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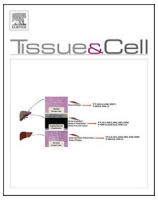

Effects of Germanium embedded fabric on the chondrogenic differentiation of adipose derived stem cells

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ABSTRACT

Osteoarthritis (OA) is a clinical state which is identified by the degeneration of articular cartilage. OA is a common condition (>500 millions of people affected worldwide), whose frequency is anticipated to continue to rise (> 110 % increase worldwide since 2019). The treatment for early-stage OA is based on a combination of therapeutic approaches, which can include regenerative medicine based on Adipose Derived Stem Cells (ADSCs). Germanium embedded Incrediwear® functional Cred40 fabric has been shown to have positive effects on OA clinically and is envisaged to give encouraging effects also on tissue regeneration. Still, the biological mechanisms underlying this therapeutic modality have not yet been fully defined. We tested the hypothesis that Germanium-embedded Incrediwear® functional Cred40 fabric could enhance chondrogenic differentiation. To this purpose, we applied Incrediwear® to human adipose-derived stem cells (hADSCs) induced to chondrogenic differentiation in vitro. Chondrogenic markers (ACAN, SOX9, RUNX2, COL2A1, COL10A1) were quantified following 21 days of treatment. We also assessed extracellular matrix (ECM) deposition (specifically Collagen and glycosaminoglycans (GAGs)) using Alcian Blue and Sirius Red staining.

Here, we provide pilot data to demonstrate that Germanium-embedded Incrediwear® functional Cred40 fabric can enhance hADSCs chondrogenic differentiation and maturity and potentially induce events of cartilage regeneration.

1. Introduction

Articular hyaline cartilage is a deeply interconnected network of chondrocytes and extracellular matrix (ECM) proteins including collagen proteins (mainly collagen II). Articular cartilage is subjected to a continuous remodeling, which is mainly attributable to the neosynthesis of ECM components by chondrocytes. Despite this, chondral tissue is known to possess low self-repair potential (Becerra et al., 2010), which is further reduced by aging. This fact increases the possibility of progressive degeneration and damage of the surface of joints cartilage. In addition to aging, traumatic lesions of articular cartilage can lead, when left untreated for a period of time, to joint function impairment and pain, which is currently defined as osteoarthritis (OA) in the clinical setting. Notably, over 500 million people suffer from OA, worldwide (Buckwalter et al., 2005), and the prevalence of OA is expected to continue to rise (> 110 % increase worldwide since 2019) along with increasing life expectancy and levels of risk factors such as obesity (Zhang and Jordan, 2010). Regenerative medicine, such as stem cell therapy and platelet-rich plasma injections, is particularly useful in the early stages of osteoarthritis when cartilage damage is still mild to moderate. However, as osteoarthritis progresses, and the damage becomes severe, other different treatment modalities are required. To manage OA in its early-stages, treatments based on the implantation of mature chondrocytes or osteochondral grafts often fail to totally restore hyaline cartilage structure and functionality (Knutsen et al., 2007; Revell and Athanasiou, 2009). This failure is mainly attributable to the poor proliferation capacity of mature chondrocytes accompanied by the propensity of these cells to de-differentiate, which has led to the proposed use of adult stem cells, in particular Mesenchymal Stem (and/or Stromal) Cells (MSCs) as a valuable cell source for articular cartilage tissue engineering (Le et al., 2020; Robert et al., 2020). Among MSCs, Adipose-Derived Stem Cells (ADSCs) have shown promise in regenerative medicine when applied to articular cartilage. Indeed, they present strong chondrogenesis potential, and can be easily obtained from

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liposuction waste following surgical interventions (Im, 2016; Wu et al., 2013; Veronesi et al., 2014). For these reasons, human ADSCs (hADSCs) have been proposed for treatment of age-induced or traumatic OA (Pak et al., 2016). We contributed to this field providing evidence that exposure to Incrediwear® functional Cred40 fabric for hADSCs cultured and chemically induced to chondrocyte differentiation *in vitro*, strongly potentiates their differentiation capability towards cartilage lineage (Iorio et al., 2022a). Mesenchymal stem cells (MSCs) are gaining recognition for their potential in treating osteoarthritis (OA), especially in early to moderate stages. Clinical trials, such as those using intra-articular injections of MSCs derived from bone marrow or adipose tissue, have demonstrated promising outcomes. It has been reported significant pain reduction and improved joint function in knee OA patients after MSC therapy. Despite these advancements, MSC treatments remain largely investigational. Regulatory bodies like the FDA and EMA are evaluating the long-term safety and efficacy of these therapies, as researchers work to standardize MSC sourcing and administration protocols.

A novel frontier in the management of OA is represented by the application of treatments based on regenerative modalities, which include the Germanium-based non-invasive wearable devices (Incrediwear® sleeves and braces). Electromagnetic Fields (EMFs), and in particular Ultra-Weak Electromagnetic Fields (UW-EMFs) have already been shown to have a positive impact on OA (Fini et al., 2013; Chen et al., 2013). As proven by previous *in vitro* experiments, this effect can be traced back to an enhancement of chondrogenesis (Iorio et al., 2022a). Also, the use of Germanium-embedded devices has been proven to exert positive effects for the treatment of different diseases including OA (Marino et al., 2019). Germanium is a relatively rare metalloid belonging to the semiconductor group of elements and is found in nature (Gapurenko et al., 2015). This nontoxic semiconductor metalloid has several applications in optics and electronics (Sutter et al., 2019). Different from metals, semiconductors like Germanium have a decreasing resistance as their temperature rises. This is because Germanium may conduct electricity better at certain temperatures because it has more "free" electrons. Theoretically, a useful technique to harness the effect of a micro electromagnetic field with transdermal action that increases circulation and mediates the inflammatory process is to incorporate pure Germanium into clothing. Previous studies have indicated that wearing clothing blended with Germanium may enhance OA clinical outcomes and Total Knee Arthroplasty (TKA) outcomes (Justice and Jacob, 2023). This technology is embraced by the proprietary Incrediwear® functional Cred40 fabric (GEF), which incorporates the semiconductor Germanium into the sleeve and brace products. Additionally, as Germanium and nanotechnology research advance, many biological processes are being shown to be mediated and promoted by Germanium, and scientists are becoming more and more interested in it due to its possible applications in biochemistry and medicine. Germanium is a substance that has steadily acquired importance in the field of biomedicine in recent years and seems to have a bright future for use.

