Fascia Insights: Deconstructing Lung Fibroblast Research

An Interview with Stephen P. Evanko, PhD

By Lina Amy Hack, Certified Advanced Rolfer® and Stephen P. Evanko, PhD, Certified Advanced Rolfer

Lina Amy Hack

Stephen P. Evanko

ABSTRACT *In this interview, Lina Amy Hack seeks to examine the constructs and results of the recent 2020 research article by Stephen P. Evanko, PhD, "A Role for HAPLN1 During Phenotypic Modulation of Human Lung Fibroblasts In Vitro" published in the* Journal of Histochemistry and Cytochemistry*. The application to Rolfing® interventions is discussed.*

Lina Amy Hack: Thanks for your time for this interview. You are both a researcher and a Rolfer. For years you maintained both a molecular biology laboratory as well as seeing Rolfing® Structural Integration (SI) clients (Evanko and Hoff 2014). In 2020 you published your recent laboratory findings in the *Journal of Histochemistry and Cytochemistry*, for this Fascia Insights we are going to deconstruct that article and discuss the broader application of biochemical research and Rolfing SI (Evanko et al. 2020). How does that sound?

Stephen P. Evanko: That sounds great!

LAH: Let's start with the basics, the title of your research article is very technical: "A Role for HAPLN I During Phenotypic Modulation of Human Lung Fibroblasts In Vitro." I have a biochemistry degree and this doesn't immediately paint a picture in my mind. So, let's deconstruct it.

To start, and correct me where I go astray, I zoom into *what* you are studying: human lung fibroblasts and the proteins they make. What is a human lung fibroblast?

SPE: These are cells that have been obtained from generously donated cadaveric lung tissue or from biopsy specimens. Commercial companies and research colleagues prepared the cells I was using. In the lungs, the fibroblasts are located underneath the gas exchanging epithelium. As their name implies they form the fibrous architectural framework, or stroma of the lung, as well as the ground substance in which they and the fibers are embedded. They are generally similar to cells in fascia, tendons, ligaments, and dermis. There are techniques used to separate them from other cell types in order to grow a pure cell population.

LAH: My understanding is that a fibroblast has a range of functioning, could we describe it as a continuum between 'healthy' fibroblasts who produce proteins into the extracellular matrix that are hydrophilic (water-loving), producing soft and supple collagen, on one side; and 'stressed' fibroblasts who produce proteins that stiffen the extracellular matrix? That either a fibroblast is producing a *gel* cellular texture or a *solid* cellular texture?

SPE: Yes, viscoelastic tissues display either more viscous, gel-like character or solid character depending upon the strain rate or the speed at which force is applied, as well as the overall composition. *Compliant* or *stiff* are also useful terms to describe the quality of the fascia. This is controlled by the cells and their responses to mechanical input, and things like the chemical environment, nutritional factors, hydration state, and pH. Ultimately, it's the combination and relative amounts of fibers, ground substance, counter ions, and water in the extracellular matrix, and the degree of cellular contractility that gives the tissue its textural quality.

This transition to a stiffer fibroblast is called the fibroblast-myofibroblast transition. It is important and necessary during normal wound healing and following other inflammatory events, where these cells facilitate wound contraction, i.e., pulling the wound edges together. It also occurs pathologically during diseases like idiopathic pulmonary fibrosis, scleroderma, and liver fibrosis. The primary driver of myofibroblast activation and the resulting fibrosis is a growth factor called transforming growth factor beta or TGF-beta.

LAH: Right, so TGF-beta is a cellular molecule that drives fibroblasts to becoming myofibroblasts. A Rolfer has their hands on this range of cellular states from compliant tissue to stiff tissue. Is it important to know why the fibroblasts change to myofibroblasts?

SPE: It's important because we as Rolfers have the potential to alter the tissue compliance to a more youthful state through our manipulations.

The myofibroblast transition can also occur as a result of mechanical loading of the tissues. Repetitive tension is one thing that will activate myofibroblasts. It is thought that this also involves TGF-beta. Tensional forces on the tissue translate to the collagen fiber matrix and to other proteins that connect the extracellular matrix to the nucleus, including other structures inside the cell. There is another protein complex, latent TGF-binding protein together with LAP (latency-associated peptide), which sequesters TGF-beta in an inactive form. In experimental studies, tension applied to the cell substrate pulls the complex open, allowing the TGF-beta to become free to dock on the cell surface receptors. This latent complex is bound to fibrillin fibers in the extracellular matrix (fibrillin fibers are different from collagen).

