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IGFBPs mediate IGF-1's functions in retinal lamination and photoreceptor development during pluripotent stem cell differentiation to retinal organoids

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Abstract

Development of the retina is regulated by growth factors such as insulin-like growth factors 1 and 2 (IGF-1/2), which coordinate proliferation, differentiation, and maturation of the neuroepithelial precursors cells. In the circulation, IGF-1/2 are transported by the insulin growth factor binding proteins (IGFBPs) family members. IGFBPs can impact positively and negatively on IGF-1, by making it available or sequestering IGF-1 to or from its receptor. In this study, we investigated the expression of IGFBPs and their role in the generation of human retinal organoids from human pluripotent stem cells, showing a dynamic expression pattern suggestive of different IGFBPs being used in a stage-specific manner to mediate IGF-1 functions. Our data show that IGF-1 addition to culture media facilitated the generation of retinal organoids displaying the typical laminated structure and photoreceptor maturation. The organoids cultured in the absence of IGF-1, lacked the typical laminated structure at the early stages of differentiation and contained significantly less photoreceptors and more retinal ganglion cells at the later stages of differentiation, confirming the positive effects of IGF-1 on retinal lamination and photoreceptor development. The organoids cultured with the IGFBP inhibitor (NBI-31772) and IGF-1 showed lack of retinal lamination at the early stages of differentiation, an increased propensity to generate horizontal cells at mid-stages of differentiation and reduced photoreceptor development at the later stages of differentiation. Together these data suggest that IGFBPs enable IGF-1's role in retinal lamination and photoreceptor development in a stage-specific manner.

KEYWORDS
IGF-1, IGFBPs, photoreceptors, pluripotent stem cells, retinal organoids

1 | INTRODUCTION

Development of the Central Nervous System, including the retina, is regulated by transcription and growth factors such as neuropeptides that coordinate proliferation, differentiation, and maturation of the neuroepithelial precursors cells.[1,2] Insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) are multifunctional polypeptides, which are essential for regulating cell growth, development, and differentiation.[3,4]

IGFs have been found in the retina of several species, such as *Drosophila*, chick, mice, and humans[1] including the human foetus older than 3 months.[5] In the eye, IGF-1 and its receptors are expressed in the sensory retina and retinal pigment epithelium during development and adulthood.[6] Work performed by our group has demonstrated an important role for the IGF-1 signaling pathway in the generation of laminated retinal tissue from human embryonic stem cells (hESCs).[7]

Most of the IGF-1/2 is bound to one of the seven IGF-binding proteins (IGFBPs), which are structurally similar proteins shown to bind IGF-1 with an equal or greater affinity than IGF-1 receptor, acting as fine tuners of IGF-1 signaling globally and locally.[3,8,9] Binding of IGF-1 to IGFBPs increases its half-life in the circulation and blocks its potential binding to the insulin receptor. IGFBP-3 is the most abundant circulating IGFBP, carrying 75% to 80% IGF-1 and IGF-2 found in the serum.[8,10] The remaining 20% to 25% of IGFs are bound to one of the other IGFBPs.[8,10] The main function of IGFBPs is to bind IGFs and either transport them to their receptors, potentiating IGFs actions, or to sequester IGFs from their receptors (due to the greater affinity for IGF than its receptor) inhibiting their action.[3,8,10,11] It has been shown that proteolysis and fragmentation of IGFBPs or the attachment of the IGFBPs to cell surface proteoglycans and/or extracellular matrix components reduces their affinity to IGFs, resulting in an increase in the amount of IGFs available for IGF-1R binding, which enhances IGF-/2 activity.[8,11] However, several IGFBPs have cellular IGF-independent actions, which are mediated via binding to cell surface proteins or nuclear localization.[8,11-14] The mechanisms underlying these IGF-independent actions are not fully understood, but may involve an interaction between the IGFBPs and different signaling pathways either at the cell surface or within the cell.[13,14]

In neonatal mice, IGF-1 is expressed in all the retinal layers and neovascular vessels extending into the vitreous (tufts); however, IGF-1R is expressed predominantly in the photoreceptors. IGFBP2, IGFBP3, and IGFBP5 are expressed in all three retinal layers and expression of some of the IGFBPs (3, 5, and 7) shows large increases in the neovascular tufts.[15] Studies performed by our group have shown localization of IGF-1R in the outer nuclear layer (ONL) and IGF-2R in the retinal ganglion cells, inner, and outer plexiform layers of the adult human retina.[7] Due to the expression of IGFBPs in the retinal vessels, multiple studies have focused on IGFBP-mediated IGF-1 functions in human retinal endothelial cells[16] in normal steady-state conditions and diabetes[17]; however, the function(s) of IGFBPs during retinal development are as yet unexplored.

