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Clinical Research Paper

Silent messengers in the nano-world: Harnessing extracellular vesicles as theranostic tools for neonatal surgical conditions☆

Augusto Zani [a, b, *]

[a] Department of Surgery, Washington University School of Medicine in St. Louis, United States
[b] Division of Pediatric Surgery, Saint Louis Children's Hospital, United States

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ABSTRACT

Extracellular vesicles (EVs) are lipid bilayer-enclosed nanoparticles secreted by all cells that mediate intercellular communication by transferring proteins, lipids, and nucleic acids. Among their cargo, microRNAs are key post-transcriptional regulators of organogenesis. In fetal lung development, EV-mediated signaling is essential for the coordination of epithelial, mesenchymal, endothelial, and immune cells. In congenital diaphragmatic hernia (CDH), disruption of these interactions leads to pulmonary hypoplasia and pulmonary hypertension, the primary causes of morbidity and mortality. Amniotic fluid stem cell-derived EVs (AFSC-EVs) have emerged as a promising cell-free therapy to restore this impaired communication. In rodent, rabbit, and human models of pulmonary hypoplasia, AFSC-EV administration rescues branching morphogenesis, promotes epithelial and mesenchymal differentiation, and attenuates macrophage-driven inflammation. Cargo analyses identified enrichment of the miR-17~92 cluster, whose regulatory role in branching and progenitor differentiation is indispensable for normal lung growth. Mechanistic studies demonstrated that RNA degradation or selective inhibition of these microRNAs abolished the regenerative effects, underscoring the central role of EV small RNAs. Single-nucleus RNA sequencing confirmed restoration of transcriptional programs in AFSC-EV-treated lungs to profiles resembling healthy controls. Together, these findings establish AFSC-EVs as potent mediators of fetal lung repair in experimental models. Beyond CDH, EVs represent a promising theranostic strategy for pediatric surgical conditions in which restoration of intercellular communication is critical.

Before we speak our first word, before we even take our first breath, our bodies are already communicating. Cells whisper to each other. Tissues negotiate. Organs align their efforts. Life, from its earliest moment, is not silent. It's a symphony of signals. Each cell plays its part, in harmony or in silence. Life depends on their ability to listen and respond. Communication is not just coordination, it is survival. And when communication fails, development falters. In the world we live in, the consequences of communication breakdowns are just as devastating. We must remember that conflict, at any scale, is not a sign of strength, but a failure of communication. But what if we could restore the dialogue? What if we could send a message, not of destruction, but of repair? This paper will discuss about extracellular vesicles (EVs), their ability to act as cellular "messengers", and their potential role as conflict mediators in conditions such as arrested lung development.

1. Lung development and pulmonary hypoplasia: *War and peace*

As a pediatric surgeon I have always been fascinated by neonatal surgery, in my view the aspect of our specialty that most clearly distinguishes us from adult general surgery. Operating on a newborn demands not only refined technical skills, manual dexterity, and composure under pressure, but also a deep sense of responsibility. It is, above all, a privilege. A privilege that can only be earned through years of rigorous training and experience. And among neonatal surgical conditions, I've always been particularly drawn to the two with the highest mortality rates: necrotizing enterocolitis (NEC) and congenital diaphragmatic hernia (CDH). When I opened my laboratory 10 years ago, I chose to dedicate my research to CDH. As I had spent the previous two decades investigating NEC both at the bench and in the clinic, I realized how little I

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* North Western Tower, Suite 11109, One Children's Place, Saint Louis, MO, 63110, United States.
E-mail address: augusto.zani@wustl.edu.

