Review

Over-Production of Therapeutic Growth Factors for Cartilage Regeneration by Protein Production Platforms and Protein Packaging Cell Lines: A Narrative Review of the Current State-of-the-Art

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Abstract: This article focuses on the current state-of-the-art in the area of cellular and molecular biotechnology for over-production of clinically relevant therapeutic growth factors and how the technology can be used for the treatment of osteoarthritis (OA). Transfected and irradiated protein packaging cell lines may be used as "cellular factories" for large-scale production of therapeutic proteins and pro-anabolic growth factors, particularly in the context of cartilage matrix regeneration. We discuss the potential for new innovations in regenerative medicine for degenerative diseases of synovial joints using mammalian protein production platforms, specifically protein packaging cell lines, for over-producing growth factors for cartilage tissue regeneration and give recent examples. Mammalian protein production platforms that incorporate protein packaging cell lines are superior to bacterial expression systems and are likely to have a significant impact on the development of new biological therapies for treating focal cartilage defects and more generally for the treatment of degenerative joint diseases such as OA.

Keywords: osteoarthritis; articular cartilage; degeneration; regeneration; therapeutic protein; growth factor; protein production platform; protein packaging cell line; transforming growth factor β (TGF-β); GP2-293 cells

1. Introduction

Growth factors (GFs) are evolutionary-conserved proteins that enhance the growth, proliferation, migration, survival, and differentiation of cells. They have the capacity to regulate the specialized function and phenotype of cells. GFs can stimulate proliferation in many cell types but there are a number of cell types, including mature neurons, that are postmitotic and cannot re-enter the cell cycle. GFs and their receptors can be grouped into 'families,' based upon shared features of amino acid sequence, and into 'superfamilies,' based upon shared structural folds. Many GF families display significant evolutionary conservation in sequence; for example, homologs of the fibroblast growth factor (FGF), epidermal growth factor (EGF), and transforming growth factor β (TGF-β) families can be found across the animal kingdom. However, higher vertebrates have larger GF families than invertebrates. For example, there are currently 22 members of the FGF gene family in the human genome, but only one in *Drosophila melanogaster* and *Caenorhabditis elegans* [1].

GFs are relatively small and stable polypeptides that are secreted by cells in the body. They are present in the extracellular matrix (ECM) as secreted or membrane-bound proteins. GFs can regulate a variety of cellular behaviors including growth, migration, differentiation, apoptosis, and survival, in both positive and negative manners. GFs produced by stem cells have an array of functions during development, and play important roles in the maintenance of tissue homeostasis and wound healing in the adult skin [2] and in other connective tissues such as articular cartilage [3]. IGF-I and basic FGF have been shown to augment articular cartilage repair *in vivo* [3].

The transforming growth factor-β (TGF-β) superfamily is encoded by 33 genes and includes TGF-β, bone morphogenetic proteins (BMPs) and activins. Recent evidence suggests that TGFs, BMPs and activins have important roles in regulating immune responses in the context of infection, inflammation and cancer [4]. TGF-β is the prototype member of the TGF-β family of growth and differentiation factors. It is the best-studied factor among the TGF-β family proteins, with its diversity of roles in the control of cell proliferation and differentiation, wound healing and immunoregulation, and key roles in pathology, for example, in skeletal diseases, fibrosis, and cancer [5]. In the synovial joint TGF-β is a pleiotropic cytokine that is important for regulation of tissue homeostasis and disease [6]. Its action on articular cartilage is particularly dependent upon the context in which it acts, eliciting seemingly opposite effects under different experimental conditions; it may counteract pathological changes in a young healthy joint, altering its signalling during ageing and may be an active participant in pathology in OA joints [6]. In the context of the present review, promotion of TGF-β activity in articular cartilage and inhibition of TGF-β activity in subchondral bone may provide new avenues of treatment for OA [7].

GFs can be produced by genetic engineering in the research laboratory setting, and exploited using biotechnology platforms for further applications and used in various clinical, therapeutic and regenerative contexts [8,9]. In this paper we focus on GFs for cartilage regeneration. We review the current state-of-the-art in the area of cellular and molecular biotechnology for over-production of clinically relevant therapeutic proteins. We propose that transfected and irradiated protein packaging cell lines may be used as "cellular factories" for over-production of therapeutic proteins and pro-anabolic growth factors, particularly in the context of regenerative medicine for treating focal cartilage defects and degenerative diseases of the joints, such as osteoarthritis (OA). These tools may be used to produce cocktails and combinations of GFs for intra-articular injection or injected in live form directly into synovial joints, combined with allogeneic primary cells (i.e. chondrocytes) or stem cells. It is also conceivable to combine different clones of genetically engineered live cells for intra-articular injections.