Germanium, and specifically GEF, has been shown in recent studies to be able to significantly reduce pain for OA patients (Marino et al., 2019).

In the last years, clinical studies have been conducted to demonstrate the ability of Incrediwear® functional Cred40 fabric to stimulate the natural healing process after injury or surgery. It has been demonstrated GEF is able to increase blood circulation through the activation of the Germanium embedded within the fabric blend by the heat released by the human body or temperatures over the threshold of 32 C°, at which point the elements present in the fabric are able to release mid-level and far infrared waves as well as negative ions. Both infrared waves and negative ions are biologically active and are able to mediate inflammatory and pain-related pathways in the human body. GEF has also been shown to increase blood flow and blood velocity by up to 22 % at rest when compared to placebo garments. Moreover, it has also been shown to release negative ions at concentrations 2–3X higher than those derivable from a negative ion-producing chamber and has shown significant results in reducing joint pain, swelling, improving range of motion in the knee in patients who underwent arthroplasty compared to the standard of care compression stockings prescribed for post-operative care (Justice and Jacob, 2023).

GEF emits electromagnetic waves in the mid and far range as well as negative ions. Delivery of infrared waves to the tissue is proposed to occur through energy absorption of intracellular water, considered as the most abundant biological chromophore in the human body. Intracellular water acts as the carrier molecule for the infrared waves energy to travel into various tissues, and the penetration depth and speed relates to the dielectric constant of the tissue. Mid and far infrared therapy is well-known to increase blood flow and lymphatic flow. Mechanisms of therapeutic benefit may include improved ion channel function, regulation of cytokine production, modulation of the anti-inflammatory nitric oxide (NO) cascade through accelerated binding of calcium (Ca^{2+}) to calmodulin (CaM). NO indeed is well-known to play a role in facilitating increased blood and lymphatic flow through endothelial smooth muscle cell relaxation. Additionally, NO is linked to down-regulation of interleukin-1 beta (IL1β) and inducible nitric oxide synthase (iNOS) in certain cell types, which leads to reduced cyclooxygenase-2 (COX-2) and prostaglandins – molecules which responsible for causing inflammation and pain under both inflammatory and traumatic conditions. Unlike systemic COX-2 inhibitors such as nonsteroidal anti-inflammatory drugs (NSAIDs), targeted infrared and negative ion therapy stimulates specifically localized reaction pathways, enabling a site-specific pain and inflammation reduction.

GEF is also proposed to reduce inflammation and oxidative stress through the release of negative ions, which are linked to a reduction of the inflammatory response biochemically through the increase of anti-inflammatory cytokines, improved ion channel function and quenching of reactive oxygen species (ROS) upon absorption of negative ions into the blood stream.

Furthermore, two clinical studies on the rate of recovery for professional athletes using GEF have been carried out in the past five years. The two studies (Stirling et al., 2019; Schwery et al., 2024) demonstrated how well Incrediwear® products work to minimize athletes' time loss. However, the research elucidating the biological action mechanisms of semiconductor or Germanium embedded fabric has been limited, and a systematic evaluation is still lacking (Anderson et al., 2017).

Based on these premises and our previous expertise in the field of hADSCs chondrogenic differentiation (Iorio et al., 2022a), we aimed to evaluate the effect of GEF on hADSCs chondrogenic differentiation and repair by using a validated *in vitro* model of chondrogenesis and evaluating changes in cell morphology, ECM deposition and expression of genetic chondrogenesis markers.

2. Materials and methods

2.1. hADSCs cell culture

Commercially available human adipose derived stem cells (hADSCs, PCS-500–011, ATCC) were routinely cultured in Mesenchymal Stem Cell Basal Medium (PCS-500–030, ATCC), supplemented with Mesenchymal Stem Cell Growth Kit for Adipose-derived MSCs (PCS-500–040, ATCC), as in (Iorio et al., 2022a). Three different hADSCs cell batches were used, meaning that three different cell types were used throughout experiments. hADSCs were cultured at 37°C, 5 % CO2 in air, and expanded as 2D cell cultures. After cell expansion, cells were harvested using Trypsin + EDTA (Life Technologies) and seeded in 6-well plates (Corning Costar) at cell seeding concentration of 0.5 $\times 10^6$ cells per well (Petroni et al., 2020; Iorio et al., 2020). hADSCs were cultured either in Basal medium (Basal) or in chondrogenic Differentiation medium (Diff) (Chondrocyte Differentiation Tool, ATCC) following manufacturer's instructions for

21 days, at 37 °C, 5 % CO2 in air. 2D cell culture medium was routinely refreshed, as in (Iorio et al., 2022a).

2.2. Treatment with Germanium-embedded Incrediwear® functional Cred40 fabric (GEF)

We used GEF, which is embedded with carbonized charcoal and Germanium. Established hADSCs 2D cultures were put under the effect of Incrediwear® Technology by directly covering the multiwells with the Incrediwear® functional Cred40 fabric and keeping them in a humidified incubator at 37°C throughout the treatment duration in order to replicate body heat and maintain cell cultures. Cultures had the final conformation depicted in Fig. 1. 2D cell cultures, under the effect of Incrediwear® functional Cred40 fabric, were kept in either Basal Medium or Chondrogenic Medium for a total of 21 days, following the cell differentiation protocol described in (Iorio et al., 2022a). Cells were examined to monitor cell morphology by means of an inverted microscope (see below). 2D cell cultures medium was routinely refreshed, as in (Iorio et al., 2022a).

