Qkine

Application note

Species-specific and optimized growth factors for the production of bovine and porcine cultivated meat and fat

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Cellular agriculture has the potential to transform food production and impact the health and well-being of future generations. Cultivated meat, fish, fat and dairy produced by culturing animal cells has immense promise but there are many challenges that must be overcome to bring these technologies to market and achieve cost parity.

The cell culture media used to grow these cells is an intense area of R&D. Bioactive recombinant proteins, especially growth factors and cytokines, are critical components in most cultivated meat cell culture media, except for some chicken and fish media. Growth factor proteins are highly potent and provide a carefully orchestrated set of biochemical signals to control cell growth and fate.

Immediate priorities for cultivated meat and fat culture media optimization are:

Cell culture media protein component optimization to support efficient intensified cell expansion, and where required maturation, in bioreactor conditions

Replacing animal-derived components, including fetal bovine serum (FBS)

Establishing a credible and robust supply chain that meets regulatory, operational, economic, and scientific requirements

Are species-specific growth factors essential to produce cultivated meat?

Many human growth factors will also stimulate animal cells, although limited data are available to help understand the relative efficacy and specificity of cross-species bioactivity. However, recently there has been a strong recommendation that cultivated meat companies use growth factors from the same species, or as a minimum use a non-human growth factor. Although, as far as we are aware, there is no formal guidance available to date, this seems to be the clear direction of travel based on feedback from submissions by industry leaders and new verbal recommendations from the FDA (USA Food and Drug Administration).

The use of species-matched bioactive proteins will undoubtedly smooth regulators' consideration, aid consumer acceptance and likely also improve efficacy, therefore helping reduce the cost of media.

There is a pressing need for properly controlled comparative data demonstrating the relative efficacy of key growth factors on animal cells. To date, this has been a challenge due primarily to the lack of availability of commercial reagents, the variable quality of those reagents that are available and the lack of open-access comparative studies using recombinant proteins of equivalent biochemical quality and well-characterized cell lines.

As a first step to addressing the sector's scientific and regulatory demands, we are developing and characterizing growth factors of identical biochemical quality from several relevant species. In collaboration with other stakeholders, we hope to accelerate culture media development and generate open-access data for porcine and bovine iPSC, ESC and MSC culture.

Do we need new species-specific forms of all the growth factors used in bovine and porcine cell culture media?

Growth factors used commonly in cultivated bovine and porcine cell culture media were analyzed to identify proteins identical between the three species and those where the protein sequence isn't conserved, which raises concerns over potential species-specific activity and regulatory needs. This analysis included sequence conservation (table 1, below) and functional

domain analysis (data not shown). These data, along with industry priorities, have been used to prioritize the development of species-matched growth factors at Qkine.

The following growth factors are used extensively in cultivated meat and fat culture media formulations and are not well-conserved between species.
Here, we focus on FGF-2, TGFβ3 and HGF.

Fibroblast growth factor 2 (FGF-2), also known as basic FGF, is crucial for the proliferation of many cell lines and for maintaining the pluripotency of stem cells.

Transforming growth factor beta 3 (TGF-β3) is used for mesenchymal stem cell maintenance and myoblast differentiation.

Epidermal growth factor (EGF) is frequently used to promote the proliferation and differentiation of mesenchymal, epithelial and other cell types

Hepatocyte growth factor (HGF) important in bovine myoblast expansion and maturation

Platelet-derived growth factor-BB (PDGF-BB), a potent mitogen that promotes proliferation and production of cell mass

Leukemia inhibiting factor (LIF) used for the maintenance or derivation of some stem cell types

Product code	Product name	Species	Human to porcine	Human to bovine
QK001	Activin A	Human	100%	100%
QK047	IGF-1	Human	100%	100%
QK045	NRG-1	Human	100%	100%
QK010	TGF-β1 PLUS	Human	100%	100%
QK072	TGF-β2	Human	100%	100%

Table 1a: Growth factors 100% conserved between human, bovine and porcine.