In this article, we have undertaken a systematic expression study of all IGFBPs in the adult human retina, while assessing in parallel which of the IGFBPs is activated or inhibited in response to IGF-1 addition in pluripotent stem cell-derived retinal organoids. Importantly, we have used an IGBP inhibitor, NBI-31772 (1-(3,4-dihydroxybenzoyl)-3-hydroxycarbonyl-6, 7-dihydroxyisoquinoline) that binds to the IGFBPs and displaces biologically active IGF-1[18,19] to assess the IGFBPs mediated IGF-1 function in generation of hESC-derived retinal organoids.

Significance statement

In vitro models such as retinal organoids provide information about molecular mechanisms that occur during retina development. Insulin growth factor binding proteins (IGFBPs) regulate cell activity in various ways, inhibiting proliferation by sequestration of insulin-like growth factors from its receptor or making them available to their receptors to facilitate signaling and enhance their function. To date, these opposite actions have been demonstrated in many cell culture systems, but a full picture of the role of IGFBPs during retinal development is lacking. This study is focused on the role of IGFBPs in human retinal organoids development, a widely used tool for undertanding human retinal histogenesis. The data from this study confirm that IGFBPs enable insulin-like growth factor 1 functions in retinal lamination and photoreceptor development during differentiation of pluripotent stem cells to retinal organoids.

2 | MATERIALS AND METHODS

A detailed description of all experimental procedures is presented in the Supporting Information.

3 | RESULTS AND DISCUSSION

To gain insights into the expression of the IGFBPs, we took advantage of a recent RNA-Seq analysis performed by our group[2] (Figure S1A), which showed a comparable expression of all IGFBPs, except IGBP-1 at the transcriptional level in adult human retina. Using immunofluorescence analysis, we observed IGFBP-2 and IGFBP-5's expression in the photoreceptor inner segment (IS), ONL, and inner nuclear layer (INL) (Figure S1B[b,e]). The highest expression of these two IGFBPs was found in the processes of the retinal ganglion cells (Figure S1B[b′, e′]). No IGFBP-1 expression was observed, corroborating the transcriptional analysis (Figure S1A,B[a]). IGFBP-3 was detected in the IS, ONL, INL, and in the ganglion cell layer (GCL) (Figure S1B[c]): the same expression, but with lower intensity was observed for the IGFBP-6 (Figure S1B[f]). IGFBP-4 was clearly detected in a few retinal ganglion cells (Figure S1B[d,d′]); a weaker signal was also detected in the IS, ONL, and INL (Figure S1D). IGFBP-7 was expressed only in the GCL (Figure S1B[g,g′]).

To assess IGFBP expression during human retinal development, we used the RNA-Seq data published by our group[2] and encompassing the 7.7 to 18 postconception weeks (PCW) of development. This analysis showed the highest expression for IGFBP2, IGFBP4, and IGFBP5 and low expression of IGFBP6 and IGFBP1 during 7.7 to 10 PCW (Figure S2A). During 12 to 18 PCW, IGFBP2, IGFBP4, IGFBP5, and IGFBP7 were highly expressed, while IGBP1