2.3. Evaluation of cell morphology

Images were acquired using an EVOS inverted microscope (ThermoFisher Scientific) with 40x magnification (Hasan et al., 2022; Iorio et al., 2022b). Images evaluation was performed by two independent operators, (CD and GB) in order to ensure unbiased accuracy, by applying the following a scoring system: N, non-differentiated; +, weak differentiation; ++, moderate differentiation; +++, strong differentiation.

2.4. Real Time PCR (RT-qPCR)

At the completion of the 21 days culture schedule, cellular RNA extraction was performed with TRIzol Reagent (ThermoFisher Scientific) as in (Duranti et al., 2021; Lottini et al., 2021), following the manufacturer protocol. Total cellular RNA content was analyzed (in both terms of both quality and concentration) using a NanoDrop2000 spectrophotometer (ThermoFisher Scientific), and 1 ug RNA was retrotranscribed using the SuperScript IV Reverse transcriptase (Invitrogen) as in (Iorio et al., 2022a).

According to (Iorio et al., 2022a) and (Duranti et al., 2023), the applied PCR protocol was carried out using a AB7500 Real Time PCR system and software (Applied Biosystems), with a final reaction volume of 25 µL containing 1 µL of cDNA, each reverse and forward primer at a final concentration of 100 µM (the applied gene-specific primer pairs are listed in Table 1) and analyzed using SYBR green chemistry (Qiagen). Samples were initially held at 50 °C for 2 minutes then 95 °C for 10 min and then amplified at 95 °C for 15 s and 60 °C for 1 min for 40 cycles. PCR amplification specificity was routinely checked by heat dissociation curves. Gene ex-pression levels were standardized to GAPDH as an internal housekeeping gene. Quantification was performed by comparative ΔCt method (Duranti et al., 2023). Samples were assayed in triplicate and analysis was performed on three different biological replicates.

2.4.1. Alcian blue staining

At the end of the 21 days culture schedule, Glycosaminoglycans (GAGs) content was assessed by Alcian Blue staining. Cells were fixed for 15 min at room temperature by 4 % Paraformaldehyde (PFA) (Tozzi

Table 1
Primers sequences used in RT-qPCR experiments.

Gene	Forward 5'-3'	Reverse 5'-3'
RUNX2 (Runt-related transcription factor 2)	GGT CAG ATG CAG GCG GCC	TAC GTG TGG TAG CGC GCT
SOX9 (Sex-determining region Y-box 9)	AGA CAG CCC CCT ATC GAC TT	CGG CAG GTA CTG GTC AAA CT
ACAN (Aggrecan)	TAC ACT GGC GAG CAC TGT AAC	CAG TGG CCC TGG TAC TTG TT
COL2A1 (Collagen type-II alpha 1 chain)	GTG AAC CTG GTG TCT CTG GTC	TTT CCA GGT TTT CCA GCT TC
COL10A1 (Collagen type-X alpha 1 chain)	CAC CTT CTG CAC TGC TCA TC	GGC AGC ATA TTC TCA GAT GGA
GAPDH (Normalizer)	ACC CAG AAG ACT GTG GAT GG	TTC TAG ACG GCA GGT CAG GT-

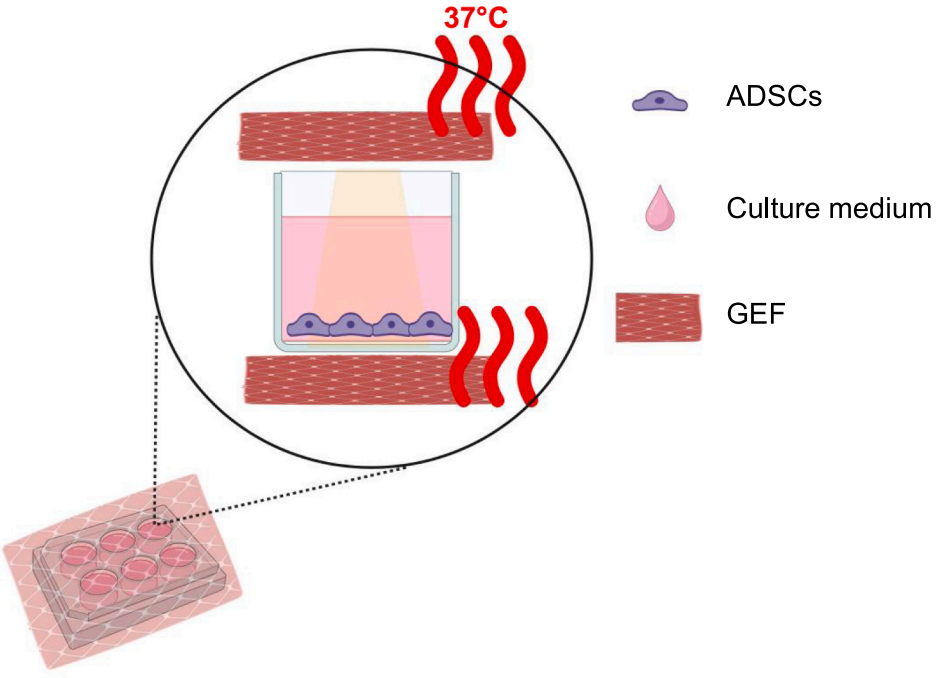

Fig. 1. 2D adipose-derived stem cells cultures conformation and exposure to Incrediwear® Germanium-embedded fabric (GEF). hADSCs= Human Adipose-Derived Stem Cells.

et al., 2020).