LAH: Okay, yes. So interesting. But then a mechanical force that stretches, shears, or torsions the matrix will spill all that TFG-beta into the extracellular space. [Cells can be but are not necessarily wounded for this to happen.] This becomes a signal to that local environment for the cells to stiffen up, and prepare for wound healing. But sometimes that cascade of molecular events happens without a direct injury, the tissue stiffens up for other reasons.

SPE: Yes, and as I mentioned, the result is fibrosis, or a build-up of collagen fibers, primarily, that leads to increased stiffness of the connective tissue. It can also be induced by repetitive motion injury, where muscular action puts tensile strains on the connective-tissue matrix. This is what we are working with all the time in our practices with our manual myofascial and neuro-fascial techniques. (Generally, collagen fibers provide tensile stiffness while the ground substance and the water it holds provide viscoelasticity, swelling pressure, and compressive stiffness.)

In the lungs, fibrosis is extremely dangerous, because it prevents normal expansion of the alveoli and movement of all of the structures in relation to each other, and may interfere with gas exchange.

LAH: When a fibroblast is switching its function between these two states, is that what is meant by "phenotypic modulation" in the title of your article?

SPE: Yes, phenotypic modulation is the switch in appearance and behavior of cells usually accompanied by changes in protein production and/or metabolism. Most fibroblasts are capable of contracting the collagen in which they reside to some extent. Depending on the tissue from which they're derived, they will be more or less contractile and display other slightly different characteristics from each other. However, during myofibroblast induction the cells become especially contractile. They develop more actin stress fibers and other muscle proteins. They secrete more extracellular matrix including hyaluronic acid and its binding proteins in the ground substance as well as collagen fibers. The cells are more actively contractile due to an increase in a specific protein called alpha smooth muscle actin which helps drive the slow progressive contraction of these cells. A sheet of fascia containing myofibroblasts essentially becomes living *shrink-wrap*, as they are the cells that are thought to be principally involved in connective-tissue contraction.

LAH: Rolfers certainly have felt that shrink-wrap fascia, really great to have that phenomenon described. So the transition, this shift from fibroblasts to myofibroblasts, if we attribute a preference to a biological process, is it better if our cellular communities have fewer myofibroblasts? The myofibroblasts

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are the cellular source of stiffer fascia in the ground substance?

SPE: Yes, theoretically, it makes sense that they are the primary source of the stiffness. Actually the ground substance is one component of the fascia comprising glycosaminoglycans, proteoglycans, and other *glycoproteins*.[1] Myofibroblasts actually make large amounts of hyaluronan (HA) and the binding proteins. These can make the extracellular matrix stiffer and more adhesive for inflammatory cells. They also form the collagen, fibronectin, elastin, and fibrillin fibers; the molecular filaments that Rolfers contact.

LAH: This is where I think we need to spend a moment with the different levels of examination that we are talking about. When Rolfers are doing a body reading, looking at tissue, the eyes are collecting information at the observation level of one meter, down to one centimeter, and we even do visual differentiation from centimeter to millimeter at times. When we touch, we are gathering information and interacting with tissue at the centimeter to millimeter size, and then even smaller, to fractions of a millimeter at times.

Your human lung fibroblasts are at the observation level of micrometer (*μ*m), correct? Which is to take one millimeter and divide it 1000 times, one of those sections would be one micrometer. How big are the human lung fibroblasts that you investigate?

SPE: These cells can be up to 100*μ*m long or about one tenth of a millimeter. So you could have ten to twenty of these cells spanning the thickness of a fingernail.

LAH: And then you take one more leap of observation, even closer to cellular events, to look at the proteins that the human lung fibroblasts are producing, that is investigating biological life at the nanometer, do I have that correct? Where we take one micrometer and divide that one up 1000 times again. You study HA and proteoglycan link protein 1 (HAPLN1), how big is the HA molecule and is it made by the human lung fibroblasts?