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actually knew about the lung. I soon became fascinated, immersing myself in articles and textbooks on lung development. Lung development is a very well-orchestrated and complex process that starts in a fetus during the embryonic phase, at around 4 weeks of gestational age, and progresses through and beyond the pregnancy via a well-regulated process of branching morphogenesis, epithelial and mesenchymal differentiation, vessel development, and air-blood barrier maturation [1–3]. Each of these processes depends on coordinated signaling between multiple cell types and tissues, ensuring that structure and function develop in harmony. In many ways, this period of precise coordination is a time of "peace", where every component works together toward the common goal of building a functional organ. When I began studying lung embryology, I focused on the pathways that are central to lung organogenesis. These pathways rely on intricate interactions among the main tissues of the lung, with constant crosstalk between epithelium and mesenchyme, epithelium and endothelium, and endothelium and mesenchyme. Throughout the five main stages of lung development, namely embryonic, pseudoglandular, canalicular, saccular and alveolar, intercellular communication remains continuous and essential. The importance of these interactions becomes even clearer when development is arrested, that is when dysregulated crosstalk results in impaired lung development. If we look at hypoplastic lungs of fetuses with CDH, we recognize that impaired communication between the epithelium and the mesenchyme yields to arrested lung *growth* and *maturation* [4]. Similarly, impaired crosstalk between epithelium and endothelium, as well as between smooth muscle cells and endothelial cells leads to prenatal vascular remodeling and altered *vascularization*, that predispose the baby to develop pulmonary hypertension postnatally [5]. Pulmonary hypoplasia represents the opposite state, a time of "war". The balanced dialogue between tissues is disrupted, signaling pathways fall into disarray, and organogenesis is derailed. As in Tolstoy's *War and Peace*, the fragile equilibrium between harmony and conflict determines the fate of the whole. In the developing lung, that balance is the difference between an organ capable of sustaining life and one destined for failure. The abnormal crosstalk across cells is not just due to disrupted growth factor signaling pathways and transcriptional factors. Over time, there has been an increased recognition that microRNAs (miRNA) are critical in all stages of lung development. miRNAs are small, single-stranded, non-coding RNA molecules that are able to silence protein translation and modulate signaling pathways [6]. They have gained attention in the scientific community as they are considered as the master regulators of the genome. For this reason, researchers have been learning how to treat diseases by harnessing the way microRNAs control gene expression at the post-transcriptional level. Interestingly, several studies showed that hypoplastic fetal lungs from human and experimental models of CDH have missing or dysregulated miRNAs, which could be used a biomarkers of disease severity or response to therapy [7–17]. In summary, fetal lung development is a complex process regulated by multiple signaling pathways and by a network of small RNA species, including miRNAs. Any therapy aimed at restoring lung development, and thereby re-establish "peace" among the tissues, must consider the multifactorial nature of pulmonary hypoplasia and therefore adopt a multi-targeted approach that includes modulation of miRNA signaling.

2. Stem cells derived extracellular vesicles: *Great expectations*

I started working with stem cells about 20 years ago during my PhD studies at the Institute of Child Health at Great Ormond Street Hospital, University College London, UK. At that time, we were exploring the use of stem cells as a novel therapeutic strategy for babies with NEC. Our hypothesis was that stem cells injected in a neonatal rat model of NEC would repopulate the sloughed epithelium and differentiate into specialized enterocytes [18]. We initially decided to use bone marrow-derived mesenchymal stromal cells (BM-MSCs), guided by promising studies demonstrating their potential in models of inflammatory bowel disease. However, BM-MSC treatment did not yield the expected regenerative outcome in our model of NEC [19]. We then turned to amniotic fluid stem cells (AFSCs), a population of cells found in the amniotic fluid that are broadly multipotent, are able to differentiate into all three germ layers, have low ethical impact, and are not teratogenic [20]. When we injected AFSCs in neonatal rats with NEC, we noticed improvement in the microscopic intestinal architecture, with an attenuation of the inflammatory profile, and most importantly, a significant improvement in survival [19,21]. Yet the degree of tissue engraftment by AFSCs was quite limited. We therefore considered that rather than repopulating the sloughed epithelium, the injected stem cells were having a beneficial effect on the damaged intestine by secreting factors with a regenerative effect. We ran the same experiments again using the condition medium, that is, the fluid secreted by stem cells. To our surprise, we obtained the same anti-inflammatory and regenerative effects, particularly a significant improvement in survival. At that time, we and other groups were facing this paradox of a low degree of stem cell engraftment in the tissue despite clear beneficial effects in the experimental models. We therefore thought that the AFSC mechanism of action was through a paracrine effect, whose factors remained unknown. Over the following years, several research groups began investigating the stem cell secretome to identify the paracrine factors that were responsible for stem cell action [22–24].