Cells were rinsed 3 times with PBS and then one time with 1 % acetic acid. Alcian blue 3 % solution (Bio-Optica) was then applied to cover the wells. Cells were incubated in Alcian blue for 15 min. Cells were then rinsed 3 times with PBS, dried in the hood and imaged.

Staining was quantified as Integrated Optical Density (IntDen) using the Fiji Software (National Institute of Health, NIH). For each sample, five randomly selected 40X whole- stained microscopic fields per condition were chosen and quantified (Kwon et al., 2016, Duranti et al., 2018; Lastraioli et al., 2020).

2.4.2. Sirius red staining

At the end of the 21 days culture schedule, Sirius Red staining was applied to assess collagen content.

Cells were fixed for 15 min at room temperature by 4 % PFA, and then stained with Sirius Red solution (Bio-Optica) for 1 h at room temperature. After washing thrice, the cells were observed and imaged. Staging was quantified as Integrated Optical Density using the Fiji Software (National Institute of Health, NIH). For each sample, five randomly selected 40X whole- stained microscopic fields per condition were chosen and quantified (Duranti et al., 2018; Kwon et al., 2016; Lastraioli et al., 2020; Lastraioli et al., 2016).

2.5. Statistical analysis

Statistical and graphical data analysis was carried out GraphPad Prism V.10. software (GraphPad Software) (Lottini et al., 2023; Sala et al., 2023).

Comparisons between groups were performed by Mann-Whitney U test or One-Way Anova depending on data distribution. For all tests, differences with $p < 0.05$ were considered statistically significant (Chioccioli Altadonna et al., 2022; Manoli et al., 2019).

3. Results

3.1. Set up of the experimental protocol

2D hADSCs cultures, under the effect of Incrediwear® functional

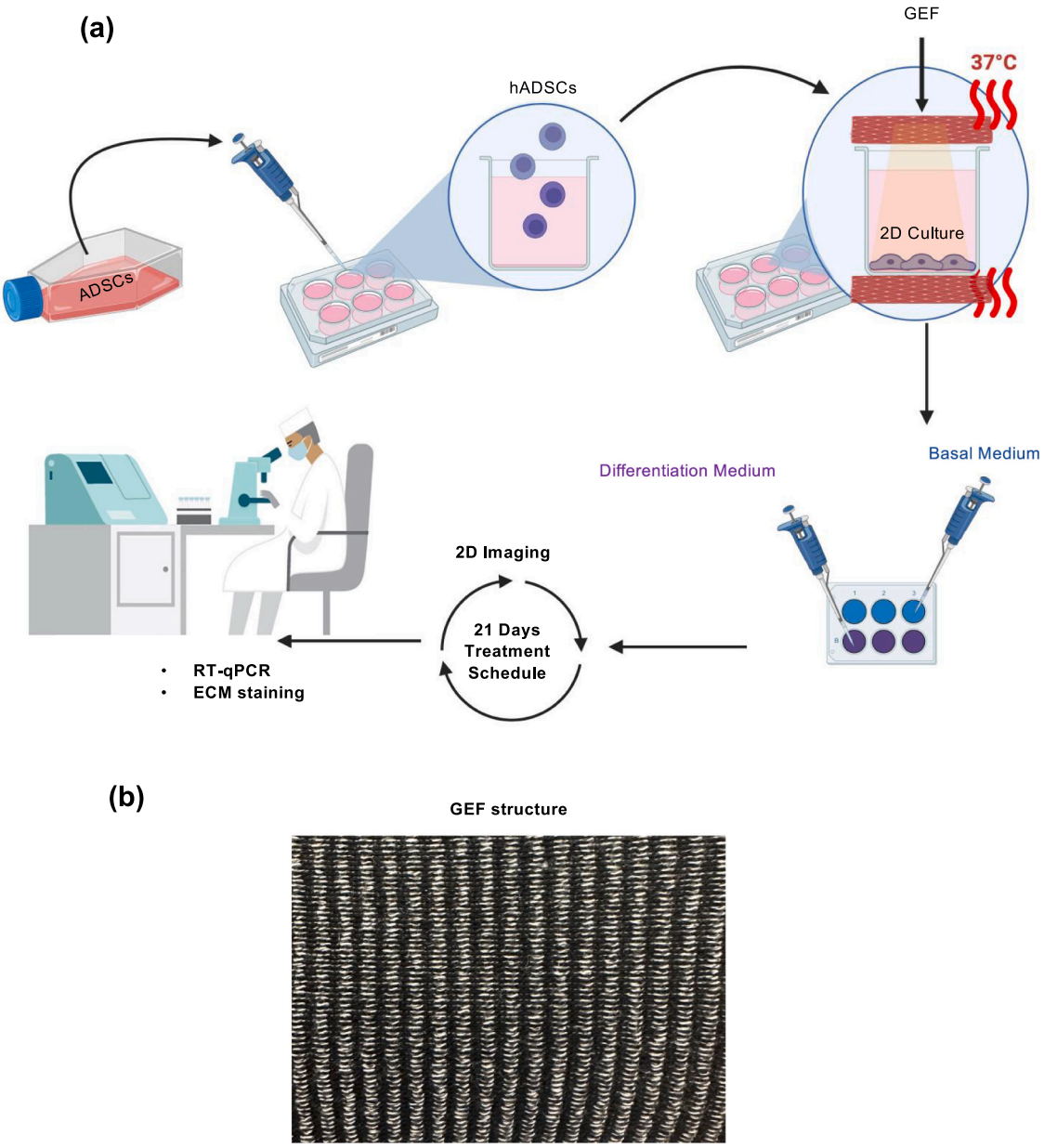

Fig. 2. Experimental Workflow (a). hADSCs= Human Adipose-Derived Stem Cells. Cred40 fabric structure (b).