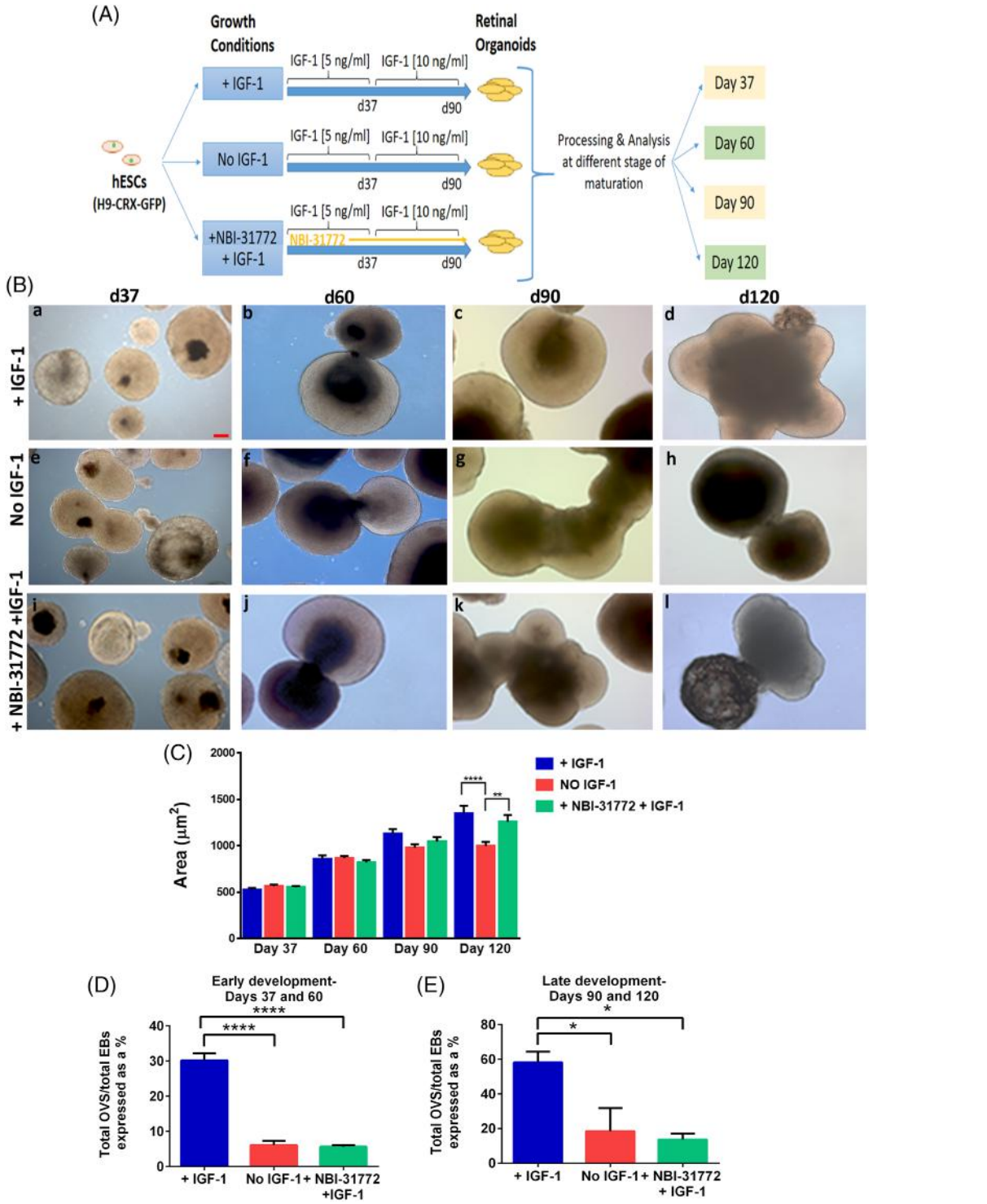

FIGURE 1 Characterization of the IGFBP inhibitor's impact during retinal organoids formation. A, Diagram of the experimental design: hESCs with CRX-GFP were cultured in three different conditions: with IGF-1 individually or in combination with NBI-31772, and without IGF-1. These retinal organoids were harvested and collected at four time points (days 37, 60, 90, and 120) of differentiation. B, Examples of the resulting culture morphology under bright-field microscopy. Morphology of human retinal organoids differentiating with IGF-1 supplement (a-d), without IGF-1 (e-h) and with the addition of IGF-1 and NBI-31772 (i-l) at day 37 (a, e, i) day 60 (b, f, j), day 90 (c, g, k) and day 120 (d, h, l) of differentiation. Scale bars = 100 µm. C, Quantitative assessment of the total EBs area (μm^2) at day 37, day 60, day 90, and day 120 cultured in three different conditions: with or without IGF-1 and IGF-1 in combination with NBI-31772. Data are shown as mean ± SEM, n = 7 to 10 organoids per group. D,E, Effects of IGF-1 and combined IGF-1 and NBI-31722 addition on the percentage of embryoid bodies (EBs) displaying visible retinal structures. Quantitative assessment of retinal structures resembling the optic vesicle (OV) at early development (D) at day 37 and 60 and past optic vesicle at late development (E) at day 90 and 120 derived from hESCs cultured with or without IGF-1 and IGF-1 in combination with NBI-31772. All values are stated as the percentage of the total population of EBs with OVs. CRX, cone rod homeobox; GFP, green fluorescent protein; hESCs, human embryonic stem cells; IGF-1, insulin-like growth factor 1; IGFBP, insulin growth factor binding protein

