tendon allograft while the other half were treated with the same allograft placed within a ECM-platelet composite sleeve (Fig. 21.2) [41]. Significant improvements in the graft tensile properties were found when the extracellular matrix-platelet composite was used. For the tensile properties, the normalized yield and failure loads were 60% higher than those of the standard ACL-reconstructed group (Fig. 21.3). Likewise, the laxity values of the reconstructed knees were significantly reduced by 28% (at 60° of knee flexion) with the addition of the extracellular matrix-platelet composite (Fig. 21.4). Histological examination found that cellular and vessel infiltration were observed in both groups, but regions of necrosis (dead tissue without cells or blood vessels) were present only in the group undergoing reconstruction without the extracellular matrix-platelet composite [41]. These data provide encouragement regarding the efficacy of the platelet-enhanced ACL reconstruction approach. Long-term studies are currently underway to evaluate cartilage health following the ECM-platelet composite augmentation of ACL grafts.

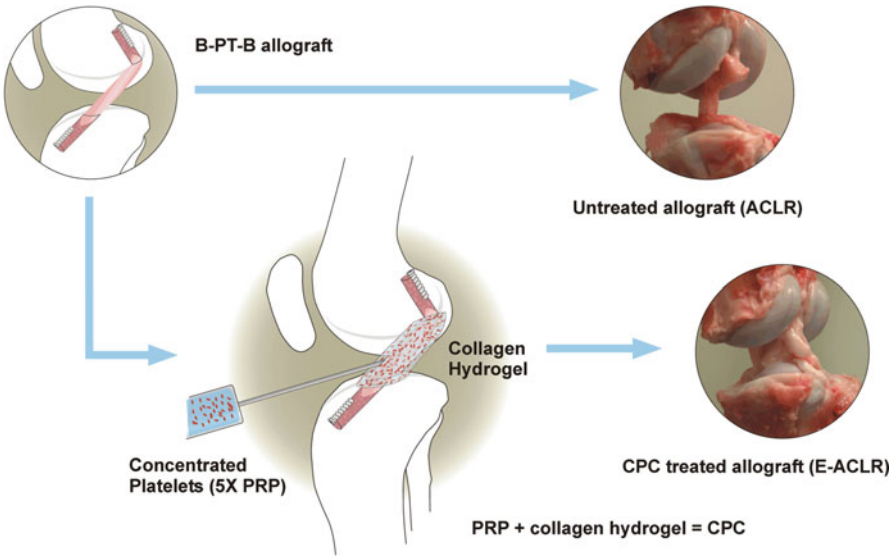

Fig. 21.2 Schematic depicting the placement of a ECM-platelet hydrogel (CPC) around an ACL graft to enhance healing (From Fleming et al. [41], copyright © 2009 by (Sage Publications), Reprinted by Permission of SAGE Publications)

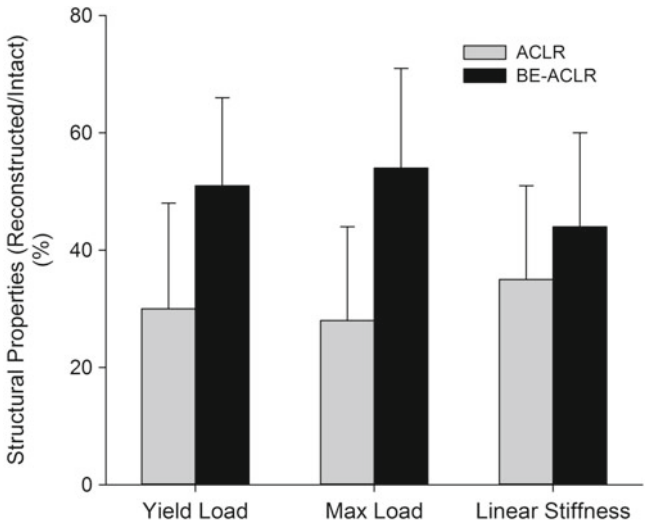

Fig. 21.3 The structural properties presented as percentages relative to the contralateral ACL intact control knee were improved following bio-enhanced ACL reconstruction (*BE-ACLR*) as compared to that of traditional ACL reconstruction (*ACLR*) (From Fleming et al. [41] , copyright © 2009 by (Sage Publications), Reprinted by Permission of SAGE Publications