Cred40 fabric were kept in either Basal Medium or Chondrogenic Medium for a total of 21 days, following the cell differentiation protocol described in detail in our previous work on ADSCs *in vitro* chondrogenic differentiation Fig. 1 (Iorio et al., 2022a). Following the 21 days differentiation period, the expression of the main chondrogenesis markers was assessed by RT-qPCR, considering the key role of the selected genes (namely SOX9, RUNX2, COL2A1, COL10A1 and ACAN) in defining chondrogenesis progression (Iorio et al., 2022a). Control samples grown in absence of the effect of Incrediwear® were included for comparison.

All the experiments were performed on three different batches of hADSCs, and each experiment was carried out in triplicate. The overall study design and workplan is schematically, as well as the structure of the used Cred40 fabric (GEF), are represented in Fig. 2A and B, respectively.

3.2. Effects of GEF on cell morphology

The morphology of hADSCs was examined throughout the 21 days of treatment period. From the images obtained by inversion microscopy at day 21, we can easily observe that cells in control conditions (Basal, meaning basal culture medium) appear different compared to those subjected to Incrediwear® functional Cred40 fabric (Basal + GEF, meaning cells cultured in basal medium under the effect of Incrediwear® technology) treatment when considered in Basal conditions with a more stretched shape and longer protrusions and appeared more interconnected (Fig. 3A).

No drastic morphological differences appeared in the differentiation set when comparing control conditions GEF-treated cultures, but a slight increase in extracellular matrix deposition was visible (Fig. 3A). It is clear how hADSC culture in chondrogenic conditioned medium (Diff) led to the deposition of extracellular matrix (ECM), generating more complex and interconnected structures where single-cell shape starts to disappear. On the contrary, cells cultured in Basal medium (Basal) maintained a fibroblast-like, single-cell appearance during the entire period of culture (Fig. 3A).

Staining of both collagen (Sirius Red) and GAGs (Alcian Blue) following the 21 days experimental schedule confirmed cells differentiation under chondrogenic medium conditions (Diff) when compared to basal medium (Basal) (Fig. 3A). Treatment with Incrediwear® functional Cred40 fabric was able to significantly increase collagen content both under basal and chondrogenic medium conditions, when compared to untreated controls (Figs. 3A and 3B). Incrediwear® functional Cred40 fabric was also able to induce a significant increase in GAGs contents was applied to chondrogenic medium (Diff) culture conditions, if compared to untreated controls (Figs. 3A, and 3B).

3.3. Effects of GEF on the expression of genetic chondrogenic markers

RT-qPCR analysis was performed to assess the effects of chondrogenic differentiation, as well as of GEF treatment, on the following main markers of chondrogenesis: SOX9, RUNX2, COL2A1, COL10A1 and ACAN. As shown in Fig. 4, it was found that SOX9 expression was not significantly changed either during induction of chondrogenic differentiation or GEF treatment. However, COL2A1 (Fig. 2C) and ACAN (Fig. 4E) expression increased when hADSC were induced to chondrogenic differentiation. For both genes, this effect was further significantly potentiated by application of Germanium embedded material in chondrogenic medium conditions when compared to untreated controls. RUNX2 (Fig. 4B) expression did not show significant differences between Basal and Diff conditions. However, its expression was significantly enhanced by Incrediwear® functional Cred40 fabric treatment in chondrogenic medium conditions.

Finally, the expression of COL10A1 (Fig. 4D) increased when hADSC were induced to chondrocyte differentiation. The application of Incrediwear® functional Cred40 fabric was however able to significantly decrease COL10A1 expression in chondrogenic medium conditions when compared to untreated controls.

4. Discussion

In this paper we provide evidence that the treatment of hADSCs with a Germanium-based device, i.e. the Germanium-embedded Incrediwear® functional Cred40 fabric which represent the basis of the Incrediwear® technology, induces the modulation the main genetic chondrogenesis markers. Specifically, results have shown that SOX9 expression did not exhibit statistically significant changes between basal and chondrogenic medium conditions, both with or without GEF treatment. This outcome is consistent with the early-stage induction of the SOX9 marker as well as with our previous study regarding hADSCs *in vitro* chondrogenic differentiation (Iorio et al., 2022a). Importantly, one of the primary early genes responsible for initiating the transition of undifferentiated progenitor cells towards chondrogenic commitment is indeed represented by this transcription factor (Barry et al., 2001). Furthermore, it has been demonstrated that OA cartilage exhibits reduced mRNA and protein expression of the cartilage-specific transcription factor sex-determining region Y box 9 (SOX9) (Kim et al., 2013). On the other hand, ACAN showed substantial modifications in the evaluated treatment conditions. Its expression significantly increased following hADSCs differentiation (Basal vs Diff) and was further enhanced by Incrediwear® functional Cred40 fabric treatment in chondrogenic medium conditions (Diff + GEF) when compared to untreated controls (Diff). This cartilage-specific GAG-containing proteoglycan is known as Aggrecan (ACAN), and it is crucial for maintaining the ECM in articular cartilage. It is regarded as a primary marker of mature chondrogenesis (Liu et al., 2007; Zhong et al., 2016), and its expression is known to decline in OA cartilage. Chondrogenic differentiation transforms mesenchymal stem cells (MSCs) into chondrocytes, crucial for cartilage formation. Key gene expression patterns during this process are vital for developing cartilage repair therapies. Early stage MSCs express transcription factors like SOX9, SOX5, and SOX6, essential for initiating chondrogenesis and activating cartilage-specific extracellular matrix (ECM) components. Intermediate stage genes, such as COL2A1 (type II collagen) and ACAN (aggrecan), are upregulated to form the cartilage matrix, with increased expression of other matrix-related genes like COMP. Late-stage maturation involves genes like COL10A1 (type X collagen) and MMP13, indicating cartilage development and remodeling. GEF-treated cultures under differentiation medium conditions (Diff + GEF) showed a significant increase in the expression of Runt-related transcription factor 2 (RUNX2) marker when compared to untreated controls (Diff). Notably, chondrocyte maturation is positively regulated by RUNX2 (Liu et al., 2007; Enomoto et al., 2000). Under differentiation conditions, cultures treated with GEF (Diff + GEF) showed a considerable decrease in the expression of hypertrophic cartilage-specific Collagen type X (COL10A1) when compared to the relative controls (Diff). Human OA cartilage usually exhibits an increase in collagen type X, α1 (COL10A1), a hallmark of hypertrophic cartilage (Liu et al., 2007; Kunze et al., 2020; Pelttari et al., 2006; Von der Mark et al., 1995). COL2A1 gene expression showed significant increase in cells treated with GEF and cultured in differentiation medium (Diff + GEF) when compared to both its untreated relative controls (Diff) and basal conditions (Basal). Collagen type II, α1 (COL2A1), the typical fibrillar collagen form specifically characterizing hyaline cartilage, is reduced during the progression of human OA (Green et al., 2015; Miosge et al., 2004). COL2A1 showed a significant increase in its expression in cells cultured in chondrogenic medium (Diff) compared to basal medium conditions (Basal).

