Title

Never let me down: Optimizing performance of serum free culture medium for bovine satellite cells

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Abstract

Cultivated meat may be a more ethical, environmentally friendly, antibiotic-free meat alternative of the future. As of now, one of the main limiting factors for bringing cultivated meat to the market is the high cost of the cell culture medium. Here, we optimize B8/B9 medium - one of the well-established serum free, fully defined medium compositions available for purchase or for preparation in-house. We show several combinations of the growth factors/myokines/hormones, which were able to substantially increase bovine satellite cells' proliferation rate, as well as treatment schemes which allow to use five to ten times lower concentrations of signaling molecules for the same effect. Additionally, we present two food grade low-price medium stabilizers, one of which exhibits superior stabilization of the B8 medium as compared to recombinant human serum albumin, and allows for its substitution, dropping the price for stabilization to approx. under 0.2% of that used in B9 medium.

Introduction

Conventional meat production and especially beef production forms the tip among the most land intensive and emission intensive food products. Since the world population and therefore the meat consumption is predicted to increase by further 73% until 2050, it will not be possible to obtain enough meat in a conventional way, as already around 80% of all agricultural land is used for animal feed[1]. One of the possible alternatives – cultivated meat – can drastically reduce land use and global warming effects.

Cultivated meat has come a long way from its first introduction in 2013 by Prof. Mark Post, with his first cultivated meat hamburger costing 250.000 Euro, using expensive and ethically questionable Fetal Bovine Serum (FBS) – containing medium – a standard those days[2]. Since then, multiple groups have been working on making its production feasible and prices low enough to compete with conventional meat on the free market. A lot of effort is still being put into the development of cultivation medium, as over 95% of the production costs are attributed to it[3,4] due to the very high prices for recombinant growth factors and other components used herein. In the first attempts, formulations known from work with other types of stem cells, e.g. iPSC cells, were used[5], but those formulations were not sufficiently effective and still overly expensive. During the last years quite a lot of progress was

achieved in this area, among other – proprietary – solutions[5,6], two renown groups have published their serum-free, fully defined medium compositions, which allow very high propagation efficiency, comparable or even superior to FBS-based medium formulations.

For the first optimized medium Stout *et al.* have taken a further development of the Essential 8 medium - called B8 medium[5] – as a basis, and supplemented it with 0.8 g/L Human Serum Albumin (HSA) for stabilization. This new medium – called B9 – was pre-printed by Stout *et al.* in May 2021, and published in June 2022[7], is shown to perform nearly as good as the 20% FBS-containing growth medium on primary bovine satellite cells (BSCs). HSA costs comprised 24.56 USD/L, and overall medium costs were in the range of 46.28 to 74.28 USD/L (excluding coating proteins), depending on the concentration of FGF-2, HSA making up to over 50% of total cost. Comparing to the Essential 8, B9 medium supports the propagation of satellite cells much more effectively at 74 USD/L, and is approx. six times cheaper than Essential 8 at about 500 USD/L[5,7].

The other serum-free, fully defined medium was published by Kolkmann *et al.*[8] in January 2022, where several non-standard growth factors and a myokine were introduced (IL-6, IGF1, VEGF, HGF and PDGF-BB – here called PDGF). These exhibit synergistic effects on BSCs proliferation and reach 97% of the efficiency of the 20% FBS-containing growth medium. To produce such effect, Kolkmann used even higher HSA concentration for stabilization – 5 g/L end concentration – for an approximal cost of 163 USD/L only for HSA. At this point, it became evident that the new trend was a more complex medium with multiple growth factors/signalling molecules orchestrating BSCs proliferation, to be able to substitute for extreme complexity of FBS. This was further complemented by stabilization of the medium, substituting the functions of extracellular matrix (ECM), which is obviously extremely important for high performance. Stabilization simultaneously presents a central proliferation potentiating factor, raising effectiveness of the medium, but is also a big cost factor, comparable or even bigger than the growth factors themselves.

The most recent paper from Stout *et al.* has addressed this latter issue, demonstrating a successful HSA substitution by in-house produced seed protein isolates[9]. Extremely low prices of the oilseed protein meals (less than 0.4 USD/kg), which were used as the starting material, do raise hope that the final prices for protein preparations will be rather low. But it is hard to assess this fact properly, as such isolates are currently commercially unavailable, and the proposed isolation method consists of alkali extraction (pH 12.5), isoelectric precipitation (pH 4.5), centrifugation, filtration, and ultrafiltration to concentrate the final protein solutions to 50 mg/mL[9]. Some of these techniques could be quite costly on an industrial scale, though the extraction process can be further optimized.

Here, we suggest several variants of the improved B9 medium as a serum free, fully defined medium composition, which can be prepared in-house. We document combinations of the growth factors/myokines/hormones, which were able to substantially increase the satellite cells' proliferation rate. We also show that, compared to a single addition of the signaling molecules, two treatments with significantly lower concentrations demonstrate much more pronounced proliferation effect. This finding underscores the importance of adjusting the application scheme, as it can potentially lead to a substantial – factor 5x to 10x – cost savings. Additionally, we present two low-priced medium stabilizers – racemic Alanine (ALA) and an emulsifier used in food industry - Methylcellulose (MC, also known as E461). They exhibit similar stabilization of the B8 medium as compared to recombinant HSA, allowing for its substitution, lowering the price for stabilization to approximately 0.1% of that used in B9 medium. Moreover, we show that combination of HSA with MC and ALA exhibits a superior stabilization effect, as compared to any stabilizer alone. As HSA can be substituted by oilseed protein isolates[9], a cost-effective triple combination of stabilizers can be produced to potentiate the propagation of muscle cells. In sum, our findings allow for a further efficiency increase during propagation phase, bringing cultivated meat closer to the market.

Results

Single addition requires much higher concentration for the same effect

Sports induces muscle hypertrophy when done regularly. Thus, taking B9 medium as a basis, we compared addition of components only once (on day 1) with adding them twice (on days 1 and 3). Interestingly, single addition does require much higher concentration for the same or less pronounced effect (**Figure 1**). For example, upon single addition of the recombinant human Hepatocyte Growth Factor (rhHGF), the concentration required for 1.5 fold improvement of the proliferation rate was 20 ng/mL (**Figure 1**B), whereas when added twice, the threshold of 1.5 fold improvement was stably reached by concentrations of 2.5 ng/mL (**Figure 1**A). The same effect but even more pronounced was seen upon Estrogen addition (**Figure 1**C-D). This underlines the importance of adjusting the application scheme, as it can potentially lead to substantial – factor 5x or 10x – cost savings on the most expensive medium components.