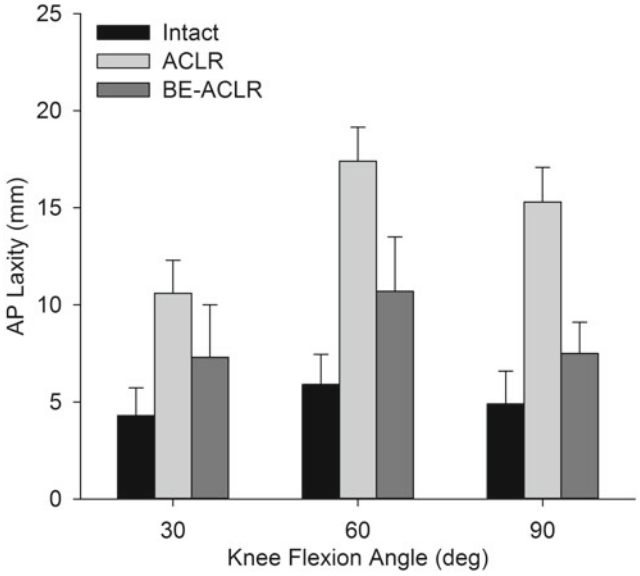

Fig. 21.4 The anteroposterior laxity of the porcine knee following bio-enhanced ACL reconstruction (*BE-ACLR*) was less than that of traditional ACL reconstruction (*ACLR*) and closer to that of the ACL intact knee (Intact) (From Fleming et al. [41], copyright © 2009 by (Sage Publications), Reprinted by Permission of SAGE Publications)

Several clinical trials have been performed to determine if the use of platelets, platelet concentrates, or platelet-derived growth factors would enhance ACL reconstruction graft healing in human patients. A recent systematic review identified eight such trials, seven of which focused on graft maturation and five on the healing between the tendon graft and the bone tunnel (Table 21.1) [42]. Four of these seven studies reported significantly better maturation "outcomes" in the grafts treated with concentrated platelets compared to those that were not [43–46]. Using MRI, Orrega et al. determined that after six months of healing, the signal intensity of the graft that was treated with a 9× platelet concentration matched that of the uninjured PCL in 100% of the patients as compared to only 78% of those that did not received the concentrated platelet application [43]. It should be noted that the graft signal intensity on MRI may be predictive of the structural properties of the graft and can potentially be used to document graft healing (see Chap. 13) [47]. However, Orrego et al. did not find any differences in clinical outcome in the two groups of patients [43]. Radice et al. also utilized MRI to evaluate the time course of graft homogenization and found that the application of the platelet concentrate (9×) reduced the time on average to achieve a normal MRI intensity value from 369 to 177 days [45]. Ventura et al. also observed a significant difference in graft homogeneity on CT scans between concentrated platelet-treated grafts (9×); however, as in the Orrego study, there were no differences in clinical- or patient-oriented outcome scores after 6 months of healing [46].

Table 21.1 Clinical studies evaluating platelet-based therapies on ACL reconstruction healing

Authors	Imaging – graft	Imaging – tunnel	Histology – graft
Vogrin et al. [48]	…without a statistically significance between both groups	…enhances early revascularization in the interface…	
Figueroa et al. [49]	…with MRI at 6 months after reconstruction, we did not find any statistically significant benefit in the APC group in terms of integration assessment and graft maturation		
Nin et al. [50]	…use of PDGF i[…] has no discernable clinical or biomechanical effect at 2 years' follow-up		
Silva et al. [51]		…use of PRP […] does not seem to accelerate tendon integration	
Orrego et al. [43]	…enhancing effect on the graft maturation process…	…without showing a significant effect in the osteoligamentous interface or tunnel widening…	
Sanchez et al. [44]			…resulting in more remodeling compared with untreated grafts…
Radice et al. [45]	…a time shortening of 48%…		
Ventura et al. [46]	…transformation from autologous QHTG to new ACL was faster in the GF-treated group than in controls		

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Using second look arthroscopy and histological biopsy analyses, Sanchez et al. reported that grafts treated with concentrated platelets (3×) showed higher arthroscopic ratings for synovial coverage, graft width, and graft tension in the platelet-treated group when compared to the controls, though this was only a trend [44]. Histology revealed that the ligament maturity index [24] was significantly greater in the platelet-treated grafts [44]. Although other studies found no significant improvement in graft performance with the supplementation of platelets [48–50], this may be partly due to the study being underpowered to detect those differences given the extent of the variability associated with this treatment. Likewise, all of the studies that evaluated osteoligamentous healing in the bone tunnel, except for one [48], found no improvements in healing at the graft-bone junction [43, 46, 49, 51]. Vogrin et al. reported that the platelets enhanced early revascularization at the interface, though how this might affect patient outcome (positively or negatively) is not known.