SPE: HA is found in a range of sizes. It's a long linear polymer of a repeating disaccharide. A single molecule of hyaluronic acid or HA can be up to 10*μ*m long. It starts to rival DNA in its size.

LAH: Ah, it is not a protein, it is a molecule in the sugar type in terms of organic chemistry. A repeating chain of two saccharide molecules bonded together, which can be as large as DNA. That is a very big size for a cellular molecule and a unique construction.

Does the fibroblast make HA? Or does the body perhaps make HA somewhere else?

SPE: Yes, HA is made by most cell types. Fibroblasts and synoviocytes [and also rapidly dividing cells; fibroblasts and synoviocytes are not particularly rapidly dividing cells in *vivo*] are rich sources. There are HA synthase enzymes which extrude the growing polymer chains directly through the cell membrane, out to the cell surface and extracellular matrix. It looks like hair growing out of the cell.

HA is one of our body's main lubricants. It's found in high concentrations with other lubricating proteins in synovial fluid and in skin. Together with binding proteins, such as large aggregating proteoglycans, they provide a gel into which the fibers are embedded. They hold water in the tissue and help create the viscoelastic properties of connective tissues.

LAH: So cool. What is so special about HAPLN1 to make it the focus of your study?

SPE: I've been interested in all the proteins that interact with HA. HAPLN1 is a smallish protein ~45kDa that stabilizes the interaction between HA and large proteoglycans. It might be thought of as a stabilizer of the ground substance. It was originally purified from cartilage where the proteoglycan-HA aggregates provide compressive stiffness. We since found out that fibroblasts and other cells make HAPLN1 but its function is only beginning to be understood. As a potential stiffener of the extracellular matrix, it's important to know more about how this contributes to myofibroblast induction and tissue mechanics.

For example, viral infection in the lungs, (such as with SARS-COV2), can induce the production of highly cross-linked HA which can further trap more inflammatory cells and is part of the viscous secretions that clog up the lungs in COVID-19.

LAH: Ah, okay, so characterizing HAPLN1 is part of your focus. Your article mentions other proteins, can you give us a rough sketch of them? Versican and others?

SPE: Versican is one of the chondroitin sulfate proteoglycans that binds to HA, forming large aggregates. Another chondroitin sulfate proteoglycan is called aggrecan, and is found predominantly in cartilage. HAPLN1 stabilizes the binding between HA and the proteoglycan in a three-way interaction, a threesome. These proteins can also be found in other places such as aorta, intestines, uterus, nerve, brain, cancers, and other sites of inflammation. Due to the high negative charge of the hyaluronic acid and the chondroitin sulfate, these molecular complexes imbibe water and create a swelling pressure.

LAH: When I read the introduction of your 2020 article, I thought "HAPLN1 is bad, it is playing a necessary part of lung fibrosis." This area of research is about lung fibrosis, scar tissue in the lung, which causes a lot of suffering. Am I on the right track to think you are kind of getting at two birds with one stone with your research? On one hand, you are studying the molecular pathways involved in the disease processes of lung fibrosis (a debilitating and at times fatal increase of stiff fascia in the alveoli of the lungs), and on the other hand, you are a Rolfer who is interested in the global mechanisms of fibrotic tissue; is this a good model to investigate fascia in general, from the perspective of the deeper cellular processes?

SPE: Yes, fibroblasts have many things in common although there will be differences between the fibroblast from the different tissues. So you can learn a lot regardless of the cell type you choose. However, specific questions regarding fascia, joint capsules, or whatever is under investigation, researchers should be using fibroblasts from that tissue for their studies. We also happened to be funded primarily for studying lung inflammation and interactions of cells of the immune system, like lymphocytes with the fibroblasts and their extracellular matrix.

It's hard to know if HAPLN1 is "bad" because it can be highly dependent on the situation, the tissue, or the disease. Also, it is only beginning to be studied rigorously.

LAH: And you take really cool pictures of three-dimensional cells as part of your workday. What I understood right away from your research publication is that you are looking at where these proteins are in the lung fibroblasts over time? You are looking at them as the cells divide to see where these proteins congregate? With special staining procedures you can makes these proteins of interest glow without killing the cell, so you can see the proteins *in vivo*.