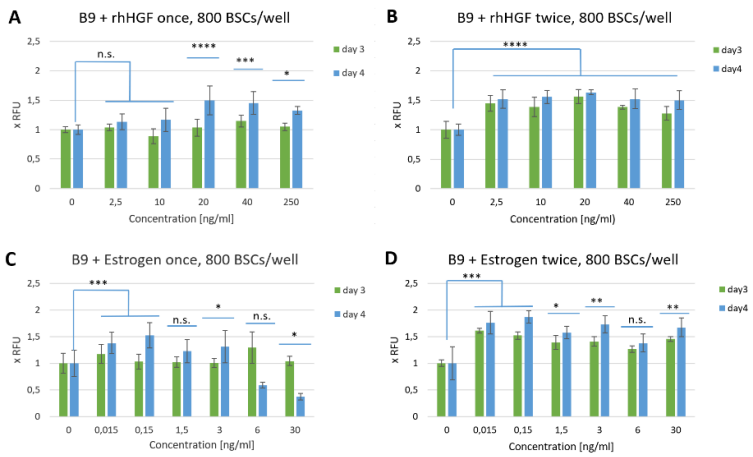

Figure 1: Comparison of single and double addition of components. *BSCs were treated either once on day 1 or twice on day 1 and 3 with indicated concentrations of the HGF (A and B) and Estrogen (C und D), Presto Blue assay was performed on days 3 and 4, and normalized to vehicle treated control (0), which contained DPBS + 0.8 mg/mL HSA. n=6 biological replicates for A and C, n=3 for B and D; statistical significance was calculated by one-way ANOVA combined with Tukey HSD for day 4, comparing all samples with vehicle treated control (0), and is indicated by asterisks, which are p < 0.05 (*), p <0.01 (**), p < 0.001 (***).*

This double addition scheme worked best for most of the components, but for one of them – recombinant human CHI3L1, a myokine activating TGFβ pathway – detrimental effect of a double addition was repeatedly observed (**Figure 2**). Such profound effects as shown on **Figure 2**A were later not reproducible, which was most probably due to stability issues of rhCHI3L1 in our standard reconstitution buffer, containing 0.8 mg/mL HSA. This emphasizes the importance of proper stabilization of medium components.

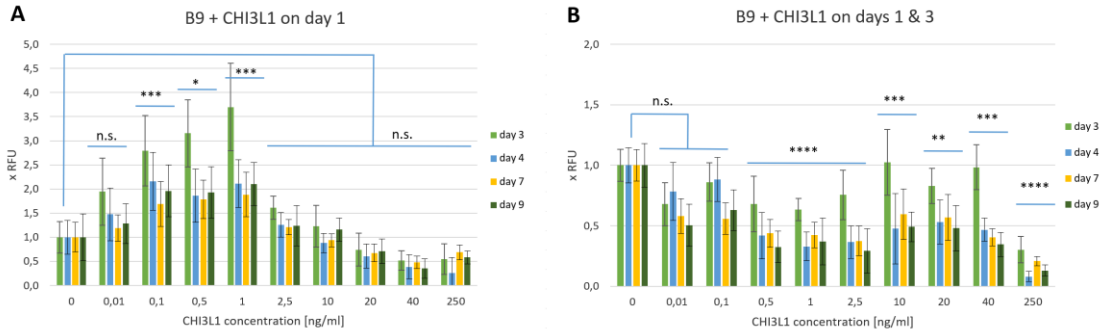

Figure 2: *Comparison of single and double addition of rhCHI3L1. BSCs were treated on days 1 and 3 with indicated concentrations of rhCHI3L1 either on day 1 (A) or on days 1 and 3 (B), and Presto Blue assay was performed on days 3, 4, 7 and 9, and normalized to vehicle treated control (0), which contained DPBS + 0.8 mg/mL HSA. n=6 biological replicates; statistical significance was calculated by one-way ANOVA combined with Tukey HSD for day 4, comparing all samples with vehicle treated control (0), and is indicated by asterisks, which are p < 0.05 (*), p <0.01 (**), p < 0.001 (***), p < 0.0001 (****).*

As for most other components checked the effect of a double addition was more pronounced than upon single addition, in all subsequent experiments we used double addition of the component(s).

Screening single medium components

Addition of single components to the serum free medium follows the traditional and most convenient approach of medium optimization, which is to try to keep the number of components low, for the purpose of making media preparation in laboratory conditions easy. This approach does not resemble conditions *in vivo*, where a very complex molecular surrounding of hundreds of active molecules in ranges of concentrations are present. But it can still be very handy for transferring findings about synergetic properties of components between different basic media compositions.

To this end, we have tried out addition of single components to B9 medium, and combinations of the resulting hits. Most of the tested components elicited a pronounced positive effect on the proliferation of bovine satellite cells, when added twice. In total, 19 components were chosen based on literature (see Supplemental Table 1 for the whole list and sourcing), from which the most prominent hits where rhHGF, Estrogen (17β-Estradiol), rbIL-6, rmWnt3a, and moderately good hits – rhIL1α, rhPDGF-BB, Testosterone (5α-Dihydrotestosterone) (**Figure 3**). (See Supplementary Figure 1 for the proliferation timelines of the rest of individual components – rhFollistatin, rhCHI3L1, rhWnt-5b, rhIGF1, rhIL-4, rhIL-13, rhIL-15, rhLIF, rhIFN-γ, rhTNF-α, rhGASP-1, EGF).

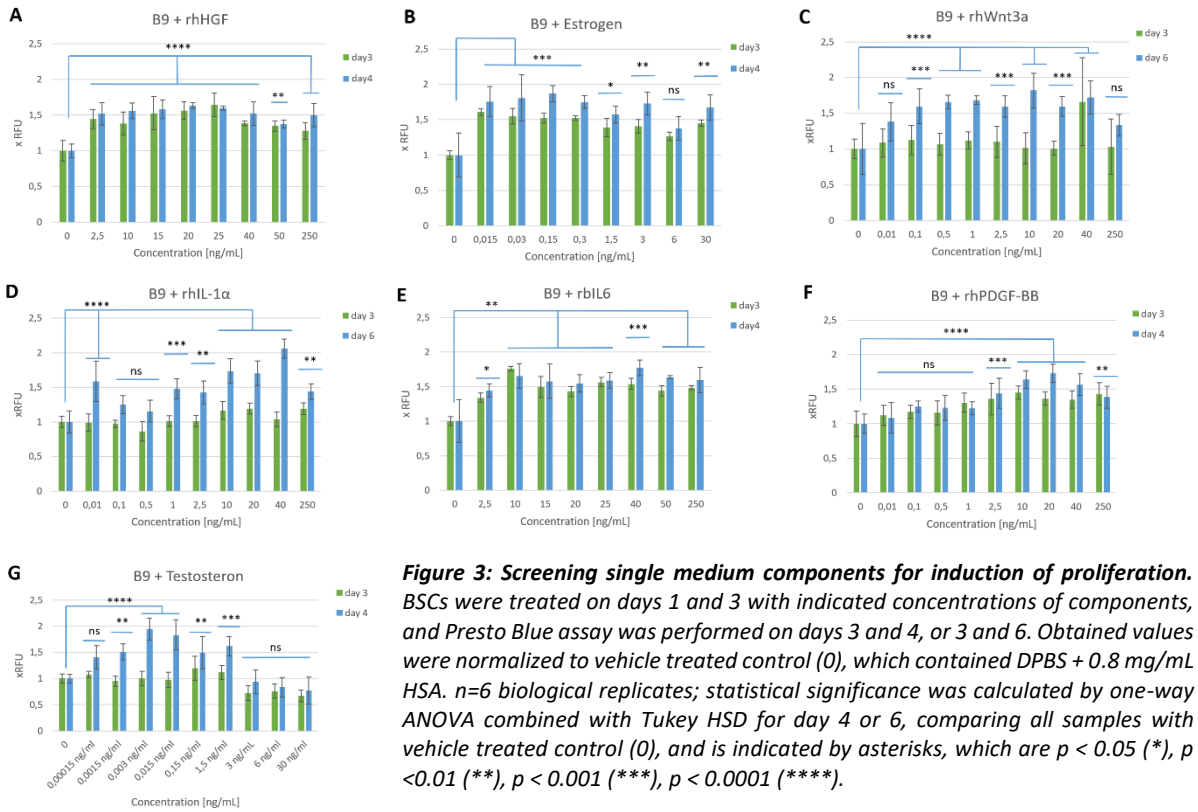

Figure 3: Screening single medium components for induction of proliferation. *BSCs were treated on days 1 and 3 with indicated concentrations of components, and Presto Blue assay was performed on days 3 and 4, or 3 and 6. Obtained values were normalized to vehicle treated control (0), which contained DPBS + 0.8 mg/mL HSA. n=6 biological replicates; statistical significance was calculated by one-way ANOVA combined with Tukey HSD for day 4 or 6, comparing all samples with vehicle treated control (0), and is indicated by asterisks, which are $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