Coming from my background studies on models of NEC where I was able to modulate the regenerative response by ASFC administration, I was fascinated to read that the same cells were able to rescue *in vitro* and *in vivo* growth, innervation, and motility of hypoplastic lungs in a fetal rat model of CDH [25]. More interestingly, another research group reported the same beneficial effects and positive outcomes when administering AFSC secretome alone [26]. It was at this point that I read about EVs being part of the cell secretome and became intrigued by their potential [27–30]. EVs are nanoparticles secreted by all cells, including plants, fungi, and prokaryotes whose first description dates back to 1967 by Wolff [31]. According to the Minimum Information for Studies of Extracellular Vesicles (MISEV), EVs are particles released from a cell, are delimited by a lipid bilayer, and cannot replicate as they have no nucleus [32]. Several terms have been used to describe these nanoparticles including exosomes, microparticles, microvesicles, ectosomes, apoptotic bodies, oncosomes. However, the International Society for Extracellular Vesicles endorses the broad umbrella term of *extracellular vesicles* [32]. In some studies, it is known that some terms are related to EV presumed biogenesis pathways, whereby *exosomes* refers to EVs coming from internal compartments of the cells and released via the multivesicular body; *ectosomes*, also known as microvesicles, refer to EVs generated from the cell surface, and *apoptotic bodies* arise during specific cellular processes, such as programmed cell death [32]. As a form of intercellular communication, EVs are secreted by a cell via blebbing, exocytosis, budding or shedding, travel for an undetermined distance, and are taken up by cells via ligand–receptor interaction, fusion, or endocytosis. To date a homing mechanism for EVs such as that of stem cells remains elusive [33].

More important than the messenger is the message. EVs carry cargo that contains bioactive lipids (e.g., sphingomyelin, ceramide, cholesterol), transmembrane proteins (e.g., tetraspanins, annexins,

antigen-presenting molecules), and genetic material (DNA and small RNA species) [34–36]. The EV molecular cargo mirrors the status of their parent cells, making EVs powerful tools to study the mechanisms underlying disease pathophysiology. Moreover, EVs are considered the paradigm of theranostics, whereby there are applications for therapeutics and for diagnostics. There are currently more than 200 clinical trials on "extracellular vesicles" or "exosomes" registered on clinicaltrials.gov, along with an increasing number of patents, an exponential number of companies in the field, and a market projected to approach $800 million by 2030 [37]. In this context, EVs represent our *Great Expectations*: they embody a new hope for the future of cell-free regenerative medicine, carrying the promise of therapies that can restore development and function in ways that stem cells alone could not, or that stem cells might achieve only at the cost of significant side effects. In parallel, EVs also herald a new era for liquid biopsies, as their cargo provides a readily accessible window into the molecular state of cells and tissues, enabling the early detection, monitoring, and stratification of disease in a minimally invasive manner.

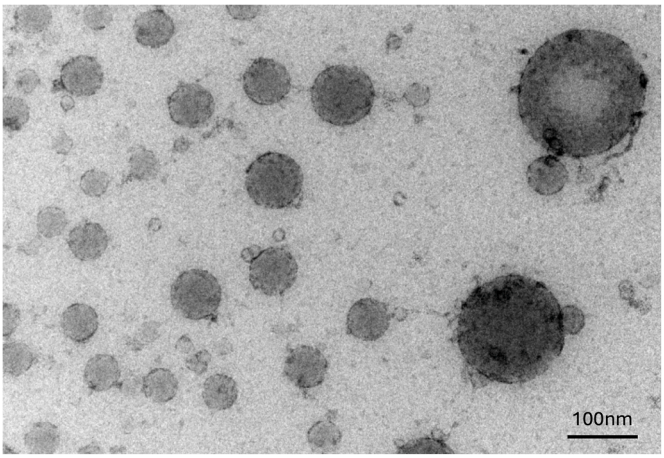

Fig. 1. Representative image of the typical double membrane morphology of AFSC-EVs obtained with transmission electron microscopy (scale: 100 nm).