and IGFBP6 continued to be expressed at low levels (Figure S2A). A recent study has shown a comparable rate of development between pluripotent stem cell-derived retinal organoids and human fetal retina.[20] We thus performed quantitative Real Time-PCR analysis of all IGFBPs in four hESC-derived retinal samples generated in the absence of IGF-1 and harvested at four differentiation time points (day 37, 60, 90, and 120), which fall within the human retinal development shown in Figure S2A. This analysis indicated that up to day 60 of differentiation, IGFBP2 and IGFBP5 were the highest and IGFBP1 and IGFBP6 were among the lowest of the IGFBPs expressed respectively within the retinal organoids (Figure S2B). From day 90 to 120 of differentiation, IGFBP2, IGFBP5, and IGFBP7 were the highest and IGFBP1, IGBP3, and IGFBP6 were among the lowest of the IGFBPs expressed respectively within the retinal organoids (Figure S2B). These results are at large in accordance with data obtained from human developmental retinal samples and show that both during embryonic development and generation of retinal organoids, IGFBPs expression changes in a dynamic manner.

Previous studies have shown that expression of IGFBPs can change in response to IGF-1 secretion. For example, IGFBP3, which is produced in the liver, increases in quantity in response to secretion of growth hormone induced IGF-1 to ensure that all IGF-1 is absorbed in circulation.[21] To assess how the IGFBPs respond to increases in IGF-1 concentration or application of the IGFBP inhibitor, NBI-31772, we generated retinal organoids from the CRX-GFP reporter hESC (H9) line[22] under three different conditions and collected samples for quantitative RT-PCR and immunohistochemistry and various time points during the differentiation process as shown in Figure 1A. At day 37, IGFBP3 was the only member of IGFBP family, whose expression was increased as results of IGF-1 addition as well as downregulated in the presence of IGFBP inhibitor (Figure S3), suggesting that at the early stages of differentiation, IGFBP3 is the most likely IGFBP that mediates IGF-1 functions. At day 60, the same trend was observed for IGFBP-5; however, at day 90 and 120, none of the IGFBPs were activated by IGFBP expression. Nonetheless, the addition of IGFBP inhibitor NBI-31772 resulted in a decrease in the expression of IGFBP7 and IGFBP5 at day 90 and 120, respectively (Figure S3). Together these data suggest that different IGFBPs are likely to mediate IGF-1 functions in a stage-specific manner during the retinal organoid formation and maturation process.

The bright-field images analysis of retinal organoids shows an increase in size throughout the course of the experiment in all the conditions (Figure 1B,C), starting with small, disk-shaped organoids at day 37 leading to polarized organoids with a prominent axis at day 90. At day 120, organoids of IGF-1 group presented numerous optic vesicle-like structures and some pigmented cells. Both the control and the NBI-31772 + IGF-1 groups showed significantly less optic-vesicle structures formation at both at the early and later stages of differentiation (Figure 1D,E). In addition, the control organoids (without IGF-1) were smaller compared to IGF-1 and NBI-31772 + IGF-1 organoids at the last point of differentiation (Figure 1C).