Presto Blue is a viability assay representing cells' metabolic activity and thus indirect in its nature[10]. To ensure that the data produced by this assay is relevant under the conditions where the added proliferation inducing compounds could cause a change in the cells' metabolic activity, the second, direct assay was used, able to assess the quantity of DNA per well, and so cell density more accurately at confluence and below – Hoechst 33258 assay. This assay can be reliably used for cell densities up to

100.000 cells/cm^2. Such combination of two assays was previously reported to function very dependably also in a high-throughput format[8]. We thus conducted Hoechst assay, which mostly confirmed our earlier findings (see **Figure 4**), except that IGF1 and HGF showed a much higher cell density than predicted by Presto Blue assay.

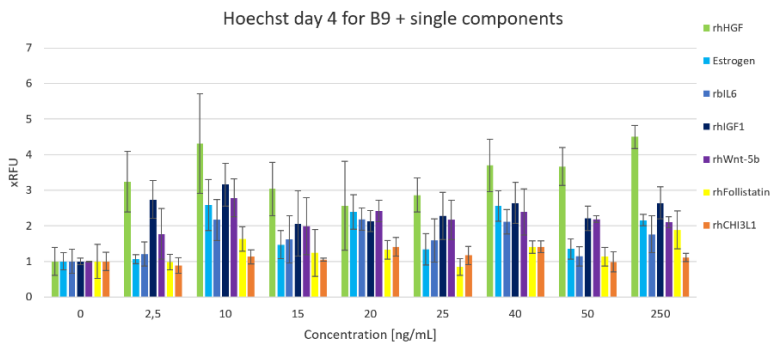

***Figure 4**: Confirmation of the Presto Blue assay results with end-point Hoechst assay for some of the tested components.* *BSCs were treated on days 1 and 3 with indicated concentrations of components, and Hoechst assay was performed on day 4 (Presto Blue assays were performed on days 3 and 4). Obtained values were normalized to vehicle treated control (0), which contained DPBS + 0.8 mg/mL HSA. n=3 biological replicates.*

Screening combinations of the resulting hits

For the screening of combinations, the lowest concentration which had had a positive effect in the screening was chosen, to avoid possible advers effects sometimes seen at high concentrations. The screening was performed as above – Presto Blue assays were performed on days 3 and 4, and additionally on days 6 or 7. As Presto Blue assay was less reliable at higher cell densities reached on days 6-8 (approx. 100 000 cells/cm^2), the significance of the observed effects was statistically evaluated by one-way ANOVA combined with Tukey HSD calculated for day 4 or at the latest day 6. Additionally, the data was confirmed by Hoechst assay at the end of the same screening round (data not shown).

We could identify following hits - see **Table 1** (see Supplementary Figure 2 for the proliferation timelines of individual combinations).

***Table 1**: Summary of the screening analysis of the single components and simple combinations based on Presto Blue screening. Darker marked components elicit more prominent effect on proliferation rate of BSCs. Unmarked components elicited least prominent effects (under 1.5 x RFU). N.A. – not analysed. * - unstable effect.*

Component	**B9 + 1 component added once**	**B9 + 1 component added twice**	**B9 + 2 components added twice (concentration = 2.5 ng/mL Estrogen (EST) = 0.15 ng/mL)**		
			+HGF	+IL-6	+ EST
Estrogen	from 0.15 ng/mL	from 0.015 ng/mL			
rhHGF	from 20 ng/mL	from 2.5 ng/mL			
rbIL-6	from 40 ng/mL	from 10 ng/mL			
rhWnt-3a	N.A.	from 0.5 ng/mL			
rhPDGF-BB	N.A.	from 10 ng/mL			
rhFollistatin	N.A.	from 10 ng/mL			
rhCHI3L1	from 0,1 ng/ml*				
rhIGF1	N.A.	from 2.5 ng/mL			
rhWnt-5b	N.A.	? from 10 ng/mL			
rhIL1α	N.A.	from 10 ng/mL			
Testosterone	N.A.	from 0.015 ng/mL			1:5

From 19 components tested, the best results upon double addition of a single component to B9 medium were shown for rhHGF (Abcam #ab245957), second best - 17β-Estradiol (Sigma #E2758-250MG), third best – rbIL-6 (Biorad #PBP021). Best combinations of two components were rhHGF + rhPDGF-BB, second best – rhHGF + rmWnt3a, third best – rhHGF + rhWnt-5b.

Further on, to determine the most potent combination of our hits, the best two-component combinations - HGF + PDGF-BB – was gradually expanded to include additional best two-component hits – IL-6, Estrogen and Wnt-5b+Wnt3a. The combination of Wnt-5b+Wnt3a was previously published to promote YAP/TAZ activation via the alternative Wnt signaling pathway[11]. Also, both were among the best hits in combination with HGF (**Table 1**), and had a positive effect when combined even without HGF (data now shown). They were thus included into the final screening together.

As shown in **Figure 5**, Presto Blue assay data suggests that in B9 medium on day 4 prominent effect was demonstrated by IL-6 addition to HGF + PDGF, though it could further be slightly improved in the combination of HGF + PDGF + IL-6 + Wnt3a + Wnt5b, as well as in the combination HGF + PDGF + IL-6 + Wnt3a + Wnt5b + Estrogen, but less so. It was surprising to see no or a negative effect from addition of Estrogen to HGF + PDGF + IL-6, because it showed significant improvement in several combinations – especially with HGF and PDGF (see Suppl. Fig. 2), but that could be resulting from the quite high concentration used in the final experiment (5 ng/ml).

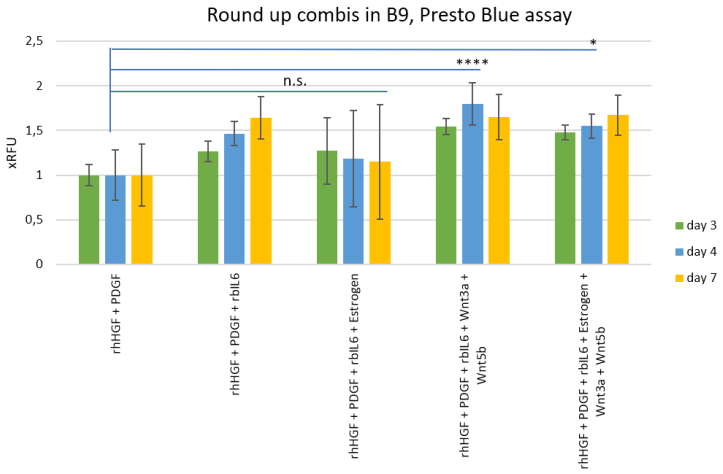

Figure 5: Final combinations of proliferation inducing hits in B9 medium. *BSCs were treated with 5 ng/mL of each component on days 1 and 3, Presto Blue assay was performed on days 3, 4, and 7. Obtained values were normalized to BSCs treated with rhHGF + PDGF. Data for n=6 biological replicates is presented; statistical significance was calculated by one-way ANOVA combined with Tukey HSD for day 4 (Presto Blue), comparing all samples to BSCs treated with rhHGF + PDGF, and is indicated by asterisks, which are $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

Non-specific stabilization of cultured medium components

Albumins are very widely used for non-specific stabilization of biologically active proteins[12–14]. But they are far from being the only known protein stabilizers – salts, sugars, amino acids and hydrogels are also used for this purpose[15]. However, sustainable stabilization properties for longer storage are often reached at non-physiological concentrations and could be causing cell death if used without adaptation. Also, growth factors can require various stabilizers at specific concentrations for a significant effect[14,16].