3. Amniotic fluid stem cell extracellular vesicles (AFSC-EVs): *Dialogues*

Given the high degree of morbidity and mortality of babies with CDH after birth, there is consensus that the prenatal period offers a window of opportunity to promote normal lung development [38,39]. Several prenatal treatments aimed at promoting lung development have been tested, but none have fully reversed the CDH hypoplastic lung phenotype. Among the available prenatal interventions, fetoscopic endoluminal tracheal occlusion (FETO) is the most extensively studied [39,40]. In this procedure, a balloon is placed within the fetal trachea to block the outflow of lung fluid, which stimulates lung expansion and growth. Although FETO increases lung size, this procedure currently does not improve lung maturation as surfactant production remains unaffected, and it does not reverse the remodeling of the pulmonary vasculature. The technique is further limited by strict eligibility criteria, the risk of preterm premature rupture of membranes, and its restricted availability to a few highly specialized centers. For these reasons, even experts in FETO have emphasized the importance of pharmacologic strategies that can more completely restore lung development, either in addition to or instead of FETO [40]. In search for a therapy that would be either an addition or an alternative to FETO, the Zani lab has been studying stem cell derived extracellular vesicles over the last 10 years. We initially compared different EV isolation techniques to separate stem cell extracellular vesicles derived from the amniotic fluid (AFSC-EVs) [41]. In several of our experiments, we have been using ultracentrifugation, which offers several advantages in terms of yield of the preparation, as well as other types of isolation techniques that are advantageous for preparation purity. In line with MISEV recommendations, we characterize AFSC-EVs using nanoparticle tracking analysis for EV size, transmission electron microscopy for EV shape, and Western blot analysis for the expression of canonical EV markers (Fig. 1) [42]. We initially tested AFSCs in experimental pulmonary hypoplasia and confirmed their potential in restoring lung epithelial cellular homeostasis [43]. We then investigated AFSC-EV administration in fetal rats with CDH and demonstrated that AFSC-EVs rescues branching morphogenesis and promotes epithelial cell and fibroblast differentiation (Fig. 2) [44]. We ran these experiments at E14.5 (pseudoglandular stage of lung development), based on previous studies that had shown a beneficial effect of AFCs and/or their conditioned medium at this developmental stage [25,26]. We subsequently confirmed that AFSC-EVs exerted similar regenerative effects by promoting epithelial and mesenchymal cell differentiation and rescuing dysregulated signaling pathways also when they were administered at the canalicular and saccular stages of lung development, timepoints that are translationally relevant stages for fetal interventions [45]. In another study, we highlighted how human and rat fetal hypoplastic lungs have impaired autophagy, a very important biological process during organogenesis, and how AFSC-EV treatment rescues the dysregulated signaling back to normal level [46]. Beyond studies focused on structural outcomes at the histological and molecular levels, we also investigated the impact of AFSC-EVs on lung function. The fetal rat model of CDH is typically non-surviving, largely because neonatal resuscitation, surgical repair, and extracorporeal life support are not feasible in this setting. To overcome this limitation, we established a tracheostomy model in newborn rats with CDH and showed that animals exposed *in utero* to intra-amniotic AFSC-EVs exhibited improved ventilation mechanics compared with placebo-treated controls [47].

When we studied the cargo content of AFSC-EVs, we initially performed proteomics analysis [42]. However, the proteins we identified were mainly involved in stabilizing RNA species rather than directly promoting lung development. We then turned to small RNA sequencing, which revealed that AFSC-EVs are enriched with microRNAs, piRNAs, long non-coding RNAs, and other small RNA species. Among these, we found a striking enrichment of the miR-17~92 cluster, a family of miRNAs known to regulate FGF10-mediated embryonic lung epithelial branching morphogenesis, as well as progenitor cell proliferation and differentiation. This cluster is indispensable for normal lung development, since its knockout in mice results in fatal bilateral pulmonary hypoplasia [48]. To connect AFSC-EV cargo with the functional response of lung cells, we performed an integrative analysis pairing AFSC-EV-enriched microRNAs with messenger RNAs that were down-regulated in the recipient cells. Importantly, we considered only miRNA-mRNA interactions that had been functionally validated and reported in the literature [44]. These analyses consistently highlighted the central role of several microRNAs, including the miR-17~92 cluster, in mediating the crosstalk between lung cells facilitated by AFSC-EVs. We then confirmed the responsibility of miRNAs in the regenerative effects of AFSC-EVs. When AFSC-EV RNA cargo was enzymatically digested, the regenerative effect of the administered EVs was completely lost [44]. Furthermore, we performed antagomir studies in which miRNAs of interest from the miR-17~92 cluster were selectively blocked in the parent cells [46].