2. Osteoarthritis (OA)

OA is a progressive and degenerative condition that causes load-bearing synovial joints to become painful and stiff [10]. According to the World Health Organization (WHO), OA is the most common type of arthritis affecting millions of people worldwide [1]. Although the main symptoms of OA are joint pain and stiffness, some patients also experience swelling (effusion), tenderness and a grating or crackling sound when moving the affected joint. OA can occur in any joint, but the disorder most commonly affects joints in knees, hips, hands and the spine. While OA is related to ageing, it is, along with many other forms of chronic disease, also associated with a variety of both modifiable and non- modifiable risk factors, including: obesity [11], sedentarism [12] and lack of physical exercise [13], genetic predisposition, bone deformities or reduced bone mineral density, occupational injuries, repeated stress and trauma in sport, certain metabolic and endocrine diseases and, importantly, the female gender, especially after menopause. . In terms of disease initiation, it is thought that there is a long and asymptomatic "molecular phase", which is followed many years later by radiographic changes and the appearance of symptoms [14]. In addition to aging, obesity, gender and genetics, inciting risk factors for osteoarthritis may include previous joint trauma or repetitive injuries or the presence of metabolic and endocrine disease [15]. There are biomechanical [16], inflammatory [17]

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[1] https://www.who.int/medicines/areas/priority_medicines/Ch6_12Osteo.pdf

95 and metabolic [18] factors that have been shown to play key roles in the initiation and progression of
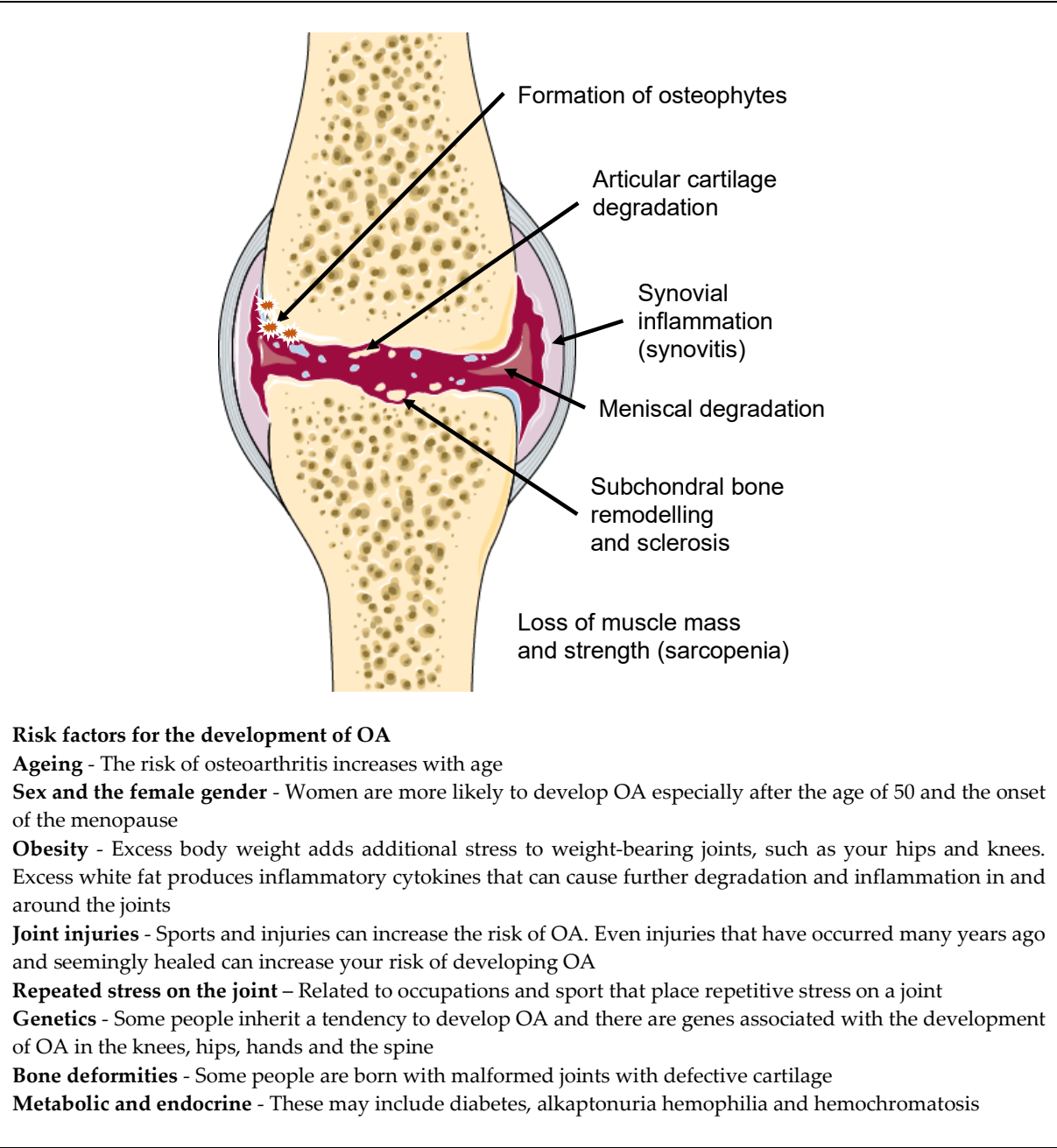96 the disease. The major risk factors for OA are summarized in Figure 1.
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98