In summary, the review of the literature suggests that the use of platelet concentrates may improve the rate at which grafts achieve a better appearance on MRI or an improved ligament maturity index on histology [43–45], though no studies have yet shown an improvement of clinical- or patient-oriented outcome at 2 years [42, 43, 46, 50] or significantly improved bone-tendon healing [43, 46, 49, 51].

The results of these preliminary studies provide credence to the concept for bio-enhanced healing of an ACL graft with the application of platelets at the time of surgery. However, there are many limitations that must be considered when reviewing these clinical studies. Of the eight clinical studies that have been published, only two meet the standards of Level 1 evidence. Many of these studies were underpowered and none of them report long-term outcomes (>2 years). Thus, the effects of these concentrated platelet preparations on cartilage health and overall knee joint function remain unknown. Also, different platelet preparations and concentrations were used across studies. It is important to note that the platelet-rich plasma resulting from the different commercial systems available produce very different preparations – different platelet concentrations, some include white blood cells, some activate the platelets with thrombin or mechanical stimulation [52, 53]. It is highly likely that the other blood constituents in some of the preparations of PRP (i.e., leukocytes, erythrocytes) are also important adjuncts to the overall healing response of the graft and need to be considered [35]. Given that the synovial fluid environment does not permit clotting to occur, how the platelets are delivered and maintained around the graft may also prove to be important. It has been shown that the healing response of the ACL itself is unaffected by the intra-articular application of platelets alone [54] and that a ECM-based carrier is required to both stabilize and activate the platelets to stimulate ACL healing [39]. Future work will be required to find the best way to deliver the blood/platelet preparation, to tune the preparation to optimize the healing response of the tendon graft, and to establish the long-term consequences of bio-enhanced ACL reconstruction on the overall knee joint health (i.e., cartilage integrity).

Cell-Enhanced ACL Reconstruction

Cell therapies are another potential option for the treatment of ACL injuries [55–57] and the enhancement of ACL reconstruction [34, 58–60]. This field is in its infancy. However, studies have been performed which document the presence of progenitor cells in ACL tissue [56, 57]. It is possible that given the right stimulus, these stem cells or ACL fibroblasts could be invoked to produce extracellular matrix products and new ligament tissue. However, the effects of different growth factor applications on adipose derived stem cells to stimulate markers of extracellular matrix production in vitro was met with only limited success in one study [55]. On the contrary, another study demonstrated that extracellular matrix production from ACL-derived stem cells could be initiated in vitro with TGF-β [61]. At this time, the optimal progenitor cell population and the stimuli required to modulate graft healing in vivo are not fully known.

Recent attempts have been made to develop cell-based adjunctive therapies for ACL reconstruction. In a rabbit model, ACL allografts were treated either with mesenchymal stem cells or PDGF-transfected mesenchymal stem cells (stem cells that have been programmed to produce PDGF) and compared to a control (ACL allograft without cell treatment) [60]. The results show that treatment with mesenchymal stem cells (with or without PDGF) accelerated cellular infiltration and enhanced collagen deposition. However, the effect of these factors on the strength of the graft is unknown as no biomechanical testing was performed. In a second study in sheep, the local application of autologous synovium-derived cells cultured with TGF-β and then embedded in a fibrin glue at the time of ACL reconstruction was found to prevent the deterioration of the tensile failure properties and eliminate regions of necrosis of the graft 12 weeks after surgery [59]. However, similar benefits were found when the grafts were treated with the fibrin glue containing TGF-β and no cells. Stem cells that were transfected to express growth factors (TGF-β and VEGF) have also been tested in large animal models as described in the growth factor discussion above with encouraging results [34].

In reviewing the literature on cell-based therapies of ACL reconstruction, it is clear that the cell enhancement is a promising approach. However, much work is still needed to define the conditions required to make this a viable option in patients.

The Future of Bio-enhanced ACL Reconstruction

Bio-enhanced ACL reconstruction may one day provide a means to improve the outcome of ACL reconstruction procedures. By understanding the principles of wound healing and then applying these principles to graft healing, it may be possible to select the right combination of growth factors, scaffolds, and/or cells that could improve healing in the synovial fluid environment. Bio-enhancement may be particularly important for improving outcomes after allograft ACL reconstruction since allografts have been shown to heal more slowly than autografts in animal models [9, 10], which in turn may be the reason why allografts fail at a much higher rate in the young and active patient [8].