SPE: Most of the images I took were of fixed dead cells treated with phosphorescent molecules that bind to HAPLN1 and versican (see Figure 1). However, you can use those fluorescent markers on living cells, do time-lapse and high-resolution microscopy on living cultured cells and tissue, *in vivo*.

LAH: Of course, that makes more sense, the cells are preserved for observation by microscopy. In your Figure 1, what are those fluorescent markers in the images in your article telling you? It looks to me like the bright spots fill the cell cytoplasm throughout, so it is helping the cell swell and have girth with water while it becomes two cells?

SPE: That is one possibility. Some have theorized that the HA inside the cells helps to push the two daughter cells apart after mitosis. Others found that the HA outside cells facilitates cell adhesion and migration. It can have multiple functions.

LAH: When a fibroblast starts to change, to have its phenotypic modulation, is that when the mitosis is taking place? Cell division? Does the cell division change the cell into a myofibroblast? And I'm still thinking – myofibroblasts are bad, they lead to the disease state of fibrosis in the tissue.

SPE: It depends on the situation. Often times, they are inhibited from proliferating when they differentiate into myofibroblasts, and sometimes the cells proliferate. TGF-beta can act bi-phasically (i.e., it can do different things depending on the concentration) and is sometimes an inhibitor of proliferation. Myofibroblasts are generally not good to have around unless you are in a wound-healing situation where they are necessary for part of the process. After the wound is healed and contracted, you want the myofibroblasts to go away. They typically die in a process called apoptosis and normal healthy fibroblasts take over. Often times they persist, presumably due to the residual tension held in the tissue. As you know, scar tissue or adhesions can become like a black hole, pulling fascia from quite distant areas.

LAH: Yes, exactly, which is why your research is so compelling. Okay, so for your 2020 article, you investigated human lung fibroblasts over time as they transitioned, via cell division, from fibroblasts to myofibroblasts. You took pictures of these cells from different angles where you were able to make the HAPLN1 glow in the images, so you could see how much was there at each step and where in the cell it was at each step of mitosis?