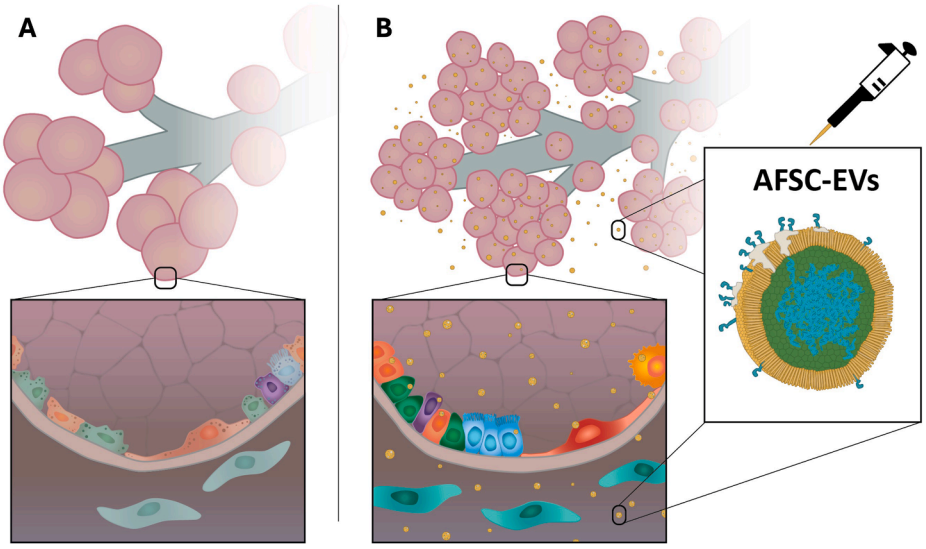

Fig. 2. A. CDH lungs have impaired branching morphogenesis with few airspaces and undifferentiated epithelium and mesenchyme. **B.** Antenatal AFSC-EV administration promotes branching morphogenesis and cell differentiation in hypoplastic lungs of fetal models of CDH.

In both scenarios, AFSC-EV regenerative effect was abolished. Taken together, these experiments demonstrated that small RNA species, and in particular microRNAs, are central to the ability of AFSC-EVs to restore lost conversations between lung cells and re-establish functional crosstalk [49].

A key question is how AFSC-EVs promote or rescue lung development in experimental pulmonary hypoplasia. Using intra-amniotic delivery and EV tracking, we demonstrated that AFSC-EVs reach the fetal lungs as well as other organs [50]. Single-nucleus RNA sequencing of fetal lungs successfully isolated all four major cell populations: epithelial, mesenchymal, endothelial, and immune cells [50]. AFSC-EV-treated fetuses with CDH had lung cell clustering similar that of healthy controls, whereas saline-treated CDH lungs displayed a multilineage inflammatory profile with marked macrophage enrichment at the end of gestation. This was confirmed at the protein level in an expanded cohort, where macrophages and pro-inflammatory cytokines such as TNF-α were elevated in untreated CDH lungs but normalized after AFSC-EV administration. Notably, increased CD68-positive macrophages and TNF-α were also identified in human fetal CDH lungs from autopsy specimens, underscoring a central role of macrophage-driven inflammation in the pathogenesis of impaired lung development. These findings suggest that inflammation is likely at the root of the established paradigm of pulmonary hypoplasia as a disorder of growth, maturation, and vascularization (Fig. 3) [51]. Importantly, AFSC-EVs appear to counteract this process by re-establishing cellular dialogues and restoring normal lung development. Lastly, we demonstrated that the AFSC-EV regenerative effects on fetal hypoplastic lungs are not limited to CDH. Pulmonary hypoplasia can also result from oligohydramnios, often caused by premature rupture of the chorioamniotic membranes. We recently demonstrated that antenatal administration of AFSC-EVs in a surgical fetal rat model of