The most promising approach for bio-enhanced ACL reconstruction, and that which has been studied the most to date, is the use of autologous derived platelets or whole blood to create a gel or composite that can withstand the degradative enzymes in synovial fluid and deliver the required growth factors with the naturally programmed dosing and timing. Some advantages of this method are that the bioactive agents are obtained from the individual patient at the time of surgery with minimal manipulation. This may reduce some of the regulatory hurdles that will be encountered with other cell-based therapies, synthetic scaffolds, and recombinant growth factors. Given that there have already been several attempts to evaluate the efficacy of bio-enhanced ACL reconstruction using platelet-based technologies in human patients, and that the results of these studies are encouraging, this method has the potential to become clinically available in the not so distant future. Nonetheless, further work is needed to better understand the intra-articular healing response of the graft to select the appropriate therapeutic agents, scaffolds, and delivery methods and to optimize dosing strategies.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Numbers RO1-AR054099 and RO1-AR056834. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Chapter 22
The Effects of Age and Skeletal Maturity on Healing of the Anterior Cruciate Ligament

Linda H. Chao and Martha M. Murray

Recent work in the development of a bio-enhanced primary repair technique for anterior cruciate ligament (ACL) injury has shown promising results in the stimulation of ACL healing at early time points using surgical placement of a substitute provisional scaffold, an extracellular matrix-platelet composite, within the wound site [1]. The application of this technique, dubbed "bio-enhanced suture repair," has been of considerable interest particularly for the treatment of pediatric patients with open growth plates, or "physes." For that population in particular, it has several potential advantages over traditional ACL reconstruction, the current gold standard of treatment for ACL rupture. Bio-enhanced primary repair of the ACL would avoid the use of large tunnels being drilled through the growth plate (as is done for standard ACL reconstruction) – these large tunnels can potentially lead to physeal injuries and growth disturbances [2]. Furthermore, bio-enhanced repair would potentially preserve the geometry of the injured ligament, the complex insertion sites, and the nerve or proprioceptive function of the ACL. However, the characteristics of patients who might be good candidates for bio-enhanced suture repair of the ACL, including factors involving age and skeletal maturity, have only recently been elucidated (Fig. 22.1).

Conventional orthopedic wisdom holds that, for fractures, "kids heal faster than adults" and basic science studies in animals support this clinical observation [4, 5].

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M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7_22,

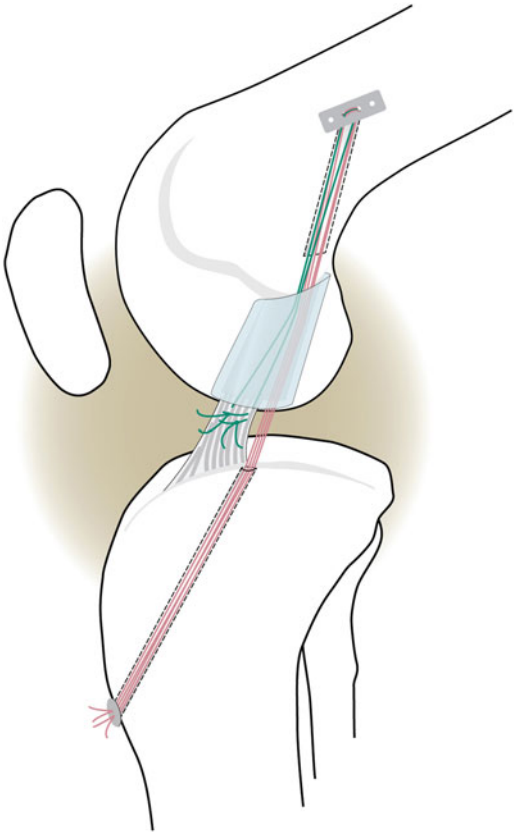

Fig. 22.1 Schematic diagram of bio-enhanced primary suture repair with an extracellular matrix-platelet composite scaffold (Used with permission from Mastrangelo et al. [3])

Recent research at the Sports Medicine Research Laboratory at Children's Hospital Boston has focused on the question, "would this be true for ligament injuries as well?" Evidence from in vivo and in vitro studies suggest that the healing capacity of the ACL depends on age and skeletal maturity.

A study was conducted to evaluate the effect of age on ligament healing. That study included skeletally immature (growth plates or physes open), adolescent (physes closing), and adult (physes closed) animals, all of which underwent ACL transection on both sides. One side was repaired using the bio-enhanced ACL repair technique, and the other side was allowed to heal naturally (no repair was performed) [6].