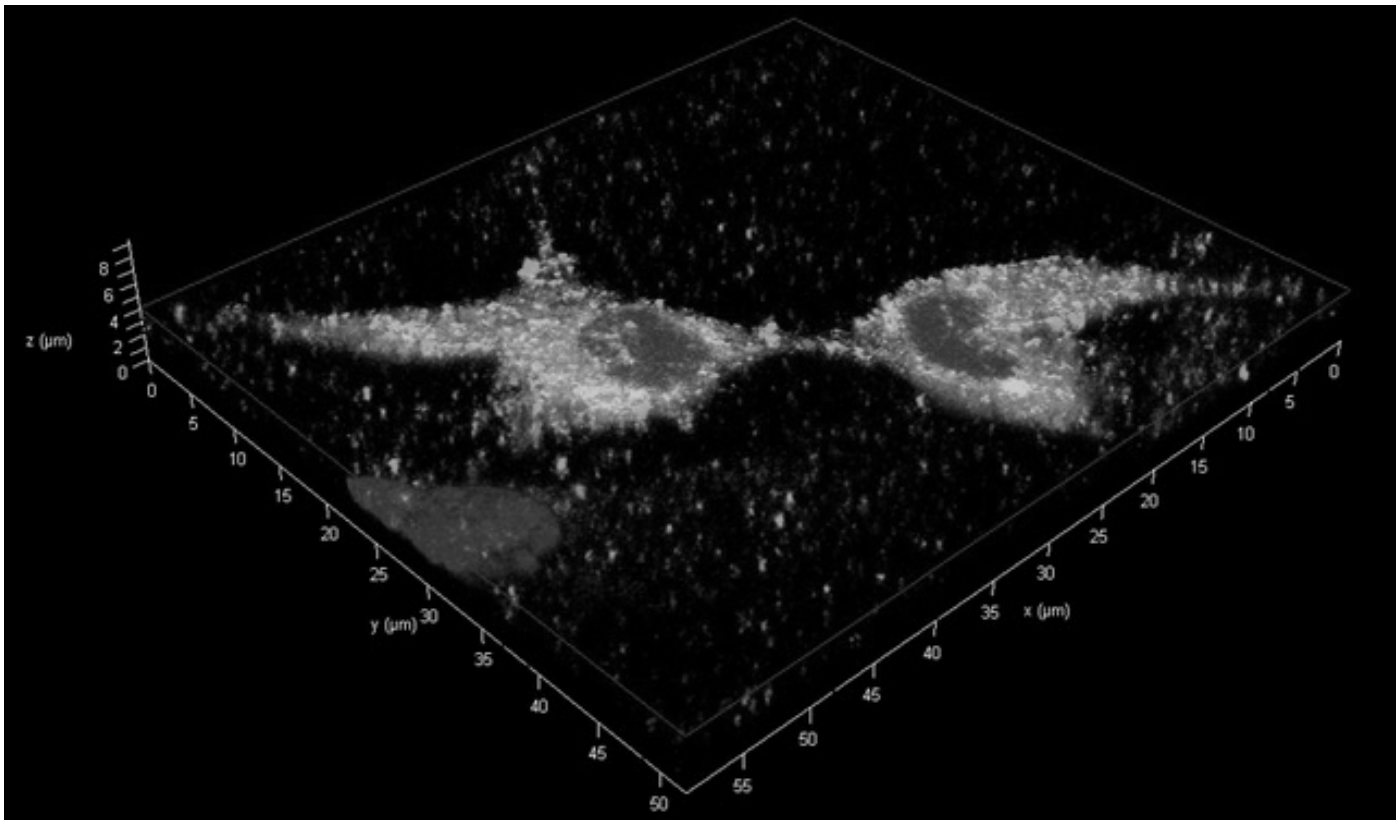

Figure 1: Mitotic lung fibroblasts. Specific monoclonal antibodies were used to identify the HA-associated proteins HAPLN1 and versican (lighter spots around the membrane of the cell and interspersed throughout the cytoplasm). HAPLN1 and versican appear as the bright spots throughout the cytoplasm of the cell. This is an oblique view of two fibroblasts that are almost completely finished with the telophase portion of mitosis. Stacked confocal microscope images were used to generate the three-dimensional appearance. Note how the bright vesicles containing the HAPLN1 and versican span the entire thickness of the cell(s). We previously found that HA is also taken up in the same way during cell division. The significance of this is not yet known.

SPE: Yes. And the fact that we find an extracellular protein associated with the nucleus is also of interest. There are now a number of proteins previously thought to be only in the extracellular matrix that also may have some function inside cells. This is quite exciting and interesting to many folks studying these proteins.

LAH: Oh yeah, now I get it. When studied biochemistry in the 1990s, the understanding was that information flowed from DNA to RNA to proteins, the arrows of the flowchart only went one direction. Now, we know, that molecules of varying types go right into the cell, into the nucleus, and affect the DNA. That is a big deal. So, you are interested in a potential feedback loop by HAPLN1 to the DNA during the phenotypic modulation, the transition from fibroblast to myofibroblast?

SPE: Exactly. Knowing more about how the extracellular matrix can impact the inside functioning of cells during these processes can help us devise better strategies to improve fibrotic situations.

LAH: And you research this cellular system because the more you can correctly describe the different proteins involved in this process, the more likely future research may be able to disrupt that biochemical pathway, produce pharmaceutical interventions to prevent lung fibrosis patients from developing this scar tissue in their lungs. *And*, it is really cool to understand fibroblasts as a Rolfer because this is one possible mechanism of change for our touch interventions?

SPE: I really enjoy peering at cells through the microscope. There's a whole other universe in there! I'm sure it helps inform about what is happening under our fingers.

LAH: Absolutely. You've written about this previously as well, in your article "Extracellular Matrix and the Manipulation of Cells and Tissues" in the 2009 *IASI Yearbook*. A very good read.

So now, with all your help here, I do understand the 'discussion' section of your 2020 publication, in a general sense. Tell me how this summary sounds, let me know where I go astray and what I missed:

1. You found HAPLN1 in the pericellular matrix (in the ground substance around the fibroblasts) of the lung tissue, which matches previous findings about HAPLN1 in the brain.

2. You found dots, or small spots, of concentrated HAPLN1 in locations where you thought the cell had moved away from, which lead you to propose that was HAPLN1 concentrated in that spot, left after that movement. So perhaps the HAPLN1 plays a role in stabilizing cell adhesion, cell stickiness.