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100
101 **Risk factors for the development of OA**
102 **Ageing** - The risk of osteoarthritis increases with age
103 **Sex and the female gender** - Women are more likely to develop OA especially after the age of 50 and the onset
104 of the menopause
105 **Obesity** - Excess body weight adds additional stress to weight-bearing joints, such as your hips and knees.
106 Excess white fat produces inflammatory cytokines that can cause further degradation and inflammation in and
107 around the joints
108 **Joint injuries** - Sports and injuries can increase the risk of OA. Even injuries that have occurred many years ago
109 and seemingly healed can increase your risk of developing OA
110 **Repeated stress on the joint** – Related to occupations and sport that place repetitive stress on a joint
111 **Genetics** - Some people inherit a tendency to develop OA and there are genes associated with the development
112 of OA in the knees, hips, hands and the spine
113 **Bone deformities** - Some people are born with malformed joints with defective cartilage
114 **Metabolic and endocrine** - These may include diabetes, alkaptonuria hemophilia and hemochromatosis
115

116 **Figure 1.** Major structural changes that occur in the joint and risk factors for the development of
117 osteoarthritis (OA).

118 **3. Growth Factors and OA**

119   GFs are important for the synthesis and maintenance of articular cartilage. The use of bioactive
120 GFs is under consideration as a potential therapy to enhance healing of chondral injuries and
121 modify the arthritic disease process [19]. The most important growth factors that are relevant to
122 cartilage homeostasis are summarized in Figure 2.

123
124 **Platelet-derived growth factor (PDGF)** regulates the secretion and synthesis of collagen;
125 **Epidermal growth factor (EGF)** stimulates cellular proliferation, endothelial chemotaxis and angiogenesis;
126 **Vascular endothelial growth factor (VEGF)** increases angiogenesis and vascular permeability;

127 | **Transforming growth factor-β (TGF-β)** stimulates the proliferation of undifferentiated mesenchymal stromal
128 | cells (MSCs), stimulates chemotaxis of endothelial cells and angiogenesis;
129 | **Basic fibroblast growth factor (bFGF)** promotes the growth and differentiation of chondrocytes and
130 | osteoblasts stimulates mitogenesis of mesenchymal cells, chondrocytes and osteoblasts;
131 | **Connective tissue growth factor (CTGF)** contributes to joint homeostasis and OA severity by controlling the
132 | matrix sequestration and activation of latent TGF-β
133 |

134 **Figure 2.** Major growth factors involved in cartilage homeostasis and the development of
135 osteoarthritis (OA).