After 15 weeks of healing, there were significant differences in the biomechanical outcomes (maximum load, yield load, and stiffness) among the age groups in both the unrepaired and repaired ligaments. When no treatment was performed, skeletally immature animals had a far more productive intrinsic healing response than the adolescent and adult animals (Fig. 22.2). When a bio-enhanced repair was performed, the skeletally immature animals improved to a small degree over the untreated case, but the adolescent animals had a remarkable improvement, showing

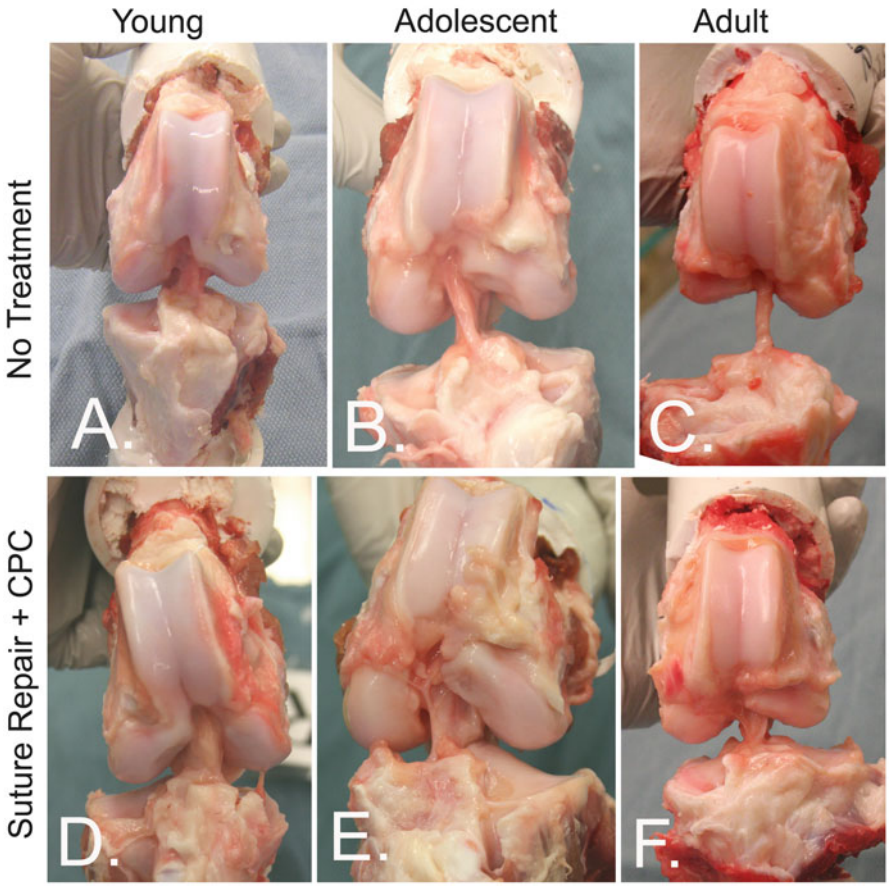

Fig. 22.2 Gross appearance of the healing anterior cruciate ligament in the young, adolescent, and adult age groups in both the untreated knees (**a–c**) and the knees treated with bio-enhanced suture repair (**d–f**). In knees treated with the bio-enhanced suture repair, organized collagenous tissue was visible in the region of the anterior cruciate ligament and appeared more robust and less lax than that in the untreated contralateral knees (Used with permission from Murray et al. [6])

over 100 % stronger repairs with the bio-enhanced repair technique. In contrast, the adult animals still had a dysfunctional healing response, even with use of the bio-enhanced repair. Histologic analysis of the tissue at 15 weeks following surgery showed a significantly greater density of fibroblasts in the adult and adolescent animals compared with that in the juvenile animals ($p<0.01$ and $p<0.001$; see Fig. 22.3), potentially indicating a delayed healing response in the adult animals, as tissue remodeling (rather than cell proliferation) should be well underway by this late time point (15 weeks).

Not only did the ligaments of younger animals heal better in their mid-substance but differences were also seen in the attachment of the ligament to the bone

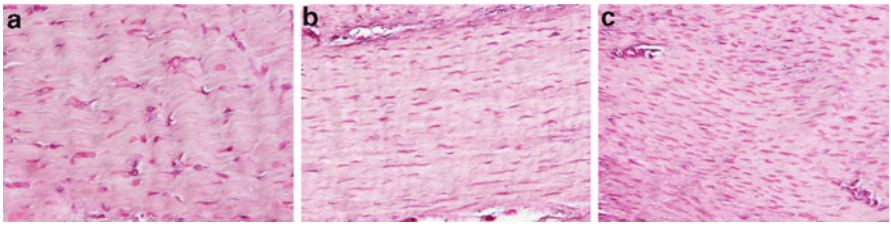

Fig. 22.3 Histologic sections of representative areas of the repair tissue in the juvenile (**a**), adolescent (**b**), and adult (**c**) groups after 15 weeks of healing. Adolescent and adult specimens exhibited notably increased cell density and smaller cell nuclei as compared to the younger animals (hematoxylin and eosin, ×400) (Used with permission from Murray et al. [6])