Thus, the action of two other stabilizers was investigated – methylcellulose (MC) and DL-alanine (ALA), which are known for their non-specific stabilization effects of FGF-2[16], are extremely cheap in comparison to HSA (see **Table 2**), and are both food components – MC is an approved food emulsifier also known as E461, and L-ALA is a natural food compound. Instead of L-ALA we used racemate to follow-up on the effects published by Benington *et al.*[16] We hypothesised that such non-specific stabilizers can achieve additional positive effect, regardless of the growth factor combination ultimately used.

Table 2: *Cost comparison of different stabilizing agents.*

Compound	Article Nr	Cost	Link	Costs/L medium
rhAlbumin	ScienCell OsrHSA	32 649 €/kg	link	0.8 g/L → 26.12 €/L
DL-Alanine, ≥99%, FCC, FG	Sigma W381810-10KG-K	60 €/kg	link	0.4495 g/L (5 mM) → 0.0275 €/L
Methylcellulose viscosity: 4,000 cP	Sigma M0512-1KG	315 €/kg	link	0.1125 g/L → 0.04 €/L

Stabilizing activity of methylcellulose (MC) and Alanine (ALA) was investigated in standard cell proliferation assays on BSCs in either B8 (without 800 mg/L HSA) or B9 (with 800 mg/L HSA) media. Several concentrations were tried known to have high stabilizing potential for FGF-2[16]. The combinations of all three stabilizers (HSA, MC and ALA) were also tested, as we reasoned it would be mimicking the very generalized content of ECM, consisting mostly of water, proteins and polysaccharides[17].

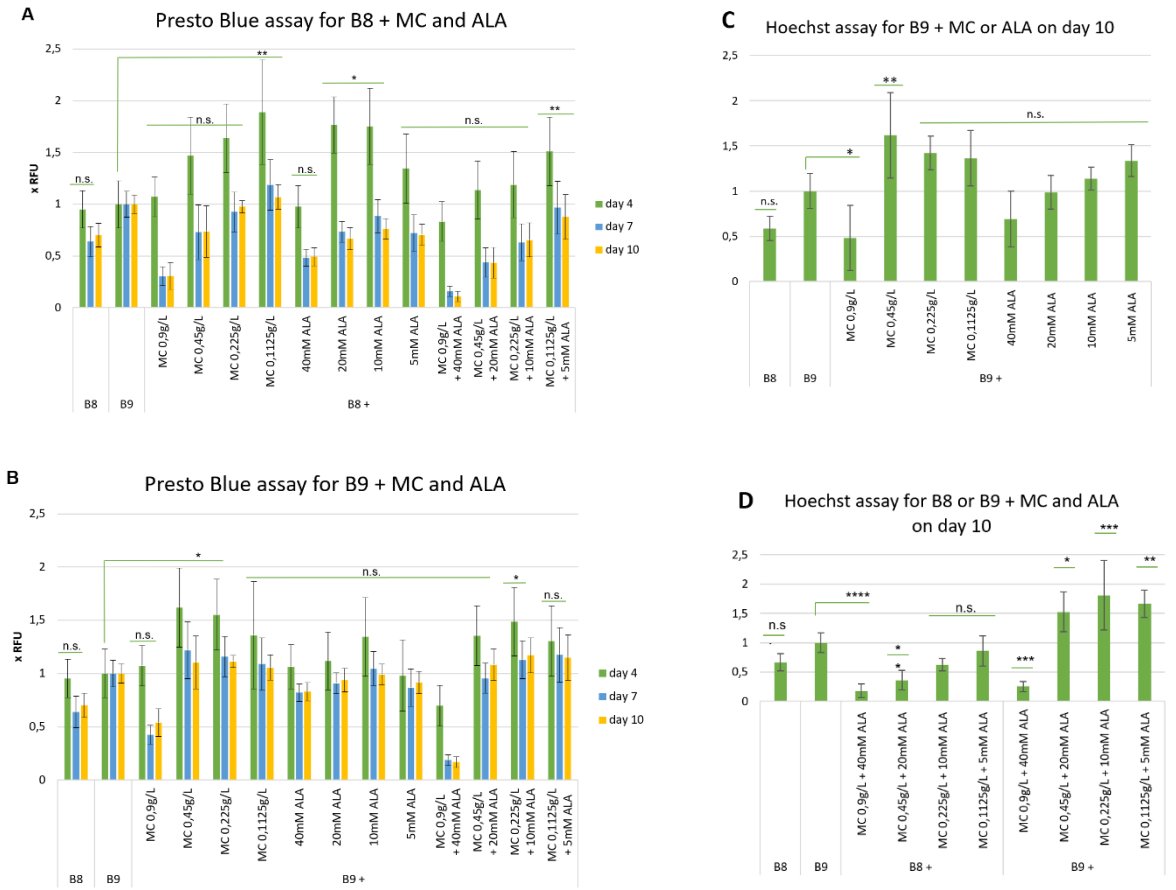

Figure 6: *Non-specific stabilization of cultured medium components. 2000 BSCs/cm² were seeded on day 0 in BSC-GM, and changed on day 1 to either B8 or B9 medium, with Methylcellulose (MC) and/or Alanine (ALA) added to indicated end-concentrations with every medium exchange. Presto Blue assay was performed on days 4, 7 and 10 (A and B), and Hoechst assay – on day 10 (C and D). Obtained values were normalized to BSCs in B9. n=6 biological replicates; statistical significance was calculated by one-way ANOVA combined with Tukey HSD for day 4 (Presto Blue) or for day 10 (Hoechst), comparing all samples to B9, and is indicated by asterisks, which are p < 0.05 (*), p <0.01 (**), p < 0.001 (***), p < 0.0001 (****).*

As evident from **Figure 6**A, in this proliferation experiment, both MC and ALA alone and in combination could stabilize B8 approximately as well as HSA at the lower concentrations used: MC = 0.1125 g/L, ALA at 10 mM and 5 mM (trend supported by Hoechst data, not shown), with MC alone performing

slightly better than ALA alone. But even more striking was the superior stabilization of B9 medium upon addition of MC or MC + ALA at the lower applied concentrations (**Figure 6**B, C, D).

Interestingly, supplementation of ALA and MC had almost no positive effect in B8 or B9 medium, as compared to MC alone. Thus, we have chosen MC alone for supplementation.