136 **3. Mammalian Protein Production Platforms**

137 Mammalian cell lines derived from human, mouse and hamster tissues (Figure 3) are excellent
138 hosts for the production of complex recombinant proteins that require extensive folding, the
139 assembly of multiple subunits and posttranslational modifications including N-glycosylation and
140 many others. Over the past 20 years the industrial demand for recombinant therapeutic proteins has
141 significantly increased. Mammalian protein production platforms and protein packaging cell lines
142 have been extensively used to produce recombinant proteins [20]. For these reasons, such mammalian
143 cells are widely used by the pharmaceutical and biotechnology industries for the large-scale
144 production of recombinant proteins [21], which may include diagnostic and therapeutic proteins,
145 peptides, antibodies and antibody fragments. Different mammalian cell platforms are used according
146 to the quantity and quality of the desired product required and the platforms can be scaled according
147 to yield requirements [22]. The most commonly used mammalian cell lines found in the research and
148 industrial therapeutic protein production settings are Chinese hamster ovary cells (CHO) [23] and
149 human embryonic kidney 293 cells (HEK-293) [24]. Some of these expression systems are transient,
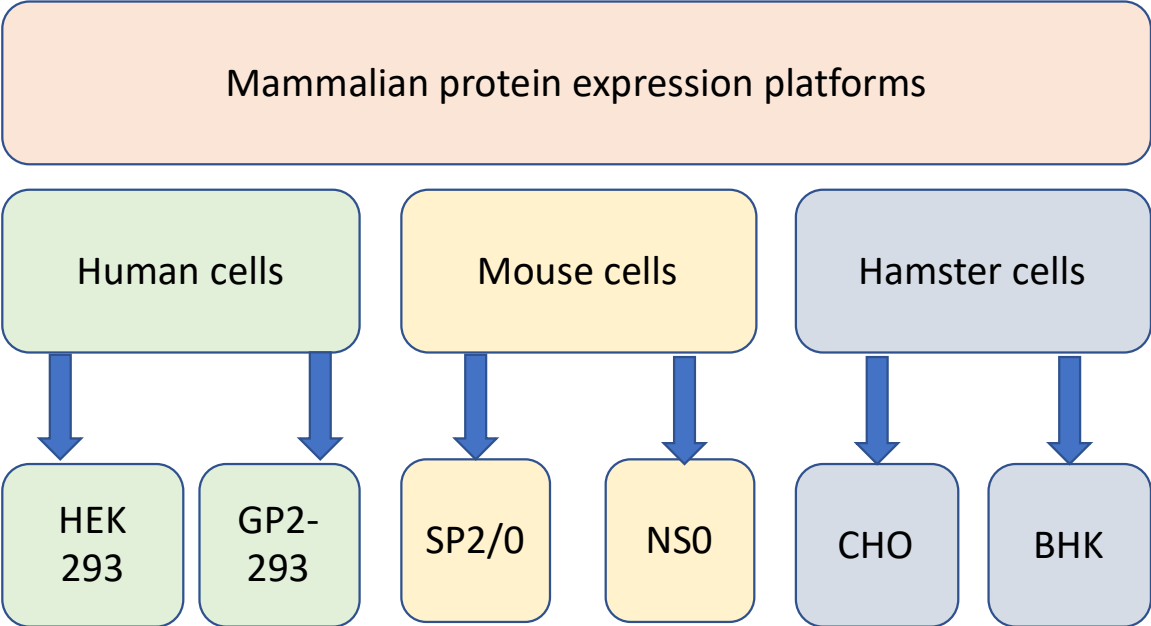150 whereas others are stable.
151

152
153 **Figure 3.** Mammalian protein production platforms using human, mouse and hamster cell lines.
154

155 **2.1. Transient Expression Systems for Recombinant Proteins**
156 Transient expression platforms for the production of mammalian proteins often use human
157 HEK-293 or hamster CHO cells [25,26]. HEK-293 is a cell line derived from human embryonic kidney
158 cells grown in tissue culture and is widely used in cell biology and biotechnology [27]. The cells were
159 derived from human embryonic kidney but the phenotypic origin of the cells is thought to be