3. The cluster of HA, versican, and HAPLN1 were imaged in the nucleus of the fibroblasts, which is pretty interesting, and suggests that it has a role in the structure and/or the function of the nucleus. Like you said, this cluster of molecules attract water, so I'm imagining the nucleus being more water filled with those three molecules present, like a water balloon? And you reported the possibility that these molecules may help fill the shape the nucleus after going through the cell division process.

SPE: That's right. Other folks have found that HAPLN1 can impact cancer metastasis by as yet unknown mechanisms, so these are important processes to understand.

LAH: The final point in your 2020 publication is quite complex, but I'd like to try to understand it. You state, "Extracellular matrix stiffness is a known regulator of myofibroblast formation" which you describe as collagen fibers dominating, producing this stiffening effect (Evanko 2020, 808). You propose that HAPLN1 may be modifying this by making the matrix more compact. It sounds to me like you think HAPLN1 is part of the molecular cascade involved in phenotypic modulation, both in the extracellular matrix and in the nucleus of the fibroblast-myofibroblast?

SPE: Yes, it is probably like several other proteins that play multiple roles in cellular physiology.

LAH: The idea that is staying with me the most after reading your 2020 article, you are the first lab group to be reporting that HAPLN1 is also in the nucleus, which is a really interesting finding for sure. Was that a good day in the lab when you saw the cleaned-up images and conclusively could say – that molecule is for sure in the nucleus?

SPE: It's always exciting when you see something new. It usually just leads to more interesting questions and another research project for someone.

LAH: Okay, wow, I find this stuff quite intriguing, how molecules' presence, absence, and/or concentration can shift the whole entire cell.

Now we know what you were studying and why you studied it. Tell us about the laboratory: you were growing cells, you bathed them in special immunocytochemistry proteins and mixtures, then you looked at them under a microscope, took pictures of those cells. What was that work environment like? Did you find it easy to switch between lab work to Rolfing SI work? Very different kind of thinking I would imagine.

SPE: Yes, a very different work environment for sure. There were fancy microscopes and PCR (polymerase chain reaction) machines sitting around on black bench tops and lots of chemical solutions on shelves above. There were small desks with a computer nearby. I enjoyed the camaraderie of working with other folks on a project and discovering new intriguing things about how the body works. The complexity of it all is fascinating.

LAH: This is very cool to me, my first career was in the lab as well, working with neural stem cells as an entry level lab technician, then later photosynthetic bacteria researcher, and right before becoming a Rolfer I was an assistant lab manager for a water quality laboratory.

After the wound is healed and contracted, you want the myofibroblasts to go away. They typically die in a process called apoptosis and normal healthy fibroblasts take over. Often times they persist, presumably due to the residual tension held in the tissue.

What I know about lab work is that there is a lot of repetition, did your protocol take a long time to develop in order to look at this cellular phenomenon?

SPE: There definitely is lots of repetition. It was only possible because of past research that I can do this work now and is directly based on the work of a lot of other people before me. They first characterized these molecules and developed techniques and reagents, including making antibodies to identify the proteins that we were interested in (like those used in the image of Figure 1).

I appreciate any interest in this kind of nerdy topic because it really does relate to what we do as Rolfers.

LAH: Absolutely, it is really important that Rolfers be empowered with this knowledge, the cellular mechanisms that our interventions affect are real, they are studied, and thanks to researchers like you, we really can understand what's happening under our hands. You've given us a lot to conceptualize, I hope we can do this again.

Stephen Evanko is a Certified Advanced Rolfer and conducted cell biology research at The Benaroya Research Institute in Seattle, Washington.

Lina Amy Hack is a Certified Advanced Rolfer based in Saskatoon, SK, Canada. She is also the Co-Editor-in-Chief of this journal.

Endnotes

1. Proteoglycan is a category of molecule known as glycosylated proteins. Generally speaking, it has a chain of amino acids forming a central protein while also having covalent bonds with one of several carbohydrate chains (polysaccharides) dangling off that central protein chain. They can be embedded in the cellular membrane with the majority of the mass of the molecule projecting into the extracellular matrix. There are many different types of proteoglycans and it is an active area of research. The primary biological function is to provide hydration and swelling pressure within tissue in order to withstand compressional forces (Yanagishita 1993)

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