As the highest concentrations chosen (MC = 0.9 g/L and ALA = 40 mM) were clearly detrimental for single stabilizers (**Figure 6**A, B, C), as well as for their combinations (**Figure 6**A, B, D), and considering that the general trend for both stabilizers and combinations was still up with the higher dilution rate, it can be assumed that further dilutions could still have an added positive effect on cell proliferation. Also, it has to be mentioned that MC at high concentrations used for preparation of stock solutions – around 5 g/L – is quite difficult to handle due to its high viscosity, which means that the two highest concentrations used are approximate.

All in all, the most prominent stabilization of B8 – on the level of 0.8 g/L HSA – was achieved by MC (0.1125 g/L) or by the combination MC + ALA (0.1125 g/L + 5 mM respectively). Most prominent stabilization of B9 – at least 1.5x better than B9 - with a triple combination of HSA (0.8 g/L), MC (0.45-0.1125 g/L) and ALA (20-5 mM). As Stout *et al.* have shown that HSA can be substituted by plant seed protein isolates[9], this could make this triple combination commercially feasible.

Considering that the chosen ALA compound – racemate as was published by Benington *et al.*[16] – is not a food grade component because it contains D-Alanine, and that the ALA + MC combination does not seem to exhibit synergistic stabilization effects, we thus reasoned that ALA could be excluded from the stabilization mixture in B8 experimental setup. Nevertheless, we still consider it reasonable to test L-ALA instead of the racemate in combination with MC in B8 and B9 media in future studies, as it is very cheap and could also be reasonably expected to potentiate other growth factors, also those not used in the presented experiment.

Stabilization of the best proliferation inducing combinations

After identifying MC as the better performing stabilizing agent, it was applied for stabilization of our best performing proliferation inducing combinations. It is known that different stabilizers can have more or less impact on different growth factors[14,16]. Thus, we compared the stabilization effect of HSA to that of MC for the best component combinations in B8 medium.

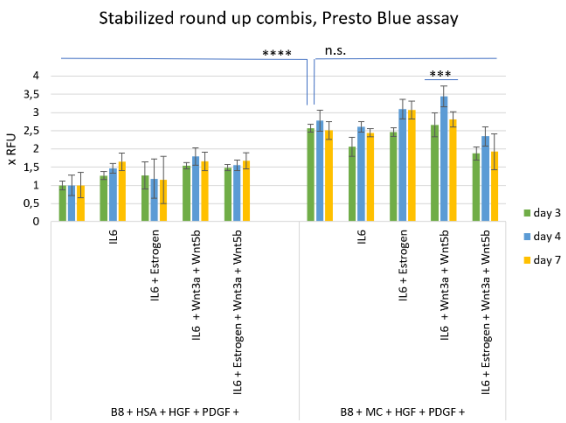

Figure 7: Comparison of stabilization effects of HSA and MC for best proliferation inducing combinations in B8 medium. *2000 BSCs/cm² were seeded on day 0 in BSC-GM, and changed on day 1 to B8 medium, stabilized with either 0.8 g/L Human Serum Albumin (HSA) or 0.1125 g/L Methylcellulose (MC). Best proliferation inducing combinations were added on day 1 and day 3 to an end concentration of 5 ng/ml, whereas stabilizers were added every time with medium exchange. Presto Blue assay was performed on days 3, 4, and 7. Obtained values were normalized to B8 + MC + HGF + PDGF. n=6 biological replicates; statistical significance was calculated by one-way ANOVA combined with Tukey HSD for day 4, comparing all samples with B8 + MC + HGF + PDGF, and is indicated by asterisks, which are p < 0.05 (*), p <0.01 (**), p < 0.001 (***), p < 0.0001 (****).*

Stabilization of any combination with MC has a significantly positive effect on proliferation, as compared to stabilization with HSA (**Figure 7**). This effect is higher than the stabilization effect of B8 with MC (**Figure 6**B, C), which could be a confirmation of its lower stabilization capacity towards less stable B8 components (Insulin, FGF2, TGFβ3, NRG1)[7] as compared to its capacity to stabilize HGF or PDGF. Additionally, as in the screening for best final combination in B9 medium (**Figure 5**), the better combination than HGF + PDGF stabilized by MC, was the combination of HGF + PDGF + IL-6 + Wnt3a + Wnt5b stabilized by MC.

It is worth noting, that the concentration of 5 ng/mL for growth factors used in the best final combination experiment could be most probably further decreased, especially when the double or triple stabilizer cocktail is applied (HSA + MC + ALA or plant seed protein extract + MC + ALA). Also, the probability that further adjustments of the treatment scheme would yield additional improvement is also quite high, as we have only tested double against single treatment schemes.

Dilution of B8/B9

In the further effort to lower the medium costs, the effect of lowering B8 content with both HSA and MC as stabilizers was tested. Therefore, the viability screen as previously described was performed, but with a B8 mixture gradually diluted in DMEM-F12. Concentration of stabilizers stayed constant.

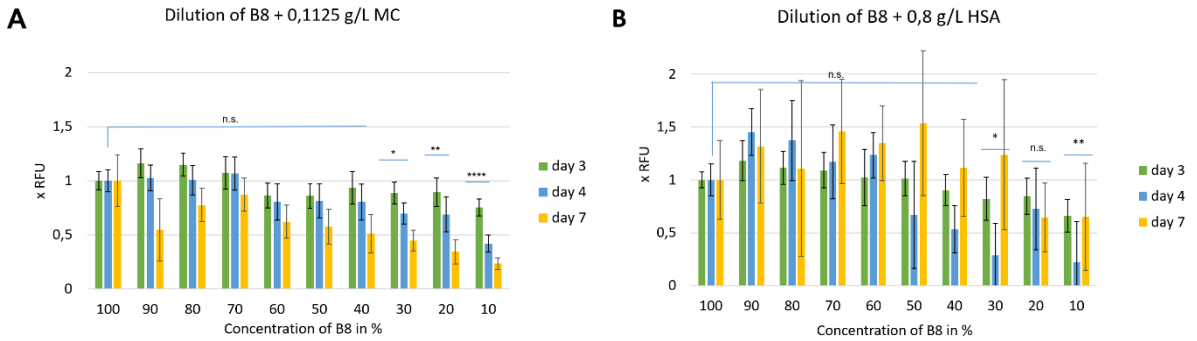

***Figure 8**: Dilutions of B8, stabilized by either HSA or MC. 2000 BSCs/cm² were seeded on day 0 in BSC-GM, and changed on day 1 to B8 medium gradually diluted in DMEM-F12 medium and stabilized with either 0.8 g/L Human Serum Albumin (HSA) or 0.1125 g/L Methylcellulose (MC). Stabilizers were added every time with medium exchange. Presto Blue assay was performed on days 3, 4 and 7. Obtained values were normalized to 100% B8. n=6 biological replicates; statistical significance was calculated by one-way ANOVA combined with Tukey HSD for day 4 (Presto Blue), comparing all samples to 100% B8, and is indicated by asterisks, which are $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).*

Statistical analysis shows that the dilution of B8 in DMEM-F12 stabilized by either 0.1125 g/L MC or by 0.8 g/L HSA does not lead to a significant drop in viability on day 4 until the concentration of B8 is below 40% (**Figure 8**). Also, the reduction by the first 30% (MC) - 40% (HSA) did not have any apparent effect. This once again underscored the importance of medium stabilization and could probably be further optimized by the double stabilizer cocktail (HSA + MC or plant seed protein extract + MC).