160 neuronal [2]. CHO cells are an epithelial cell line derived from the ovary of the Chinese hamster [23].
161 CHO cells are used in diverse biological and medical research applications [28]. CHO cells are also
162 used commercially for the production of clinically relevant therapeutic proteins. Glycoengineering
163 of CHO cells is a thriving area of research focusing on enhanced glycosylation capabilities for highly
164 glycosylated proteins [28]. However, many of these expression systems are "transient", meaning that
165 they can only be manipulated acutely to drive over-expression of a desired protein for a given period
166 of time. Therefore, they enable the rapid production of milligram to gram quantities of protein on a
167 flexible scale within a few weeks. Detailed description of the workflow involved in the development
168 of such tools is beyond the scope of this review article but in essence it involves the transfer of the
169 gene of interest into an expression vector, over production and purification of the recombinant
170 protein, quantification of the protein, determination of protein integrity, and specific functional
171 studies, if necessary [29]. However, there are industrial applications that require sustained and stable
172 production, packaging and secretion of proteins.
173
174 **2.2. Stable CHO Cell Line Development**
175   It is possible to develop stable cell lines that continuously produce a target protein of therapeutic
176 value. Establishing highly productive clonal cell lines with constant productivity over 2–3 months of
177 continuous culture is extremely challenging, but possible and has already been achieved [30].
178 Transfected CHO DG44 cells are often used as a model for this purpose. They are cultivated under
179 several rounds of methotrexate selection [31]. Monoclonal CHO-derived cell lines may be generated
180 by subcloning pools of the most productive cells, and clone stability is confirmed. For example, stable
181 cell lines have been designed to produce recombinant monoclonal anti-tumor necrosis factor α (TNF-
182 α) antibody [32]. Once the clone has been established, the cells may be frozen and archived or shipped
183 and distributed to other locations and banked at multiple sites.
184
185 **2.3. Mammalian Protein Production Platforms for Large Scale Production of Therapeutic Proteins**
186   Mammalian protein production platforms are currently being employed as indispensable
187 "cellular factories" for the   large-scale production of humanized therapeutic antibodies and proteins
188 [33]. Mammalian cell expression systems can now support the large-scale production of proteins,
189 especially of those of clinical relevance and human origin [34]. Over the last few decades these
190 platforms have gradually evolved and found new applications in biology and medicine. Protein
191 production platforms have had a profound impact in many areas of basic and applied research, and
192 an increasing number of biological drugs are now recombinant mammalian proteins made using
193 these tools [35]. Recombinant proteins and a vast array of monoclonal antibodies are now produced
194 in mammalian cell lines instead of bacterial expression systems (i.e. *Escherichia coli*) to ensure that
195 proper protein folding and post-translational modifications, which are essential for full biological
196 activity, are properly introduced in the most appropriate eukaryotic and "mammalian" context.
197 Mammalian cell expression systems are the dominant tools for producing complex biotherapeutic
198 proteins [36]. As already mentioned, various mammalian expression systems are also being used for
199 protein and glycoprotein production and recent cellular engineering strategies have been developed
200 to increase glycoprotein productivity [37], a crucially important feature that bacterial expression
201 systems do not possess.
202
203 **4. Gene Therapy for OA**
204   Most of the early experimental progress in the area of gene therapy for OA was made with gene
205 transfer to the synovium, a tissue that is particularly amenable to genetic modification by a variety
206 of gene vectors, using both *in vivo* and *ex vivo* protocols [38]. However, despite the importance of
207 targeting inflammatory pathways in the synovium to treat the synovitis associated with OA, the main
208 focus of regenerative therapy joints has been cartilage regeneration. The focus so far has been upon
209 the transfer of genes whose products enhance synthesis of the cartilage ECM, or inhibit its

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[2] https://www.hek293.com/

210 breakdown, although there is certainly room for finding novel and alternative targets, which may
211 include cytoprotective factors and molecular chaperones. Recombinant adenovirus associated
212 vectors may be used to directly transfer candidate gene sequences in human articular chondrocytes
213 *in situ*, providing a potent tool to modulate the structure of OA cartilage. Although very few
214 preclinical animal studies in OA models have been performed thus far, equine models of OA have
215 been proposed for proof of concept studies in translational models [39]. Several gene therapy clinical
216 trials have also been carried out in patients with end-stage knee OA based on the intraarticular
217 injection of human juvenile allogeneic chondrocytes overexpressing a cDNA encoding TGF-β1 via
218 retroviral vectors [40].
219   Unfortunately, it has not been possible to build a convincing clinical case for targeting
220 interleukin-1 (IL-1β) as a key mediator of cartilage loss in OA, as the clinical trials conducted by
221 AbbVie for targeting IL-1β have produced generally disappointing results. Nevertheless, the
222 therapeutic effects of IL-1β receptor antagonist (IL-1Ra) gene transfer have been confirmed in three
223 different experimental models of OA [38] and it is likely that targeting IL-1β may still be a viable
224 solution for targeting the more inflammatory phenotypes of human OA [41]. Gene therapy may be
225 combined with cell therapy for developing innovative new treatments for OA.