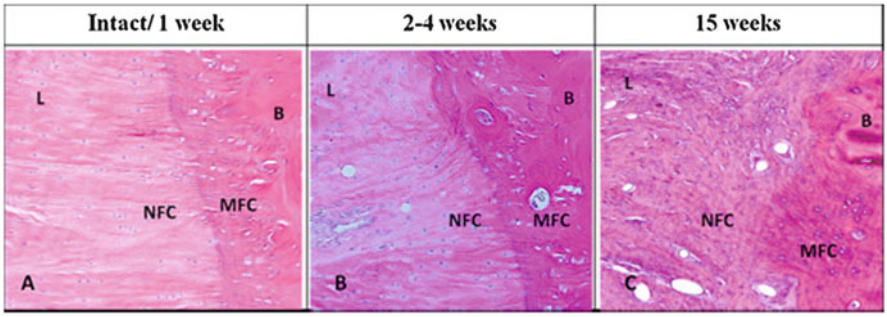

Fig. 22.4 Representative photomicrographs of the ACL insertion site in the adult animal after 1, 4, and 15 weeks of healing using bio-enhanced ACL repair technique. Distinct layers of the direct insertion are represented: ligament (*L*), non-mineralized fibrocartilage (*NFC*), mineralized fibrocartilage (*MFC*), and bone (*B*). Only minor changes are seen in the insertion site from 1 to 4 weeks. By 15 weeks, the fibrous tissue becomes relatively acellular and disorganized with partial loss of the parallel arrangement of collagen fibers, and the chondrocytes in the fibrocartilage zones appear apoptotic and flattened, with the loss of distinct lacunae and increasingly disorganized arrangement of the cells (Used with permission by Haus et al. [7])

(the "insertion site") as a function of age [7]. Animals of different ages underwent an ACL transection, and the appearance of the insertion sites of the injured ACLs were evaluated at 1, 2, 4, and 15 after injury. In that study, we found that the healed ligaments of skeletally immature and adolescent animals had a relatively reliable series of changes that were seen at the insertion site. These included early loss of the collagen alignment and normal fibrocartilage zones of the ACL insertion site between 2 and 4 weeks (Fig. 22.4), followed by restoration of both the collagen alignment and the fibrocartilaginous zone by 15 weeks after injury and repair. In contrast, the adolescent and adult animals that did not go on to heal the ACL had early degradation changes, but no recovery of the insertion site at the 15-week time point. These results suggested that functional healing of the ACL was required for recovery of the ACL insertion site and that younger animals were more likely to have productive changes seen in both the mid-substance and insertion site of an injured ACL [7] (Fig. 22.5).