The effects of IGF-1 and NBI-31772 + IGF-1 supplementation in culture were investigated by immunofluoresccence analysis with specific retinal cell marker proteins. At day 37, only IGF-1 treated organoids (Figure 2A[a-e]) showed a neural retina laminated structure, characterized by the presence of an apical layer of retinal progenitor marked by the VSX2 expression and a basally located layer of putative retinal ganglion cells expressing SNCG and HuC/D markers. Although some of these markers were present in the other two groups, no characteristic retinal lamination was observed (Figure 2A[f-o]). The quantification analysis showed a significantly higher number of proliferating cells (Ki-67$^+$ cells) in the control and NBI-31772 + IGF-1 treated groups compared to the IGF-1 group (Figure 2B), suggesting an early exit from the cell cycle in the IGF-1 group, which may promote photoreceptor differentiation, exemplified by a higher number of Recoverin$^+$ cells (Figure 2B). The control group was also characterized by a significantly higher number of retinal ganglion cells, which were distributed throughout the retinal organoids. In accordance with the transcriptional analysis, IGFBP-3 expression was increased in the IGF-1 treated group compared to the control; furthermore, it expression was undetectable in the IGFBP inhibitor-treated organoids (Figure 2A). This suggests that in the absence of exogenous IGF-1, the expression of IGFBP-3 is lowered, possibly through negative feedback or increased proteolysis. As a number of IGFBP-3 proteases have already been identified, further studies are needed to investigate the IGFBP-3 breakdown products and the likelihood of decreased IGFBP-3 expression by increased proteolysis.[23] The absence of IGFBP-3 in the inhibitor condition was surprising as additional IGF-1 was added to these organoids, IGFBP-3 was expected to be present. The lack of signal could be due to several reasons including constant protein inhibition which could have influenced expression levels of the protein itself or other members in the pathway. Future research is needed to determine if this is the case. Furthermore, the IGFBP-3 antibody used here binds to a central sequence of IGFBP-3[24] but the exact sequence is unavailable, this could impact the results if it binds in the same place as the inhibitor.

At day 90, retinal organoids of all the groups contained neural retinal structures with laminar organization (Figure 3A). Numerous SNCG$^+$ retinal ganglion (Figure 3A[c,f,i]) and AP2α^+ amacrine cells (Figure S4B,E,H) were observed in the basal layer of the retinal organoids. An ONL of photoreceptors (Figure 3A) was found in all conditions with a striking increase in the number of RCVRN$^+$ cells compared to day 37, as shown in the quantification plot (Figure 3B). At day 90, the quantification analysis did not show significant differences in numbers of photoreceptors or interneurons, except Prox1$^+$ horizontal cells, which were more abundant in the NBI-31772 + IGF-1 treated group (Figure 3B). We think that this effect may be due to downregulation of IGFBP-7 in this group (Figure S3). IGFBP-7 has a 100-fold lower affinity for IGF-1 and IGF-2, but high affinity for insulin (500-fold more elevated compared to other IGFBPs[25]). This IGFBP7-IGF-1/2 independent activity may be the underlying reason for the increased number of Prox-1$^+$ horizontal cells in the NBI-31772 + IGF-1 group; however, this needs further investigation.

At day 90 of differentiation, Ki-67$^+$ cells were present as the neural retina was still developing (Figure 3A[c,f,i]); however, no difference was observed between all the three groups (Figure 3B). A few bipolar cells detected by the marker PKC-α (Figure S4A,D,G) were detected, forming a numerically small proportion of the retina's interneurons as