ECM components staining further confirmed ADSCs differentiation under chondrogenic medium conditions. Furthermore, a significant increase in both GAGs and collagen deposition following GEF treatment under chondrogenic medium conditions was confirmed in hADSCs. Interestingly, the proposed treatment was able to induce a significant increase in collagen deposition by hADSCs also under Basal conditions.

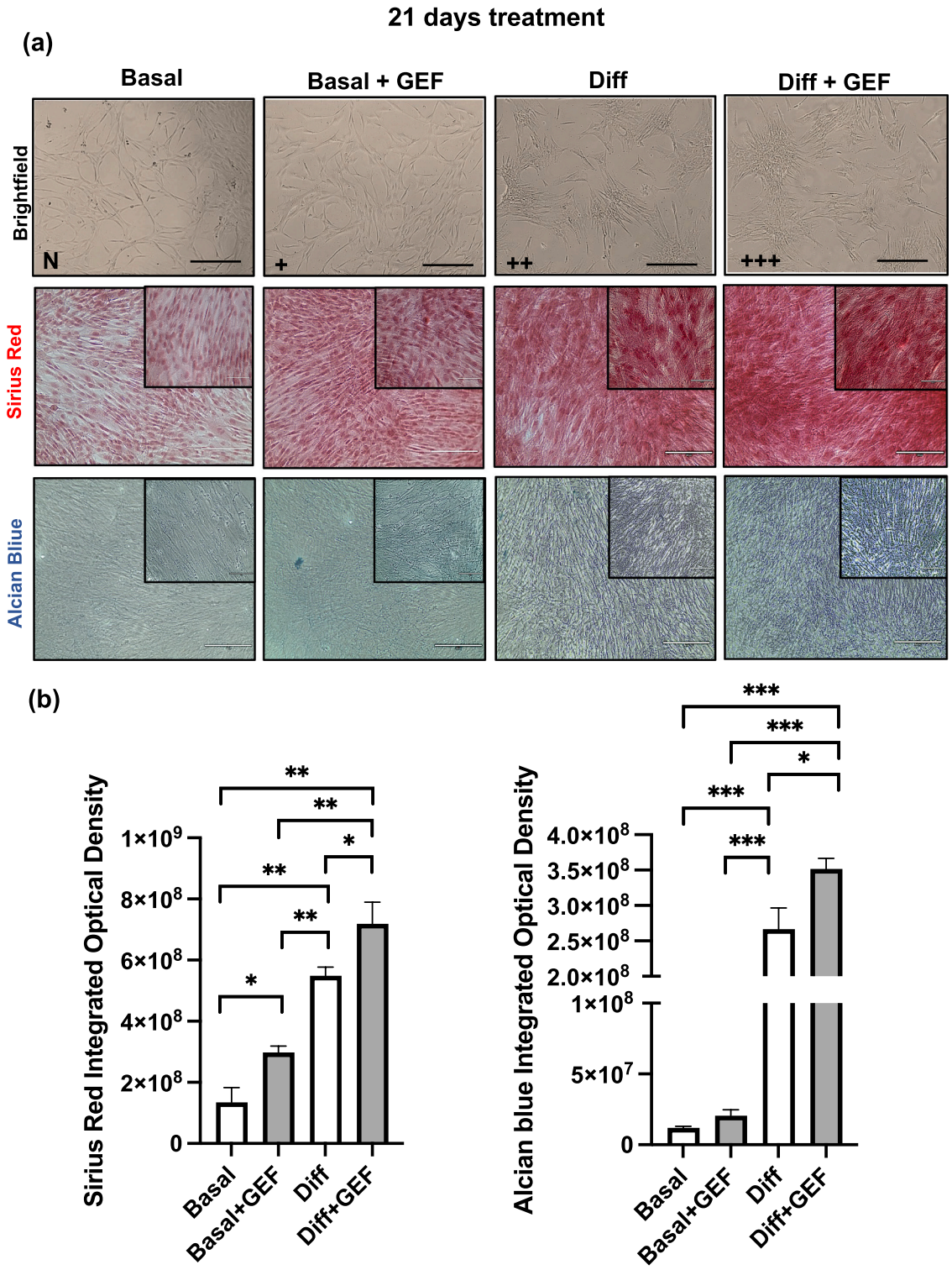

Fig. 3. Morphological examination and ECM staining (Alcian Blue and Sirius Red staining) of hADSCs 2D cultures after 21 days of culture (a). Cells have been examined under different conditions. Basal, which corresponds to cells cultured in basal adipose stem cells medium; Basal+ GEF, corresponding to cells cultured in basal adipose stem cells medium under the influence of Incrediwear® Germanium-embedded fabric; Diff, corresponding to cells cultured in chondrogenic medium; Diff + GEF, which corresponds to cells cultured in chondrogenic medium under the influence of Incrediwear® Germanium-embedded fabric. Black scale bar = 100 μm. White Scale bar = 400 μm. Scoring System: N, non-differentiated; +, weak differentiation; ++, moderate differentiation; +++, strong differentiation. Sirius Red and Alcian Blue staining quantification (b) as Integrated Density (* = $p < 0.05$; ** = $p < 0.025$; *** = $p < 0.01$, One-Way Anova). Experiments are means of three different biological replicates assayed in triplicate and expressed as Mean±SEM. GEF = Incrediwear® Germanium-embedded fabric.