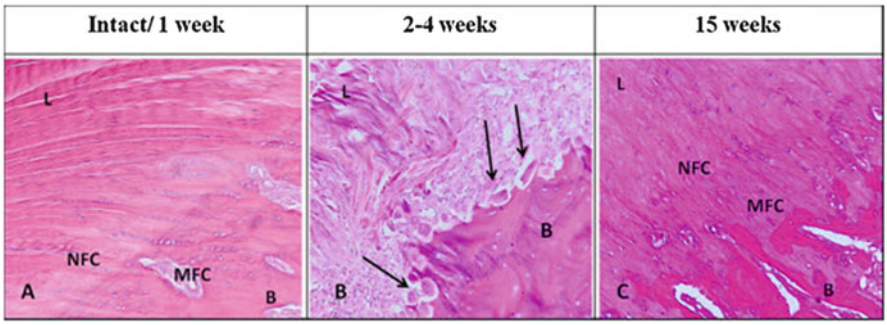

Fig. 22.5 Representative photomicrographs of ACL insertion sites from skeletally immature animals (with open growth plates) at 1, 4, and 15 weeks after bio-enhanced ACL repair surgery. Distinct layers of the direct insertion are represented: ligament (*L*), non-mineralized fibrocartilage (*NFC*), mineralized fibrocartilage (*MFC*), and bone (*B*). (**a**) At 1 week, a four-zone insertion site is similar to the intact control group, with minimal changes seen. (**b**) At 2–4 weeks, changes in both the fibrous tissue and in the fibrocartilage zone can be seen. In the fibrous tissue, there is an increase in fibroblast density, capillary invasion, and loss of collagen alignment. In the fibrocartilage zone, osteoclasts appear (*black arrows*), and there is a loss of the distinct non-mineralized and mineralized fibrocartilage layers as well as a loss of the tidemark. (**c**) By 15 weeks, the ACL insertion site appears more organized. In the fibrous tissue, although the cell density remains elevated, the collagen organization is improved over the 4-week time point. In the mineralized and non-mineralized fibrocartilage zones, there is also improved organization of chondrocytes than that seen earlier and the osteoclasts have disappeared (Used with permission by Haus et al. [7])

So why do younger animals heal better than adolescent or adult animals? If we think about the basic biology of wound healing, we can come up with a few hypotheses to test. For example, the increased strength in the immature animals might be due to earlier cell migration into the wound site or earlier blood supply coming in to help the cells be more productive. If more cells are present earlier in the wound site, that could be due to either increased cell migration into the wound site or that the cells multiply faster once they are in the wound site. These types of hypotheses were posed and tested and the results are summarized in the next section.

To begin to understand the cellular level mechanisms for the differences in ACL healing, we elected to first look at the cellular and tissue changes in a cut ACL for both skeletally mature and skeletally immature animals, focusing on how many cells and blood vessels had come into the wound site at time points up to 4 weeks after ACL injury [3]. In this study, skeletally immature, adolescent, and adult animals underwent bilateral ACL transection and bilateral bio-enhanced ACL repair. The knees were allowed to heal and the healing ligaments were retrieved at 1, 2, and 4 weeks after surgery for histologic analysis. We found that the wound site of immature animals had far more cells early on, and that the adult wound sites lagged behind by 2 weeks in terms of regaining cell numbers [3]. These results agree with the finding of the earlier study that showed significantly higher fibroblast density in adult animals than skeletally immature animals at 15 weeks, suggesting that adult animals do indeed exhibit delayed wound repopulation by fibroblasts in the first few weeks of healing as compared to immature animals. So perhaps one of the reasons

adults do not heal as quickly as immature animals is that the wound site gets populated more slowly in the adults.

Why were the adult cells slower to populate the wound site? To determine the mechanisms that might be different between immature and adult animals, two in vitro studies were performed to evaluate cell migration and cell proliferation, respectively. Using Boyden chamber assays, the migration potential of human and animal ACL fibroblasts from skeletally immature individuals was compared to that of older individuals [8, 9]. Results from these studies demonstrated that the cells from the younger animals both migrated and proliferated faster than the cells from the adolescents or adults. These results are consistent with the results of earlier in vitro studies of fibroblast proliferation, which showed that fibroblasts from older donors exhibit decreased replication rates and lower cell yields at cellular confluency [10].

These results suggest that as individuals grow older, their cells exhibit lower rates of cell migration and proliferation – factors that could decrease the capacity of a wound to heal following injury. Skeletally immature individuals may have a more functional healing response than older individuals owing to earlier and easier repopulation of the wound site with active ACL fibroblasts, due to both increased migration and proliferation potential of cells in younger individuals.

There is a growing body of evidence that there are differences even at the protein level within cells that can account for the superior healing of skeletally immature patients as compared to older individuals. Age-dependent differences in ACL cell metabolism, collagen gene expression, and the ability of the cells to respond to growth factors in PRP have been examined in vitro [11]. ACL cells obtained from skeletally immature, adolescent, and adult animals were cultured in a collagen type I hydrogel with or without platelet-rich plasma (PRP) for 14 days and evaluated for cell viability and for collagen gene expression, to test the hypotheses that (1) ACL cell survival and collagen gene expression in a 3D scaffold are dependent on age and (2) the effects of PRP on these measures in cultured cells are also age dependent. The results of the study suggested that cells from adult animals cultured without PRP had a significantly lower apoptotic rate (19 %, $p=0.001$) and 25 % higher cellular metabolic activity ($p=0.006$) when compared to adolescent animals [11]. Cells from the adolescent group had lower overall cellular metabolic activity but higher expression of types I and III collagen mRNA than the other two groups, and cells from skeletally immature animals exhibited a significantly higher rate of apoptosis than cells from older animals [11]. However, addition of PRP to the cultures resulted in significantly increased cellular metabolic activity, reduced apoptotic rate, and stimulation of collagen production in the cells from the immature and adolescent animals, but there was less of an effect for the adult animals [11].