Differentiation with and without MC stabilization

Finally, to test the possible influence of MC on differentiation of BSCs, their ability to differentiate into muscle fibres with and without MC in the growth and in the differentiation media was compared. For that, BSCs were grown in B8 stabilized with either HSA or MC, and, upon reaching confluence, the medium was changed to serum free differentiation medium also stabilized with either HSA or MC.

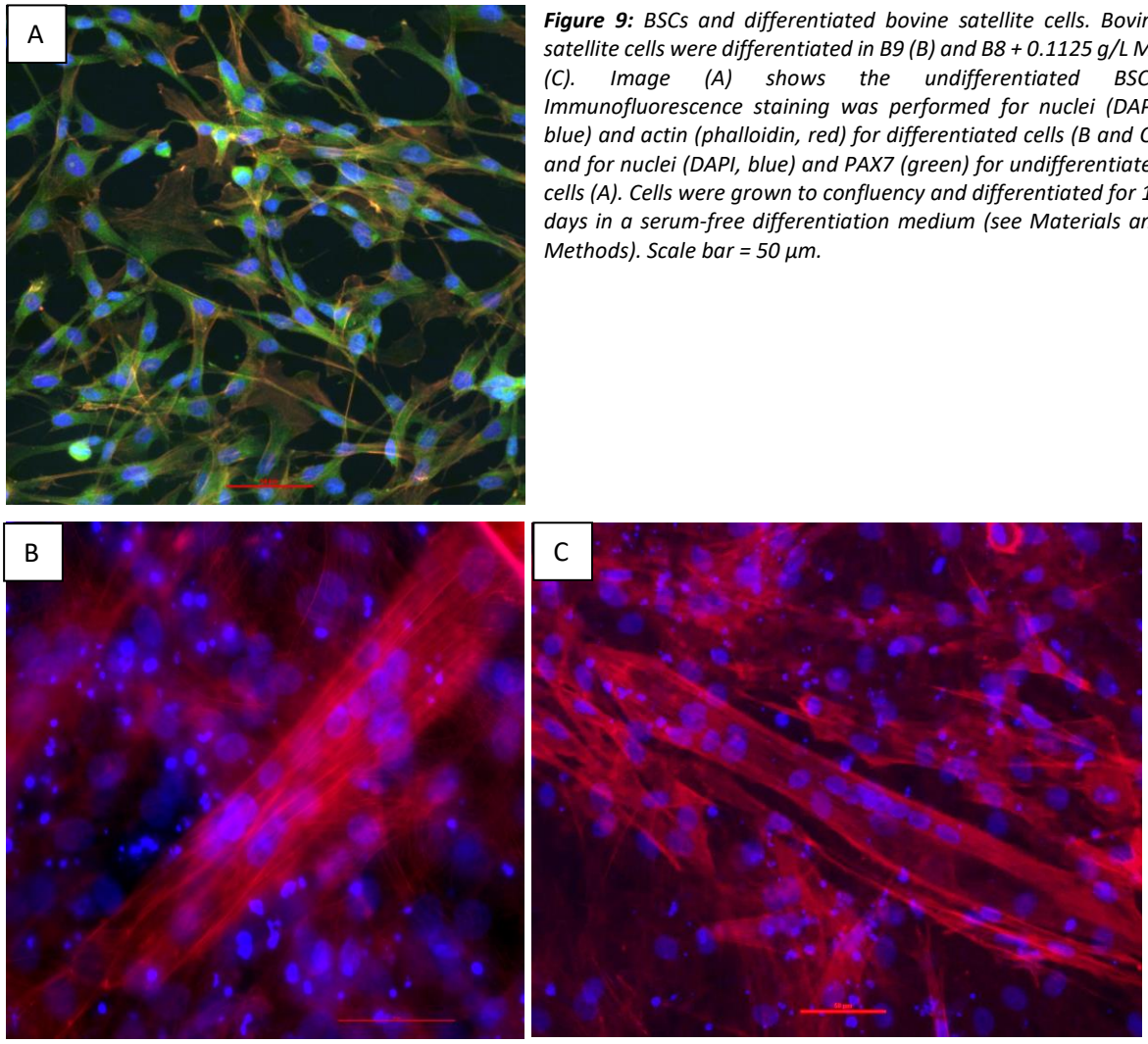

Figure 9: *BSCs and differentiated bovine satellite cells. Bovine satellite cells were differentiated in B9 (B) and B8 + 0.1125 g/L MC (C). Image (A) shows the undifferentiated BSCs. Immunofluorescence staining was performed for nuclei (DAPI, blue) and actin (phalloidin, red) for differentiated cells (B and C), and for nuclei (DAPI, blue) and PAX7 (green) for undifferentiated cells (A). Cells were grown to confluency and differentiated for 14 days in a serum-free differentiation medium (see Materials and Methods). Scale bar = 50 µm.*

We could not see any significant difference in the samples differentiating in the medium with HSA or with MC. Both differentiation setups yielded some short muscle fibres upon reaching confluency, and in both setups their quantity, length and thickness was rising upon several days in differentiation medium. We thus concluded that the presence of MC does not have any adverse effects on the cells' differentiation process.

Discussion

Development of the serum free medium for BSCs expansion has come to a new level in the last two years, as several medium compositions were presented demonstrating efficiency comparable or even higher than the Fetal Bovine Serum containing medium compositions. This was facilitated by observing the importance of using not only the right combination of growth factors, but also after introducing medium stabilization. Both Stout *et al.* and Kolkmann *et al*. use well known and very widely applied Human Serum Albumin for medium stabilization. In case of B9 medium containing 5 ng/mL FGF-2, about 53% of the whole B9 medium costs are comprised by HSA, or 33% if 40 ng/mL FGF-2 is used (see Supplementary Table 3 from Stout *et al.*[18]). In the medium formulation of Kolkmann *et al.* 5 g/L HSA are used, making it approx. 163 USD/L only for stabilization[8]. Recently, in a new paper Stout *et al*.

presented the possibility to substitute HSA with seed protein isolates, which is commercially not available, but can be produced in-house[9].

In this study we have shown that other known protein stabilizers can be used in cell culture – Methylcellulose (MC) and DL-Alanine (ALA)[16,19] – instead of HSA for the same stabilization effect (MC slightly better than ALA), or in a combination with HSA for a superior (over 1.5x) stabilization over HSA alone. Our results are in line with the FGF-2 stabilization study from Benington *et al.*, who demonstrated that a combination of two stabilizers – MC + ALA or MC + HSA – performs much better than any component alone[16].

Superior effect of the combinations could be based on the macromolecular crowding setup we had in our experiments, where high concentrations of "inert" macromolecules (MC + HSA) are mimicking the ECM environment, with its limited space containing only considerably restricted amounts of free water[20]. It is also known that MC sequesters hydrophilic proteins, preventing their aggregation and subsequent decay, and in our setup proteins are additionally stabilized by HSA through ionic, electrostatic and hydrophobic interactions[12,14]. We also used DL-ALA in the present study as amino acids with no net charge, e.g. alanine and glycine, are often used as cryoprotectants of proteins, providing stability through weak electrostatic interactions[21].

There is a great range of other protein stabilizers used in medicine and research, which comprise salts, sugars, amino acids, polymers, and last but not least – proteins (e.g. HSA)[14,15,21]. Best combination for every single target protein could be quite specific and not always cell culture compatible. We hypothesize that MC, structurally resembling glycosaminoglycan chains of ECM proteoglycans[17], together with high concentration of HSA as "inert" ECM proteins, could reasonably mimic the ECM – better than MC or HSA alone. It is thus definitively worth investigating if the expensive HSA could be substituted with rapeseed protein isolate, reported by Stout *et al.*[9], in the triple or double stabilizer combination with MC and ALA, to be able to indulge the joys of superior stabilization at a very low price. Also, additional cell culture compatible stabilizing components could further improve this effect.