Product code	Product name	Species	Human to porcine	Human to bovine	Qkine R&D pipeline
QK011	EGF	Human	~85%	-	Porcine EGF (Qk064) Bovine EGF (available on request)
QK025	FGF-2 (145 aa)	Human	~99%	~99%	100% conserved between bovine and porcine FGF-2 (145 aa) (Qk040)
QK027	FGF-2 (154 aa)	Human	~99%	~99%	Bovine/porcine FGF-2 (154 aa) (Qk056)
QK013	HGF (NK1)	Human	~95%	~93%	Porcine HGF (NK1) (Qk061) Bovine HGF (NK1) (Qk060)
QK036	LIF	Human	87%	88%	Porcine LIF in development Bovine LIF in development
QK044	PDGF-BB	Human	~95%	91%	Bovine PDGF-BB in development
QK054	TGF-β3	Human	~98%	~99%	Porcine TGF-β3 in development Bovine TGF-β3 in development

Table 1b: Growth factor proteins not conserved between human, bovine and porcine.

Species-specific FGF-2 (bFGF) supplementation improves maintenance of pluripotency in porcine iPSCs and reveals differences in the efficacy of the two lengths of FGF-2.

FGF-2 is commonly used to regulate cell proliferation and differentiation in pluripotent stem cell (PSC) culture. FGF-2 is incorporated in the growth medium of ESCs and iPSCs to maintain cell pluripotency as either a short form (145 aa) or a longer form (154aa), which comprises the core structured region and an N-terminal extension. Human FGF-2 is often used in animal stem cell growth media regardless of the cell species of origin.

Perhaps unusually, the bioactivity of FGF-2 is highly functionally conserved between species (data not shown). The amino-acid sequence of bovine and porcine FGF-2 is identical and differs from the human protein by two amino acids.

Porcine induced pluripotent stem cells (piPSCs) can self-renew and differentiate into fat and muscle cells. These characteristics make them ideal candidates for cultivated meat applications. Developing an optimal growth media for porcine iPSCs is essential in maintaining pluripotency during long-term cell culture. Porcine iPSCs require FGF-2 supplementation in their growth medium to maintain stemness.