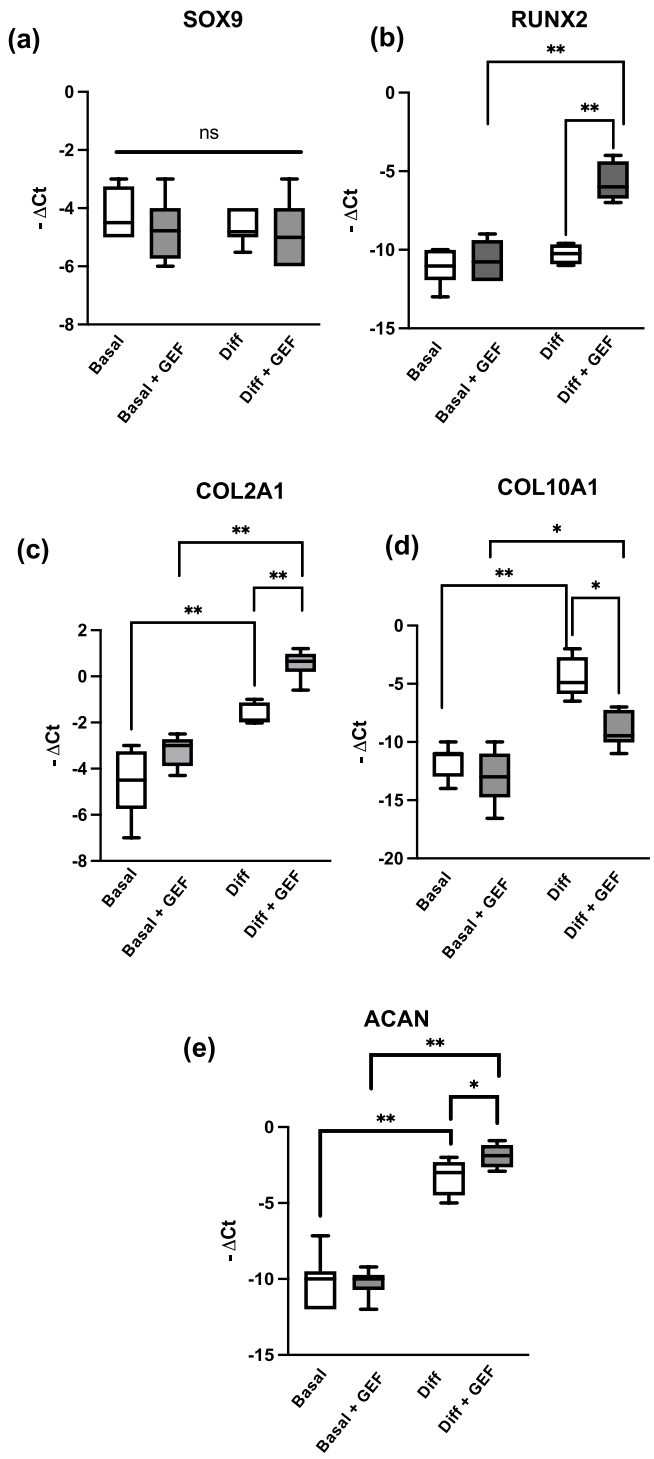

Fig. 4. Expression analysis of chondrogenic differentiation markers in different culture and treatment conditions. Basal, which corresponds to cells cultured in basal medium; Basal + GEF, which corresponds to cells cultured in basal adipose stem cells medium under the influence of Incrediwear®, Germanium-embedded fabric (GEF); Diff, corresponding to cells cultured in chondrogenic differentiation medium; Diff + GEF, corresponding to cells cultured in chondrogenic differentiation medium and under the influence of Germanium-embedded fabric. SOX9 (a), RUNX2 (b), COL2A1 (c), COL10A1 (d) and ACAN (e) expression levels were assessed by RT-qPCR. Experiments are means of three different biological replicates assayed in triplicate. Results are expressed as relative gene expression (-ΔCt). Box and whiskers plots (95 % CI). * = $p < 0.05$; ** = $p < 0.025$; *** = $p < 0.01$ (Mann-Whitney U test). ns= not statistically significant. GEF= Incrediwear® Germanium embedded fabric.

Taken together, this evidence further suggests how hADSCs chondrogenic differentiation might be enhanced by the proposed Germanium-embedded fabric treatment, not only from a genetic point of view, but also from a histological and molecular point of view. The results emerging from ECM staining are also coherent with the modulation of the chondrogenesis markers COL2A1 and ACAN.