All four strategies presented in this study could significantly lower medium price without letting down the BSCs propagation efficiency, even rather improving it. In **Table 3**, impacts on medium price and on potential cell yield of single optimization strategies are presented, calculated from a conservative assessment of their effects based on Presto Blue data. Optimization coefficient was calculated by dividing expected yield by medium costs, starting from initial B8/B9 (high FGF-2) values. Its values present the efficiency of the medium formulation per unit of its price (the higher – the better).

All described strategies are calculated to be more economical than the original composition of both B8 or B9 media, with an exception of Wnt – containing variants, due to the very high price for Wnt3a (see optimization coefficient in **Table 3**). Most cost-efficient according to this prediction will be B9-based medium with 30% less B8 components, but with initial 0.8 g/L HSA concentration, and with rhHGF, rhPDGF-BB and IL-6. Recombinant bovine IL-6 was used in this study, but human IL-6 performed better than bovine IL-6 in Kolkmann *et al*. 2022[8], and it is more economical, and thus was used for the calculations of optimization coefficients.

Nevertheless, it must be mentioned that among optimized B8 media, the highest optimization coefficient was calculated for the variant with rhHGF, rhPDGF-BB, IL-6 and additional estrogen. For the B9-based double-stabilized medium such component combination can turn out superior, as estrogen was consistently one of the biggest hits on its own (**Figure 1**), as well as in combinations with other components (Supplementary Figure 2), also at much lower concentrations than 5 ng/ml used in the final screening. Also, its combination with testosterone[22] (Supplementary Figure 2) was very promising. Altogether, we would expect a further prominent improvement from this hormone, or from

combination of both estrogen and testosterone, which are both also very cost-effective components, in a double-stabilized medium.

rhHGF, rhPDGF-BB with or without IL-6 was recently published by Kolkmann *et al*.[8] in yet another medium composition, and thus seems to be quite a universal proliferation inducing combination, that will most probably also be showing high performance in other satellite cells' propagation media.

Table 3: Calculation of the effect of optimization strategies on medium price and cell yield. *The effects on potential cell yield were calculated based on the conservative approximation of Presto Blue assay data. Self-made B8 and B9 prices were used.*

Medium component	Supplier, Cat. #	End concentration	Price/Unit (Bulk)	Price €/L medium	Price €/L medium, after following optimization				
				Initial	B8/B9 dilution, MC ALA stabilization applied	+ HGF + PDGF	+ HGF + PDGF + IL-6	+ HGF + PDGF + IL-6 +Wnt3a + Wnt5b	+ HGF + PDGF + IL-6 + Estrogen
HiDef8 500X	HiDef8	2.2 ml/L	1330 € / 5 L	266	186.2	186.2	186,2	186.2	186.2
Self-made B8[5]			49.72 €/L	49.72	34.80	34.80	34.80	34.80	34.80
Self-made B9[5]			74.28 €/L	74.28	52.00	52.00	52.00	52.00	52.00
HSA	ScienCell #OsrHSA	0.8 g/L	32 649 €/kg	24.56	24.56	24.56	24.56	24.56	24.56
Methylcellulose	Sigma M0512-1KG	0.1125 g/L	315 €/kg		0.04	0.04	0.04	0.04	0.04
DL-Alanine	Sigma W381810-10KG-K	0.4495 g/L (5 mM)	60 €/kg		0.0275	0.0275	0.0275	0.0275	0.0275
rhHGF	Abcam ab245957	5 µg/L	225 €/ 25 µg			45	45	45	45
rhPDGF-BB	GenScript Z02529	5 µg/L	2165 €/1 mg			11	11	11	11
rbIL-6*	Biorad PBP021	5 µg/L	200 €/ 5 µg						
rhIL-6*	Peprotech 200-06	5 µg/L	2600 €/1 mg				13	13	13
rmWnt3a	R&D 1324-WN-002	5 µg/L	270 €/2 µg					670	
rhWnt5b	R&D 7347-WN-025/CF	5 µg/L	469 €/ 25 µg					93.8	
Estrogen (17β-Estradiol)	Sigma E2758-250MG	5 µg/L	28.40 €/ 250 mg						0.000568
Costs based on B8 (high FGF-2), €/L medium				49.72	34.87	90.87	103.87	867.67	103.87
Costs based on B9 (high FGF-2), €/L medium				74.28	59.43	115.43	128.43	892.23	128.43
% from initial B8 price				100	70.14	182.77	208.91	1745.12	208.91
% from initial B9 price				100	80.01	155.40	172.90	1201.17	172.90
Expected cell yield, %, B8				-	100	250	250	300	300
Expected cell yield, %, B9				100	120	300	450	495	N.A.
Optimization coefficient, B8**				**-**	**2.868**	**2.751**	**2.407**	**0.346**	**2.888**
Optimization coefficient, B9**				**1.346**	**2.2**	**2.599**	**3.504**	**0.555**	**N.A.**

*Recombinant bovine IL-6 was used in this study, but human IL-6 performed better than bovine IL-6 in Kolkmann *et al*. 2022[8], and it is more economical.
**Optimization coefficient was calculated by dividing expected yield by costs based on B8/B9 (high FGF-2) value (the higher - the better).
- - B8 without stabilizers does not support proliferation of BSCs for longer than 3-4 days.
N.A. - data not available to assess the yields of this combination.

In summary, this study presents four strategies for enhancing the efficiency of BSCs propagation, potentially raising the yields to 450% as compared to initial B9 composition. It also gives clues as to further promising lines of optimizations. Future research has to be conducted to further minimise the costs for growth factors – which can be directed to an even better and cheaper stabilization of the medium, or towards protein engineering of the growth factors themselves, or also towards adaptation of more complex treatment schemes. All these approaches will further greatly cut down the prices for growth factors.

Materials and methods

Isolation of bovine satellite cells (BSCs)

Primary bovine satellite cells were isolated based on methods established by Stout *et al*.[23]

A sample the size of ~0,5 g of skeletal muscle tissue was extracted from a 19 months old, castrated Simmental Ox (*Bos taurus*) at Marcher Fleischwerke GmbH. The muscle probe was transported in transport medium on ice and immediately further processed, where it was cut up into small pieces and digested in 0.2% collagenase II (Worthington Biochemical #LS004176, Lakewood, NJ, USA; 275 U/mg) for 45-60 min until the paste was homogenized. Digestion reaction was brought to a halt with BSC growth medium (P-GM). The cells were filtered twice, counted using an Invitrogen Countess Automated Cell Counter and plated at a density of 100.000 cells/cm^2 onto uncoated tissue-culture flasks. Throughout the incubation over 24 h at 37 °C with 5% CO_2 adherent cells attached at the surface of the tissue-culture flask and BSCs stayed in suspension and were transferred to coated flasks at a density of 2000 cells/cm^2 with 1.5 µg/cm^2 recombinant human Vitronectin (FisherScientific #15134499). The BSCs were left untouched for three to four days before P-GM was changed every two days until a maximum of 70% confluence was reached and the cells were frozen in FBS with 10% Dimethyl sulfoxide (DMSO, Sigma #D2650) or passaged for screening or differentiation by using 0.25% trypsin-EDTA (ThermoFisher #25200056).