5. Cell Therapy for OA

228   A detailed discussion of cell and stem cell therapy for OA is beyond the scope of this review.
229 The readers are referred to a series of excellent research and review articles that cover this topic [42,43]
230 including several comprehensive reviews from our own group [44] that discuss the potential for
231 using primary chondrocytes [45] adipose, bone marrow and synovial mesenchymal stromal cells
232 (MSCs) [46–48], menstrual blood-derived stem cells [49] and induced pluripotent stem cells (iPSCs)
233 [50]. The most important point to make is that MSCs derived from patients with advanced OA exhibit
234 attenuated chondrogenic activity, suggesting that these cells may be poor candidates for cell-based
235 therapies for OA [51].

6. Therapeutic Growth Factors for the Treatment of OA

238   A number of GFs have been proposed as novel biological agents for cartilage regeneration [52].
239 GFs represent a broad range of biologically active agents that are capable of activating and
240 stimulating the growth and repair of damaged tissues as well as protecting cells from premature
241 death. They therefore offer a very promising avenue for both treatment and further study, especially
242 in the context of OA. As previously stated, cartilage degradation and subsequent OA is more
243 common in people aged over 50 years, but people of any age have a significantly increased risk of
244 cartilage and joint damage that may lead to post-traumatic osteoarthritis after sports or other joint
245 injuries. GF treatments offer potential benefits to prevent OA (especially at the earliest stages of
246 disease pathogenesis) later in life, as well as being an immediate consideration after sports injury,
247 when the prevention of further damage is a priority.

7. GP2-293 Protein Packaging Cells in Invossa

250   Invossa, developed by Kolon is a revolutionary cell and gene therapy for the treatment of knee
251 OA. Invossa is undoubtedly the current state-of-the-art cell and gene therapy platform for the
252 treatment of OA. In this product transfected and irradiated protein packaging cell lines are used as
253 "cellular factories" for over-production of high quantities of therapeutic TGF-β1. Invossa is actually
254 a unique combination of cell and gene therapy targeting knee OA through a single intra-articular
255 injection of joint-derived chondrocytes, irradiated GP2-293 cells (derived from HEK293 cells and,
256 most importantly, the biological GFs that they acutely overproduce to possibly promote anabolic
257 repair and regeneration in the diseased joint" as a future possibility in the treatment for OA. The
258 general concept for Invossa is presented in Figures 4 and 5.