The Incrediwear® technology, and particularly the Germanium embedded fabric (GEF) that was used in the present paper, has already been reported to produce medical effects on the clinical setting. Specifically, they were able to provide notable reduction in pain for OA patients (Marino et al., 2019). Furthermore, two clinical investigations on the rate of recovery for professional athletes using GEF have been carried out in the past five years. The two studies demonstrated how GEF can help athletes lose less time due to injury recovery during the course of the sport seasons (Stirling et al., 2019; Schwery et al., 2024). The Incrediwear® technology is intended to facilitate improved joint function, a quicker recovery from surgery and injuries, and a reduction in pain, discomfort and edema. GEF has been shown to improve circulation to lessen swelling and inflammation, ease pain, regain movement, and hasten the healing process following surgery or physical activity (Stirling et al., 2019; Schwery et al., 2024; Lee et al., 2018). Unlike compression products, Incrediwear® products are non-compressive and only rely on activation of the semi-conductors embedded in the fabric blend to increase blood flow through the release of infrared electromagnetic waves and negative ions. The technology incorporates semiconductor elements into a fabric blend that releases infrared waves and negative ions when directly stimulated by the wearer's body heat (Justice and Jacob, 2023; Lee et al., 2018). The infrared waves activate cellular vibrations that increase blood flow and speed and the negative ions reduce oxidative stress and support cellular function. Increased circulation facilitates the healing process by delivering more oxygen and nutrients to the targeted area, optimizing the organism's natural healing process, ultimately enhancing and accelerating recovery.

The effect on chondrogenic differentiation shown in the present paper should be included in the biological effects exerted by the Incrediwear® technology, and more broadly to the effects of Germanium-embedded materials and textiles. Whether this effect can be related to the modulation of ionic currents (Santini et al., 2023), which in turn modulate cellular processes (Becchetti et al., 2022; Arcangeli et al., 2023; Duranti and Arcangeli, 2019; Iorio et al., 2019; Arcangeli et al., 2024; Levin, 2021), is still a point of debate and merits further studies.

It is however already clear that understanding how to alter the bioelectrical and physiochemical signals that regulate cell differentiation might provide us a potent new toolkit in the biomedical settings (Dimitri et al., 2022).

Membrane proteins are indeed susceptible to the electric fields produced at the plasma membrane level due to specific features (Becchetti et al., 2022). Reevaluating cell signaling in the context of protein interactions with membrane electrostatic profiles indicates that this essential component of cell biology involves a significant electrical component.

Moreover, the processing of morphogenetic information by bioelectrical networks is able to regulate gene expression, allowing cells to collectively choose their large-scale development and morphology. Recent developments in the modeling and study of bioelectric circuits and channelopathies have provided new insights into how the cellular tissue components operate to provide and maintain developmental structural order at the organ level (Kwon et al., 2016). These developments offer a promising view of bioelectric signaling-based therapies as valuable tools in wound healing, regenerative medicine, and synthetic morphology. This could be potentially accomplished by identifying and activating specific modular patterns utilizing unique stimuli, which are transient bioelectric signals produced by modulating the conformational state of existing channels. The induction of novel bioelectric states during tissue regeneration has been already

demonstrated *in vivo* and *in vitro* and has been directly linked to modifications in cells morphology and function (Kwon et al., 2016).

It is well known that electrical stimulation (ES) directs the growth and regeneration of numerous tissues (Enomoto et al., 2000; Dimitri et al., 2022). For what concerns the setting of our study, ES has been also demonstrated to be able to specifically induce *in vitro* chondrogenesis of mesenchymal stem cells (even in the absence of exogenous growth factors) by stimulating both bone morphogenetic proteins (BMPs) and transforming growth factors (specifically TGF-β) signaling (Wohlrab et al., 2001).

Moreover, several ion channels (in particular, the ones related to calcium signaling) have been already identified in human chondrocyte and their regulation has been demonstrated to be able to modulate the function of this specific cell type (Jahr et al., 2015).

In this context, numerous biomechanical, bioelectric, and physicochemical strategies acting on the state of cellular ions have already been shown to affect chondrocyte metabolism and could be therefore applied to regenerative medicine techniques targeting different types of cartilage pathologies ranging from traumatic local damage (in younger patients and athletes) to OA (in the elderly). The most promising strategies aiming to improve cell-based chondral defect repair are indeed currently represented by therapeutic devices based on electrical, magnetic, and mechanical tissue stimulation (Oliveira et al., 2021).

It is also worth mentioning that this evidence regarding ion channel function in cartilage regeneration, coexist in Incrediwear® technology with the well-known effects of photo biomodulation through treatment with infrared waves. The latest represent a promising approach in treatmet of cartilage disorders due to its ability to reduce inflammation, OA progression and extracellular matrix (ECM) degradation both *in vitro* and *in vivo* (Oliveira et al., 2021).

Overall, optimizing conservative treatments represents a crucial factor in postponing the need for surgical joint intervention as OA and cartilage traumatic lesions are still major sources of morbidity in the worldwide population. Our study highlights the role of Incrediwear® as a promising non-invasive therapeutic approach for cartilage repair in the regenerative medicine field. Despite the limitations in the study and the need to extend it to a wider level, the obtained results warrant future investigations to elucidate the exact molecular and cellular mechanisms underlying Incrediwear® technology effects on hADSCs chondrogenic differentiation and to further explore its promising role in osteoarthritis on a biological level.

5. Conclusions

Our results indicate that the treatment of hADSCs with Incrediwear® functional Cred40 fabric is able to induce a significant modulation in the expression levels of some of the main genetic markers of chondrogenesis (namely SOX9, RUNX2, COL2A1, COL10A1 and ACAN) specifically inducing healthy cartilage markers up-regulation and pathogenic cartilage markers down-regulation, as well as modifications in cell morphology and ECM deposition indicative of chondrogenic differentiation *in vitro*.

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CRediT authorship contribution statement

Rossella Colasurdo: Investigation. **Valentina Devescovi:** Investigation. **Annarosa Arcangeli:** Writing – review & editing, Writing – original draft, Validation, Supervision, Funding acquisition, Data curation, Conceptualization. **Claudia Duranti:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Giacomo Bagni:** Writing – original draft, Methodology, Investigation. **Jessica Iorio:** Supervision, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Not applicable.

Informed Consent Statement

Not applicable.

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