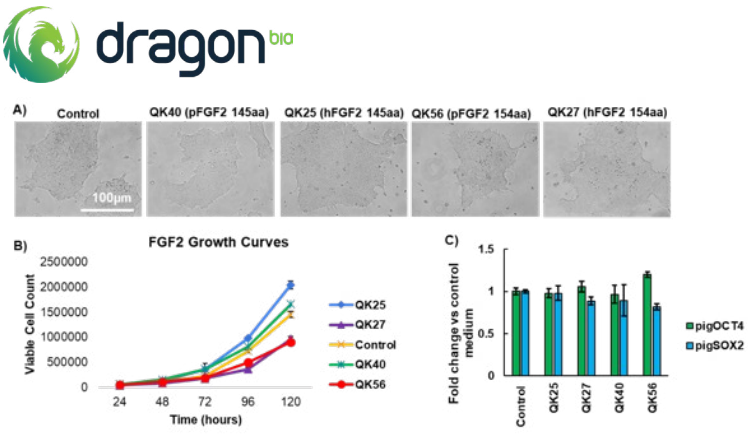

Figure 1: piPSCs cultured with FGF-2 variants. piPSCs were cultured in medium supplemented with 100ng/ml Qkine FGF-2. A) Brightfield microscopy images. B) Growth curves. C) Gene expression analysis of pluripotency markers (OCT4/SOX2). piPSCs were clump passaged using reLEsR on Geltrex coated 6-well plates. piPSCs were cultured for a minimum of 5 passages in porcine iPSC medium supplemented with FGF-2 at 100ng/ml (QK025, Qk027, Qk040 and Qk056). At passage 5, growth curves were set up for piPSCS cultured with each growth factor. piPSCs were dissociated into single cells using accutase and seeded at a density of 10,000 cells per well of a Geltrex coated 24 well plate, in 500µL of culture medium containing 1x revita cell supplement. The cells were fed daily over 5 days with 500 µL of culture medium containing each FGF-2 variant. Triplicate cell counts were performed daily over 5 days and growth curves established. After 5 passages in medium containing FGF-2, piPSCs were harvested and RNA extraction performed using NEB Monarch Total RNA miniprep kit. 2µg of RNA was converted to 2µg CDNA using the Applied biosystems high-capacity cDNA reverse transcription kit. cDNA samples were diluted to 5ng/µl for qPCR and 10ng of cDNA was used per well of a 96 well PCR plate. Primers against porcine GAPDH (housekeeping), porcine OCT4 (pluripotency) and porcine SOX2 (pluripotency) were ordered from IDT. qPCR was performed using the Quantstudio 1 real-time PCR system. Delta-delta CT

analysis was performed to calculate the relative fold change in gene expression of the markers OCT4/SOX2 in Qkine growth factor supplemented medium vs
control piPSC growth medium.

In this study, we observed that both porcine and human Qkine FGF-2 variants demonstrate bioactivity conducive to the successful culture of piPSCs. Culturing piPSCs with either human or porcine FGF-2 isoforms resulted in the maintenance of normal colony morphology, as depicted in Figure 1. Interestingly, supplementation with both human and porcine 145aa FGF2 variants led to enhanced growth rates of piPSCs compared to those cultured in control piPSC medium. However, the growth rates of porcine iPSCs were notably slower when exposed to standard maintenance medium supplemented with human or porcine 154aa longer FGF2 isoforms. Despite these variations, the expression of pluripotency markers OCT4 and SOX2 remained stable across all piPSC cultures supplemented with Qkine FGF-2 growth factors. Notably, the 154aa porcine FGF2 variant (Qk056) exhibited the highest OCT4 to SOX2 ratio, supporting the potential benefits of utilizing porcine-specific growth factors in piPSC culture medium. Please note that control piPSC culture media already contains human FGF-2 and further work is required to fully elucidate the mechanisms of the observed change in cell proliferation.

Thermostable bovine/porcine FGF-2 retains bioactivity after 7 days in culture allowing reduced frequency of media changes and improving compatibility with process scale-up

FGF-2 is inherently unstable and prone to proteolytic degradation and aggregation. This fundamental biochemical instability, and therefore low functional half-life in culture media (<10 h), is an important contributor to the need for frequent media changes and challenges in improving homogeneity during stem cell proliferation. The quality of the pluripotent stem cell culture has an important impact on the subsequent cell yield from differentiation and maturation processes.

Recombinant bovine/porcine FGF2-G3 protein is a thermostable engineered form of FGF-2 that supports the development of optimized species-specific serum-free culture media. The use of human thermostable FGF2-G3 for improved iPSC feeding schedules is well documented, and the utility of this protein for improved scale-up and impact on homogeneity and controllability of cell mass production during bioprocessing scale-up is under investigation by the cellular agriculture community. However, protein tag-free forms of bovine/ porcine FGF2-G3 compatible with manufacturing scale-up have not previously been available. In this study, the bioactivity of bovine thermostable FGF-2 was shown to be equivalent to wild-type bovine/porcine FGF-2 in a quantitative reporter assay (Figure 2).

To assess the extension of the functional half-life of bovine/ porcine FGF2-G3, the protein was incubated in conditioned media for 48 h to mimic cell culture conditions prior to bioactivity analysis. Thermostable bovine/porcine FGF2-G3 retains full activity after pre-incubation with conditioned media at 37°C for 48 hours in contrast to wildtype protein activity, which degrades in culture.