Primary chondrocytes from allogeneic donor

+

Irradiated GP-293 cells transduced with retroviral vector over-expressing TGF-β1

Focal Cartilage Defect
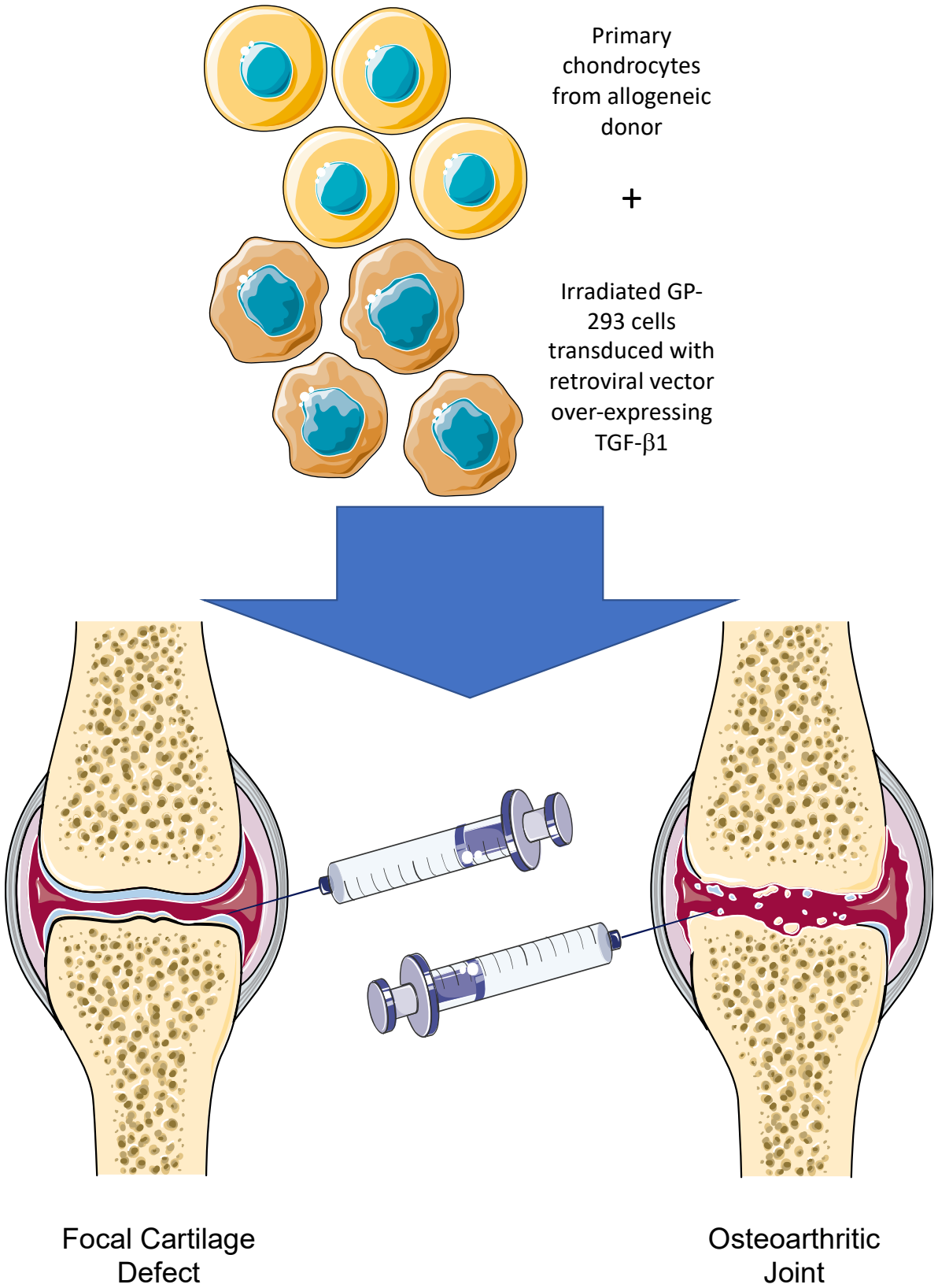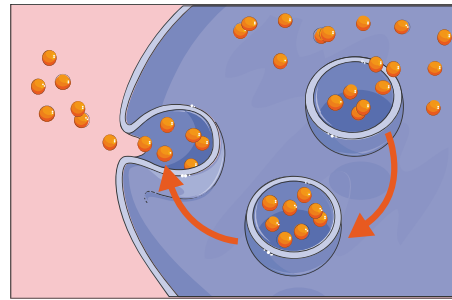
Osteoarthritic Joint

Figure 4. The intra-articular injection concept for Invossa, a novel cell and gene therapy targeting knee OA through a single intra-articular injection of joint-derived primary chondrocytes, irradiated GP2-293 that produce TGF-b1, the biological growth factor that promotes anabolic repair and regeneration.

Production and secretion of therapeutic
GFs by protein production platforms
and protein packaging cell lines such as GP2-293
cells
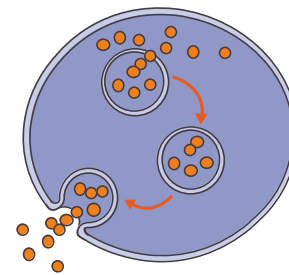
Release of extracellular vesicles
containing recombinant therapeutic
GFs

267
268
269 **Figure 5.** Production and secretion of GFs by GP2-293 cells in Invossa.
270