To begin to understand why biologic stimuli such as PRP might be more effective in younger animals, we then looked at what growth factor receptors were present on the cells in the different age groups. Interestingly, we found that the number of growth factor receptors found on ACL cells was dependent on the age of the animal the ACL cells were obtained from, with younger animals having cells with more growth factor receptors [12].

This finding is especially important in the context of bio-enhanced ACL repair using a extracellular matrix-platelet composite in that the multitude of growth factors released by the platelets, such as platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF), are important in the promotion of wound healing [13]. Previous studies showed that these growth factors are constantly released by platelets as a function of platelet number and that the levels released per platelet are independent of age [14–16].

Thus, while growth factor concentrations may remain constant over time and not vary significantly with age, the number of growth factor receptors may decrease with age and may be responsible for the differences in response to PRP as a function of age. In a recent study, correlations between mRNA expression levels and age were found for most of the receptors studied, which included FGF receptor ($p<0.01$), PDGF receptor ($p=0.09$), TGF-β receptor 1 ($p=0.01$), TGF-β receptor 3 ($p=0.08$), and VEGF receptor 2 ($p=0.05$) [12]. Furthermore, in a multivariate model of VEGF receptor 2 expression and biomechanics (strength of the repaired ligament), younger age resulted in improved healing ligament strength, suggesting the age dependence of successful healing response with bio-enhanced repair [12].

Summary

The pediatric patient faces special challenges when sustaining an ACL tear. On the one hand, there is the challenge of avoiding potential damage to the physes and subsequent growth disturbances. On the other hand, younger patients and animal models demonstrate enhanced intrinsic healing capabilities as compared to adults. Recently, important advances have been made in the development of a bio-enhanced technique for primary ACL repair that could be ideal for pediatric patients with open physes susceptible to damage and growth disturbances by traditional ACL reconstruction. The mechanism of action of the bio-enhanced scaffold and age-dependent effects on ligament healing remains active areas of study.

Acknowledgement Research reported in this chapter was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number RO1-AR054099. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Conclusion

The Future of ACL Injury

"Bones" had a job I totally envy. He was the doctor on Star Trek, and whenever someone was hurt, he came in with his tricorder, waved it over the patient and it told him the diagnosis. Even better, he could push a second button on the device, wave it over the patient again, and the problem would be fixed. No wonder a $10 million prize has recently been offered to anyone who can develop a working tricorder.

Unfortunately, we do not have that technology yet. While we have ways to accurately diagnose ACL tears via physical examinations and magnetic resonance imaging, we still need to perform extensive surgery to replace the torn ligament. When I think about the future of ACL surgery, I think about the "tricorder" solution – can we develop a noninvasive way to treat ACL injuries?

Steps along that path include making surgery less invasive. Our profession has already made great strides in this direction. The advent of arthroscopic surgery – where much of the operation can now be performed through small incisions using a camera to see inside the joint – has been a major advance. The use of smaller incisions to harvest both hamstring and patellar tendon grafts as well as the invention of fixation devices that can be used to secure the graft without direct visualization have also made our surgery easier on patients.

Enhancing primary (suture) repair of the ACL using a biologic scaffold is the next logical step. This technique avoids the need for graft harvest, thus maintaining the normal anatomy of the extensor mechanisms of the knee as well as the hamstring musculature (without the risks of infection or elevated failure rates associated with allografts). The specifics of this technique will undoubtedly improve as we see how patients respond to this new therapy, and we learn more about the biology of the joint and its response to this treatment. Improved understanding of the individual types of blood cells, their role in the wound healing process, identification of the complex array of interacting growth factors in the injured joint, and a better understanding of all the pathways that stimulate matrix production and organization will

M.M. Murray et al. (eds.), *The ACL Handbook: Knee Biology, Mechanics, and Treatment*, DOI 10.1007/978-1-4614-0760-7,

all help to improve the repair process for the ACL and other commonly injured intra-articular structures including the posterior cruciate ligament, menisci, and the shoulder rotator cuff tendon.

It is my hope that one day, we will be able to stimulate repair of the ACL with techniques that are even less invasive and more effective at stimulating the regeneration of this ligament. Perhaps someday, an ACL injury will not feel like a death sentence for an athlete. When Emily tears her ACL in the future, she will be carried off the field and may only need a simple injection into her knee, possibly guided by ultrasound or another imaging modality, to assure proper placement of a scaffold and the introduction of the right biologic stimuli. A tricorder would be even better. Perhaps the final frontier will be in the prevention of these injuries. I expect we will see advances in all of these areas in the near future, and it will be an exciting time as we boldly go where no one has gone before.

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