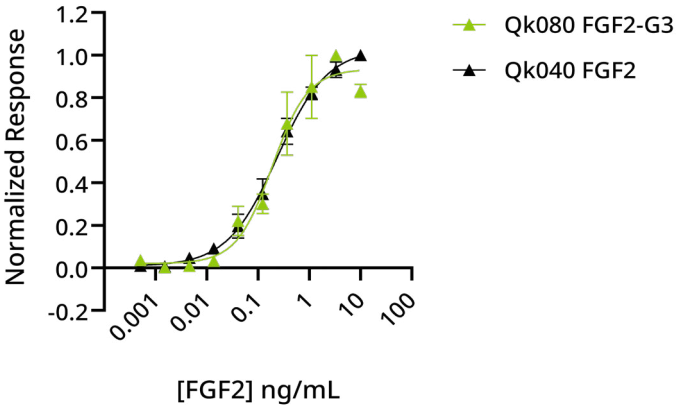

Figure 2: Wild-type bovine/porcine FGF-2 (black) and bovine/porcine FGF2-G3 (green) have equivalent bioactivity. Qk080 bovine/porcine FGF2-G3 (EC50 0.190 ng/ml) and Qk040 WT FGF-2 (EC50 0.237 ng/ml)

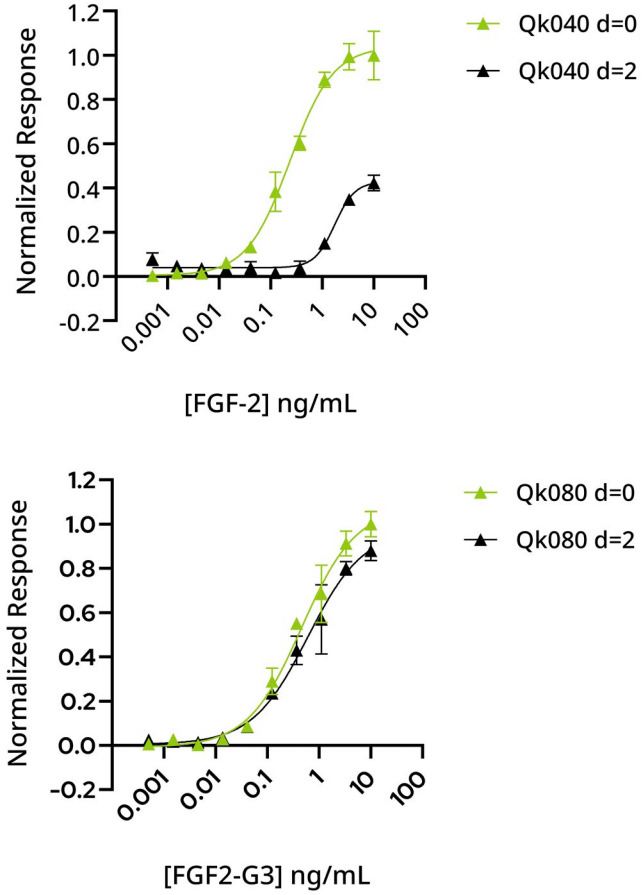

Figure 3: Comparison between Qk040 wild type (WT) FGF-2 at 0 hours (green) (EC50 0.237 ng/ml) and 48 hours (black) (EC50 1.75 ng/ml. Comparison between Qk080 FGF2-G3 at 0 hours (green) (EC50 0.464 ng/ml) and 48 hours (black) (EC50 0.606 ng/ml) bovine/ porcine FGF2-G3 activity is determined using a serum response element luciferase reporter assay in transfected HEK293T cells. Cells are treated in triplicate with a serial dilution of FGF2-G3 for 3 hours. WT FGF-2 (Qk040) and FGF2-G3 (Qk080) were diluted in conditioned media and incubated at 37 °C. Samples were taken at 0 and 48 hours. FGF-2 activity was assayed in triplicate using the Promega serum response element luciferase reporter assay (*) in transfected HEK293T cells. Results were normalized to the maximum response for 0 hours.