271   The human GP2-293 cell line is one of the key components of Invossa. These cells carry out the
272 vital function of over-producing the crucially important TGF-β1. The GP2-293 cells have been used
273 throughout the whole developmental process from the first production of the Master Cell Bank
274 (MCB) to the next step, which is the development of the working cell bank and the final product
275 formulation. As mentioned earlier, GP2-293 is a HEK 293-based retroviral packaging cell line used
276 for large-scale protein production and packaging. It is a cellular platform for over-production of
277 therapeutically relevant human proteins. This is the first time that such a human protein production
278 platform has been employed in the context of OA treatment and cartilage regeneration. The GP2-293
279 cells are transformed with adenovirus type 5 DNA and engineered to express the MoMuLV Gag and
280 Pol proteins. Effectively these cells are a protein producing tool and "cellular factory". Native patient
281 derived chondrocytes simply do not have the capacity to over-produce TGF-β1 in sufficiently high
282 quantities for effective cellular therapy and regenerative applications. Transduced and irradiated
283 GP2-293 cells may be transformed cells but since they have lost their capacity for proliferation, they
284 cannot proliferate. Therefore, the GP2-293 cells in Invossa cannot survive and proliferate in the joint.
285 These cells will simply carry out their transient function as radiation inactivated transfection models,
286 protein packaging tools and "cellular factories" for over-production of therapeutic TGF-β1.
287 Therefore, the cells cannot survive for more than a very short period after being injected into the joint.
288 The Korean Food and Drug Administration and the Ministry of Health in South Korea have stated
289 they are not concerned about the safety of Invossa, noting that cells no longer survive 44 days after
290 administration. Furthermore, no drug-related side effects were identified from those subject to
291 clinical trials[3]. After the cells carry out their TGF-β1 production duties, they will die and their remains
292 will be cleared by joint resident inflammatory macrophages through the process of phagocytosis
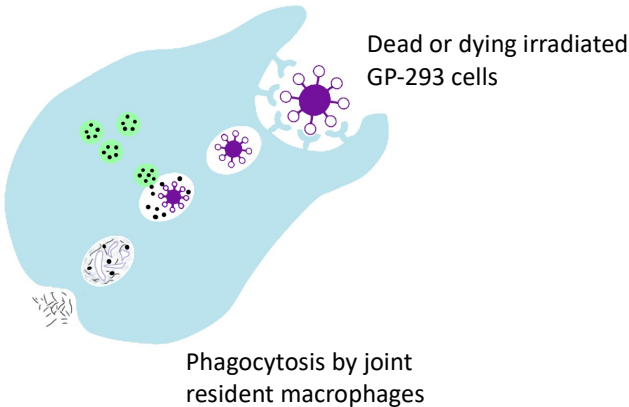293 (Figure 6).

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[3] http://www.businesskorea.co.kr/news/articleView.html?idxno=32318

Dead or dying irradiated GP-293 cells

Phagocytosis by joint resident macrophages

294
295 **Figure 6.** Phagocytosis and destruction of dead GP2-293 and their cellular debris by joint resident
296 macrophages.
297
298   The scientific basis for the use of mammalian cell transfection models is clear in the development
299 of Invossa. There is a well-established literature on the use of HEK-293 cells and their GP2-293
300 derivatives as transfection and cell culture models for protein production. The efficacy and safety of
301 HEK-293 cells and their GP2-293 derivatives in regenerative medicine has not been extensively
302 reviewed but the prospects for future use of transfection tools in regenerative medicine and cell
303 therapy is very positive, especially since native and untransformed cells do not have the appropriate
304 regenerative capacity.
305

306 **8. Conclusions**

307   Therapeutic strategies that combine cell and gene therapy have significant potential for clinical
308 development. However, cell and gene therapy rely on protein production platforms and protein
309 packaging cell lines for over-production of the desired therapeutic proteins. Most of the emerging
310 biological agents in the current drug development pipelines are produced using mammalian protein
311 production platforms. Protein production tools are essential for large-scale production of therapeutic
312 proteins and growth factors. These tools may be used to generate functional native and mutant
313 proteins with appropriate folding, assembly and posttranslational modifications. In most cases these
314 platforms have been used to produce therapeutic proteins that are then purified, characterized and
315 incorporated into products and derivatives. However, there is potential for using protein packaging
316 cells in new therapies that include live cells. The concept for Invossa has been reviewed as the current
317 state of the art in this area. Further developments in this area may include combinations of cell clones
318 that over-produce several growth factors and cell and gene therapies that may be used to target other
319 joint tissues and the intervertebral disc in the spine. The realization that many primary, aged and
320 senescent chondrocytes and MSCs possess feeble regenerative properties means that future
321 regenerative medicine and tissue engineering strategies for the joints and the spine could use primary
322 allogeneic cells or stem cells combined with mammalian protein production platforms to drive the
323 production of therapeutic proteins and pro-anabolic growth factors. Advances in the field of
324 molecular and cellular biotechnology are likely to have a positive impact on tissue engineering and
325 regenerative treatments for the musculoskeletal system.

326 **Author Contributions:** Conceptualization, A.M and P.M-V. writing—original draft preparation, A.M and P.M-
327 V.; writing—review and editing, A.M and P.M-V.

342 **Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the
343 study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to
344 publish the results.

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