Confirmation of BSCs identity and differentiation

After isolation, undifferentiated BSCs were identified by staining for Paired-box 7 (Pax7) marker. BSCs were cultured in BSC growth medium (BSC-GM) on a cover glass until they reached 70% confluence, fixed with 4% paraformaldehyde (FisherScientific #AAJ61899AK) for 30 min, washed in DPBS, permeabilized for 15 min with 0.5% Triton-X (Sigma #T8787) in DPBS, blocked for 45 min with 5% goat serum (ThermoFisher #16210064) in DPBS with 0.05% sodium azide (Sigma #S2002), and washed with DPBS with 0.1% Tween-20 (Sigma #P1379). Primary Pax7 antibodies (ThermoFisher #PA5-68506) were added at a dilution of 1:500 in blocking solution containing 1:100 Phalloidin 594 (ThermoFisher #A12381) to cells and incubated at 4 °C overnight. Further, cells were washed with DPBS + Tween-20 and incubated with secondary antibodies for Pax7 (ThermoFisher #A-11008, 1:500) for 1 h at room temperature, washed with DPBS + Tween-20 and mounted with Fluoroshield mounting medium with DAPI (Abcam #ab104139). Visualization and imaging were performed by the Core Facility Imaging at Medical University Graz to validate the satellite cell purity of the isolated cell population.

Isolated BSCs were differentiated for seven days. After growing cells to confluency in BSC-GM, the medium was changed to Differentiation Medium (DM) and then 50% of the medium was regularly changed (every two to three days) until BSCs reached differentiated state and were fixed and prepared as previously described. Primary Antibody for myosin heavy chain (MHC) (Developmental studies hybridoma bank #MF-20, Iowa City, IA, USA; 4 µg/mL, developed by Fischman, D.A. from Cornell University Medical College, was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242) were diluted in phalloidin 594 (1:100) and incubated at 4 °C overnight. Secondary Antibody for MHC (ThermoFisher #A-11001, 1:1000) was added the next day and mounted with Fluoroshield mounting medium with DAPI.

Media composition

Skeletal muscle tissue sample was transferred into transport medium consisting of DMEM + Glutamax (ThermoFisher #10566016) and 1% Antibiotic-Antimycotic (ThermoFisher #15240062) after extraction.

For Isolation of BSCs, Primocin growth medium (P-GM) was used containing DMEM + Glutamax (ThermoFisher #10566016), 1% Primocin (Invivogen #ant-pm-1), 20% fetal bovine serum (FBS; ThermoFisher #26140079) and 1 ng/mL human FGF basic/FGF2/bFGF (R&D Systems #233-FB-025/CF). After passage one, P-GM was changed to growth medium (GM) where Primocin is switched out with 1% Antibiotic-Antimycotic (ThermoFisher #15240062). Serum free differentiation medium was prepared according to Messmer *et al*.[24]

For short-term growth analysis, cells were plated in 96-well tissue culture plates (Greiner #655180) containing BSC-GM with 1.5 µg/cm^2 recombinant human Vitronectin (FisherScientific #15134499). After incubation for 24 h, cells were washed with DPBS (ThermoFisher #14190250) and BSC-GM was changed to screening medium - B8 medium containing DMEM/F12 (ThermoFisher #11320033), HiDef-B8 medium aliquots (Defined Bioscience #LSS-201) and 1% Antibiotic-Antimycotic, supplemented or not supplemented with 0.8 g/L HSA . According to the screening method, defined concentrations of medium components (Suppl. Table 1) and stabilizers (Table 3) were added. All added medium components were reconstituted and diluted in DPBS containing 0.8 g/L HSA (Sigma #A9731-1G), highly concentrated aliquots were stored at -80°C.

Screening

BSCs were thawed in 5 mL GM and centrifuged at 200 x g for 3 min. The supernatant was discarded, and the cells were washed with 5 mL DPBS. After another centrifugation step and removal of the supernatant, the cells were carefully resuspended in 5 ml BSC-GM and counted with Invitrogen Countess Automated Cell Counter.

BSCs (800 cells/well = approx. 2000 cells/cm^2) were plated in 96-well tissue culture plates (Greiner #655180) containing 100 µl BSC-GM with 1,5 µg recombinant human Vitronectin (FisherScientific #15134499)/cm^2. After 24 h of incubation (day 1) at 37°C and 5 % CO_2, the cells were rinsed with DPBS (ThermoFisher #14190250), BSC-GM was removed and screening medium (B8 or B9) and screening components were added (see the list of components and their sourcing in Supplementary Table 1). On indicated days Presto Blue assay was performed. On day 3 after Presto Blue assay screening components were added again into the fresh medium. Over the weekend cells were covered with 200 µl medium, instead of 100 µl. On the last day, the cells were rinsed with DPBS and frozen at -80°C.

Presto Blue assay

To measure the metabolic activity, which correlates with the number of cells, Presto Blue assay was performed in 96-well plate format according to the manual. Shortly, 10 µL Presto Blue reagent (ThermoFisher #A13262) was added to the remaining 90 µl of medium in each well and incubated at 37°C for one hour. After incubation, the medium was transferred to a fresh 96-well plate (Greiner #655101) and the fluorescence at 560 nm (excitation) and 590 nm (emission) was measured with a plate reader (BioTek SynergyMx). Depending on the time point of the assay, cells were covered with 100 or 200 µL of fresh B8 or B9 medium and further incubated.

Hoechst assay

In order to verify Presto Blue assay data, we used Hoechst 33258 assay to assess the quantity of DNA per well, which directly correlates with the cell number.

On the last day of every screening, the screening plates were frozen at -80°C (see Screening). After thawing to room temperature, 100 µL ddH_2O were added per well and the plates were incubated at 37°C for one hour. The plates were then again frozen at -80°C and thawed to room temperature. 100 µL of Hoechst dye reagent - consisting of 25 µL Hoechst 33258 stock solution (10 mg Hoechst 33258

(ThermoFisher #H1398)/mL in DMSO + ddH_2O (1:4)) in 10 mL TNE buffer (10 mM Tris, 2 M NaCl, 1 mM EDTA, 2 mM sodium azide, pH 7.4, were added. After 15 min of incubation at room temperature, the fluorescence was measured at 352 nm (excitation) and 461 nm (emission).

Statistical analysis

For statistical analysis of Presto Blue and Hoechst assays, the data was tested for significance using one-way ANOVA with post-hoc Tukey's test using the online tool "One-Way ANOVA Calculator and Tukey HSD" by Statistics Kingdom (https://www.statskingdom.com/180Anova1way.html). The following parameters were used: significance level (α): 0,05; outliers: included; effect: medium; effect type: f; effect size: 0,25; rounding: 4. Statistical significance was reached when $p < 0,05$ (*), $p < 0,01$ (**), $p < 0,001$ (***) and $p < 0,0001$ (****). Non-significant results ($p \geq 0,05$) were marked as "n.s.". The error bars in the graphs represent the standard deviation.

Competing interests

The authors declare no conflict of interest.

Author contributions

Lisa Schenzle: conceptualization, investigation, visualization, data analysis, draft writing. Kristina Egger: conceptualization, investigation, visualization, data analysis, draft writing. Aleksandra Fuchs: conceptualization, resources, writing- reviewing and editing, supervision, funding acquisition. Harald Pichler: resources, reviewing and editing, supervision.

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