Our results demonstrate that FGF2-G3 has an increased functional half-life from <10 h (wild-type) to >48 hours, similar to that observed with the human thermostable FGF2-G3 protein suggesting bovine/porcine FGF2-G3 is an appropriate alternative to human thermostable FGF-2 for cultivated meat media optimization. Please note, although engineered forms of proteins like FGF2-G3 offer unique promise for bioprocess optimization and scale-up, the use of engineered proteins may present additional regulatory hurdles. However, the transformative impact on cell yield, feeding schedules and homogeneity of cell cultures may warrant this additional dialogue with the regulators.

Overview of TGF-beta family proteins and the impact of species-specific growth factors on porcine iPSC culture

The TGF-beta superfamily includes Activin A, TGF-β1-3, GDFs, GDNF, and BMPs. This protein family plays a crucial role in governing many developmental and physiological processes. Activin A and TGF beta family proteins are often essential in cell culture media used in bovine and porcine cell culture.

There are three closely related TGF-β proteins, TGFβ1, TGFβ2 and TGFβ3 that have distinct physiological roles, tissue distribution and developmental expression profiles in vivo. Distinct genes encode TGF-β 1–3 proteins, which are expressed in unique, occasionally overlapping, patterns and serve different functions in vivo. TGF-β proteins are potent signaling molecules that act primarily through the Smad 2/3-mediated pathway and perform crucial roles in cell proliferation, growth, differentiation and motility.

TGF-β1 is 100% conserved in human, porcine and bovine, and used in many commercial ESC and iPSC media including Essential 8 and mTesR that form the basis of many culture media used in cultivated meat development. In contrast, TGF-β3 is not conserved between species but is also used extensively in serum-free media
including B8 and the bovine variant, Beefy 9, and many mesenchymal and adult-derived animal stem cell media.

It is important to note that this family of proteins are inherently difficult to manufacture due to their structural complexity. The active form of the protein is a disulfide-bonded dimer, and monomeric forms of the protein are not bioactive. The need for
this family of growth factors is likely to eventually dominate the cost of goods related to bioactive recombinant protein media components as companies move to >50 l scale and then pilot phase.

Several innovative approaches are being explored to address this challenge. These include genetic approaches to remove or reduce the requirement for these proteins; co-culture and utilizing the secretion of these proteins by mammalian cells during culture, and developing optimized proteins and manufacturing processes to address the supply chain capacity and costs.

The first step to improving the supply chain is developing reliable species-specific proteins and building on these data to develop engineered forms with improved manufacturing yield or biological properties. There is also potential to innovate in the protein manufacture process to tailor it specifically to the needs and scale of the cultivated meat industry.

Porcine TGF-β3 was manufactured using an animal-origin-free microbial fermentation process. The biological activity of this protein was compared directly with human TGF-β3 in a quantitative luciferase reporter assay on a human cell-line and showed equivalent bioactivity. This is promising and suggests a like-for-like substitution of human TGF-β3 with the porcine protein may be possible.

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CLUSTAL O(1.2.4) multiple sequence alignment

sp|P15203|TGFB3_PIG|298-409        ALDTNYCFRNLEENCCVRPLYIDFRQDLGWKWVHEPKGYYANFCSGPCPYLRSADTTHSS 60
sp|P10600|TGFB3_HUMAN|301-412      ALDTNYCFRNLEENCCVRPLYIDFRQDLGWKWVHEPKGYYANFCSGPCPYLRSADTTHST 60
tr|A6QP91|A6QP91_BOVIN             ALDTNYCFRNLEENCCVRPLYIDFRQDLGWKWVHEPKGYYANFCSGPCPYLRSSDTTHST 60
                                   ************************************************:*****:

sp|P15203|TGFB3_PIG|298-409        VLGLYNTLNPEASASPCCVPQDLEPLTILYYVGRTAKVEQLSNMVVKSCKCS 112
sp|P10600|TGFB3_HUMAN|301-412      VLGLYNTLNPEASASPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSCKCS 112
tr|A6QP91|A6QP91_BOVIN             VLGLYNTLNPEASASPCCVPQDLEPLTILYYVGRTPKVEQLSNMVVKSCKCS 112
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Figure 4: Multiple sequence alignment of porcine, bovine and human TGF-β3 demonstrate a ~98% sequence conservation between human and porcine TGF-β3, with a ~99% conservation between human and bovine TGF-β3. Figure 5: Human and porcine TGF-β3 activity was determined using a TGF-β3 responsive firefly luciferase reporter in HEK293T cells. EC50 of human TGF-β3 =39.53 pg/ml and porcine TGF-β3 = 37.51 pg/ml. Cells are treated (in triplicate) with a serial dilution of TGF-β3 for 6 hours. Firefly luciferase activity was measured and normalized to the control Renilla luciferase activity.

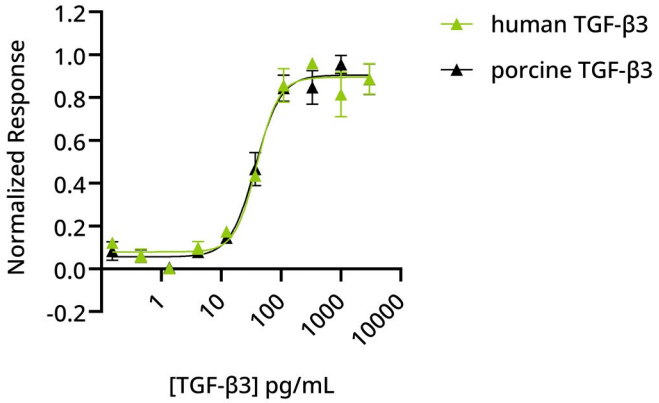

Figure 5: Human and porcine TGF-β3 activity was determined using a TGF-β3 responsive firefly luciferase reporter in HEK293T cells. EC50 of human TGF-β3 =39.53 pg/ml and porcine TGF-β3 = 37.51 pg/ml. Cells are treated (in triplicate) with a serial dilution of TGF-β3 for 6 hours. Firefly luciferase activity was measured and normalized to the control Renilla luciferase activity.

Qkine TGFβ1 maintains pluripotency in porcine iPSCs

A piPSC cell line from Dragon Biotechnologies was used to validate the efficacy of Qkine human/porcine/bovine TGFβ1 and human TGFβ3 in culture. Porcine induced pluripotent stem cells (piPSCs) when exposed to either human/bovine/porcine TGFβ1 or human TGFβ3 during culture, demonstrate typical colony morphology and exhibit enhanced growth rates compared to piPSCs cultured under standard conditions with standard medium (Figure 3A-B). The expression levels of pluripotency markers OCT4 and SOX2 remain stable following TGFβ1 supplementation, as depicted in Figure 3C.

However, importantly, the introduction of human TGFβ3 leads to a decrease in OCT4 expression, suggesting that human TGFβ3 in isolation of piPSCs may not adequately sustain pluripotency or may induce differentiation. Equivalent bioactivity of human and porcine proteins suggests that it may be possible to directly substitute porcine TGF-β3 in media that currently contain human TGF-β3 to develop a species-matched culture media formulation. Further work using porcine cell lines is required to determine the relative efficacy in porcine cell culture, which will be facilitated by the commercial availability of this growth factor.