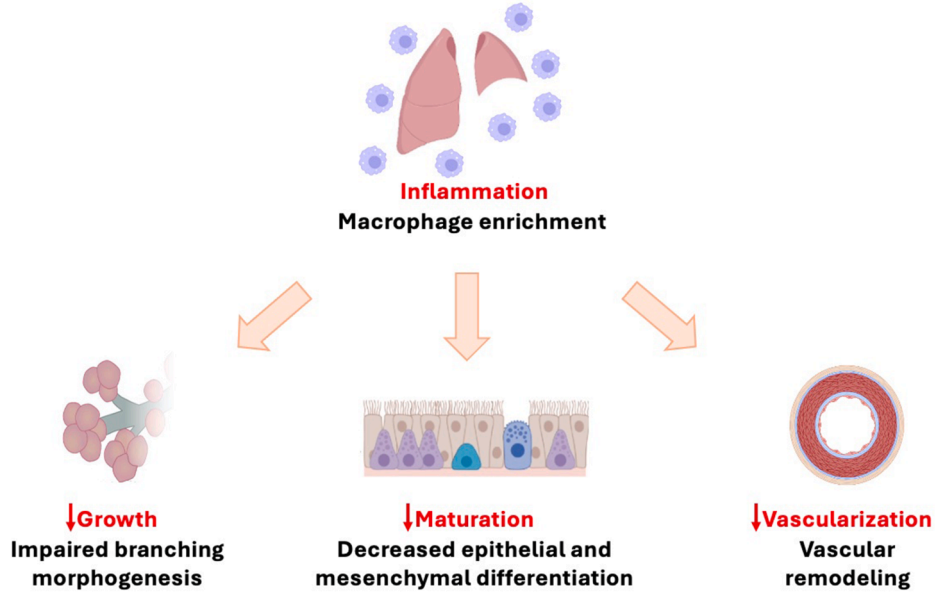

Fig. 3. The new paradigm of pulmonary hypoplasia in CDH: inflammation is at the root of arrested lung development (Created with BioRender.com).

oligohydramnios rescues branching morphogenesis and airway progenitor cell patterning through the release of miRNAs that regulate the TGF-β signaling pathway [52]. These findings suggest that AFSC-EVs hold broader therapeutic potential for multiple causes of pulmonary hypoplasia, opening the door to their use as a unifying strategy for fetal lung regeneration.

Our initial studies were mainly performed in fetal rats, which is considered the most robust model of CDH. Similar regenerative effects on the hypoplastic fetal lung growth were also observed in hypoplastic fetal mouse, rabbit, and human lung models. Current efforts are focused on validating these findings in a large-animal model to assess the safety, feasibility, and translational potential of EV-based fetal therapies. Several key questions remain, including the dosing regimens needed to achieve efficacy without off-target consequences, the frequency of administration, and the most practical and effective delivery route [51]. Equally important will be the establishment of reproducible EV cargo composition, particularly with respect to miRNA and protein content, as this will be critical both for experimental reproducibility and for meeting regulatory standards. Resolving these issues will be fundamental to moving EV-based strategies from experimental promise toward clinical application.

4. Conclusions: *Brave new world*

While our work has focused mainly on CDH, the potential applications of EVs in pediatric surgery extend well beyond this condition. EVs can mediate repair in congenital anomalies, support cellular regeneration, enhance wound healing, and modulate inflammation and vascular remodeling. They also offer opportunities for monitoring disease progression in settings such as ischemia-reperfusion injury, neuropathic pain, and cancer. Importantly, the possibility of using EVs from accessible body fluids such as urine, blood, saliva, or breast milk as less-invasive alternatives to tissue biopsies opens new avenues for diagnosis, therapeutic monitoring, and personalized interventions. Together, these approaches position EVs as “silent messengers in the nanoworld”, versatile theranostic tools that bolster the promise of transforming care across a spectrum of pediatric surgical diseases.

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