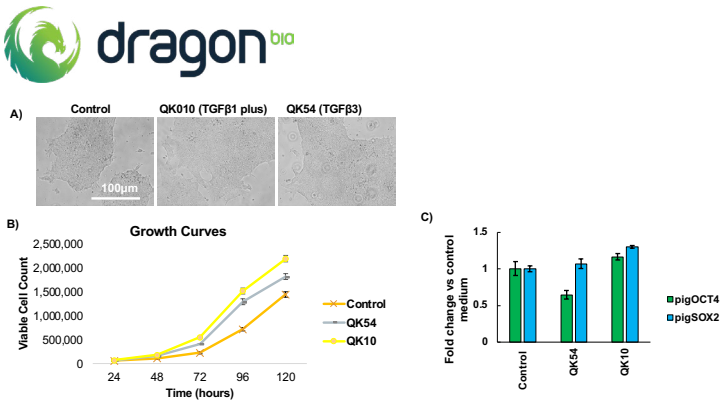

Figure 6: piPSCs cultured with TGFβ1 and TGFβ3. piPSCs were cultured in medium supplemented with 2ng/ml Qkine TGFβ1 or TGFβ3. A) Brightfield microscopy images. B) Growth curves. C) Gene expression analysis of pluripotency markers (OCT4/ SOX2).

HGF exhibits species-specific activity, suggesting published data using human HGF on bovine cells may warrant re-evaluation

HGF is an important growth factor in bovine myoblast expansion and maturation media. Previously, Qkine developed an optimized animal-free native sequence active isoform of human HGF, HGF NK1, as all existing commercial HGF proteins were produced using animal or human cell protein expression systems, limiting their application in translational studies.

To extend species-specific HGF availability, bovine and porcine HGF NK1 were produced. The activity of human, bovine and porcine HGF NK1 proteins was compared using a reporter assay on human cells. Bovine HGF NK1 and porcine HGF NK1 have reduced bioactivity compared to human HGF NK1 in human cells, suggesting species differences in HGF (NK1) activity.

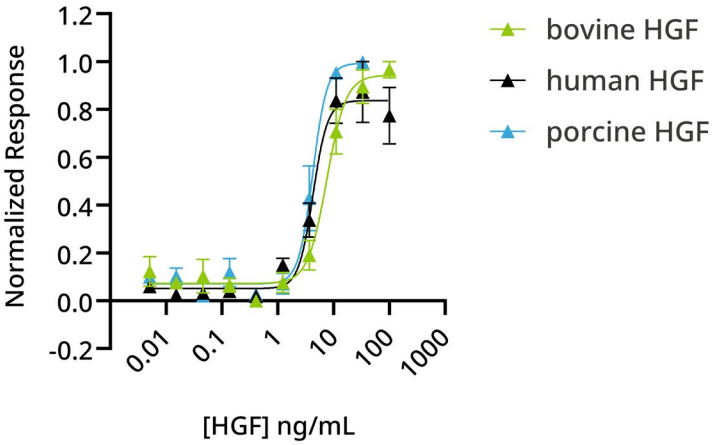

Figure 7: human and porcine HGF NK1 activity was determined using the Promega serum response element luciferase reporter assay (*) in HEK293T cells. EC50 human HGF (NK1) = 1.56 ng/m; EC50 porcine HGF (NK1) = 3.86 ng/ml and EC50 bovine HGF (NK1) = 7.71 ng/ml. Cells were treated in triplicate with a serial dilution of HGF NK1 for 3 hours. Firefly luciferase activity was measured and normalized to the control Renilla luciferase activity.

Bioactivity assay data from human cells suggests HGF NK1 exhibits species-specific differences. Further experimental work using bovine and porcine cells is required to determine the impact on cultivated meat process development.

Cultivated meat, fish, fat and dairy are rapidly developing fields with the potential to impact global sustainability. Qkine is committed to supporting the cellular agriculture sector by extending the range of commercially available species-specific growth factors and supporting development of open collaborations, a scientific research base and credible long-term supply chain.

For more information

Please contact our team: **customerservice@qkine.com** if you would like to discuss commercial or academic collaborations, supply agreements or any aspects of growth factor optimization for cultivated meat, fish, fat, dairy and other products.

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