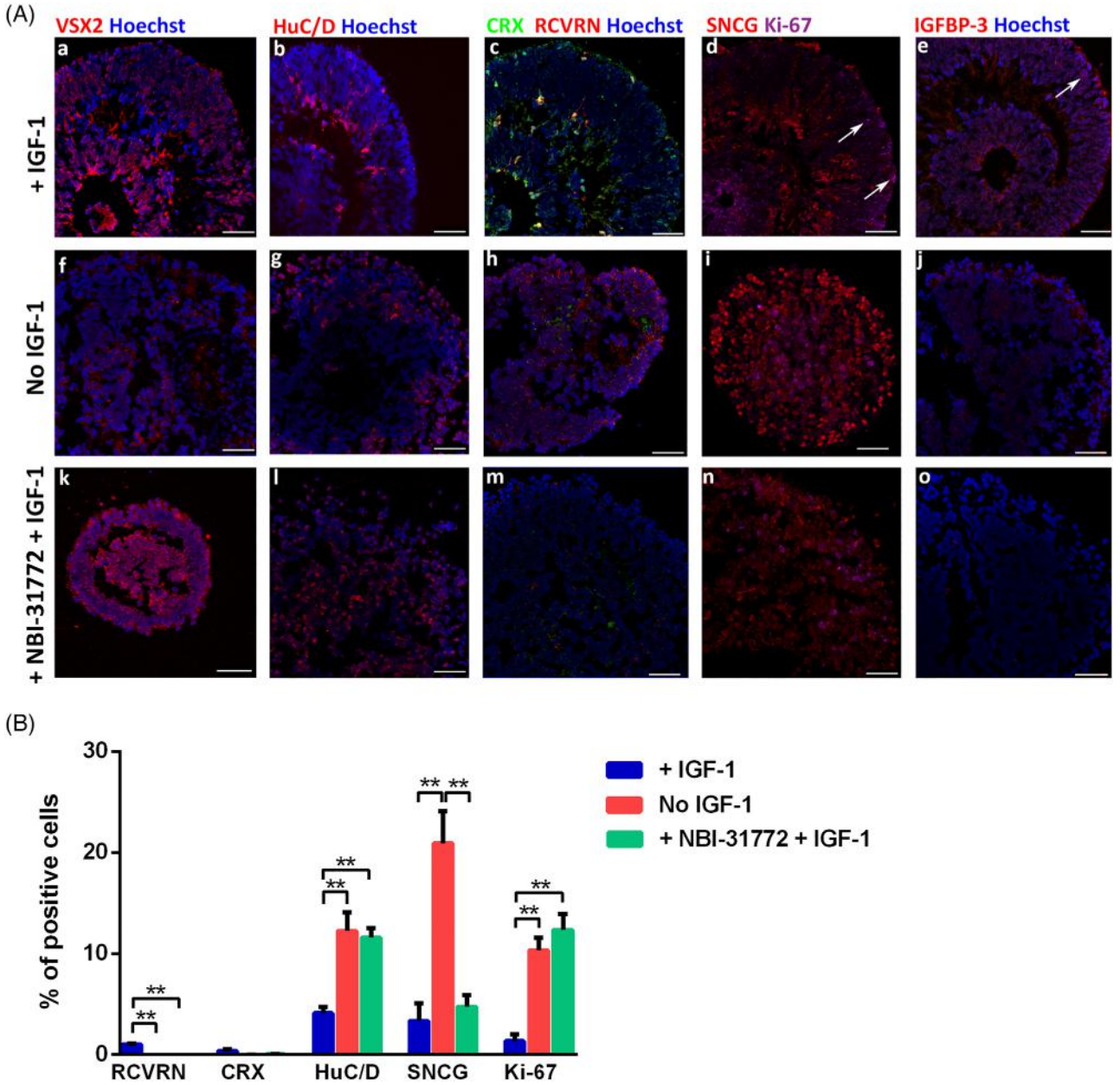

FIGURE 2 The impact of IGFBP inhibition in the early stages of retinal organoid differentiation: day 37. A, Immunohistochemical analysis of human retinal organoids harvested after 37 days of growth, in three different culture conditions: with IGF-1 (a-e), without IGF-1 (f-j), or with IGF-1 and NBI-31772 (k-o). Antibodies used against neural retinal progenitors: VSX2 (red); ganglion and amacrine cells: HuC/D (red); photoreceptor precursors: RCVRN (red); ganglion cells: SNCG (red); proliferating cells: Ki-67 (purple, white arrows), and IGFBP-3 protein (red, white arrow). Hoechst nuclear stain (blue) and endogenous GFP (green) also shown. B, Immunohistochemistry quantification analysis. Data are shown as mean ± SEM (n = 5). Scale bars = 50 μm. CRX, cone rod homeobox; GFP, green fluorescent protein; IGF-1, insulin-like growth factor 1; IGFBP, insulin growth factor binding protein; RCVRN, Recoverin

confirmed by the quantification analysis (Figure 3B). Müller glia cells were observed to span the whole retinal structure similar to the adult retina organization (Figure S4C,F,I).

By day 120, IGF-1 and NBI-31772 + IGF-1 treated organoids were larger and with better lamination and stratification compared to retinal organoids grown without IGF-1 (Figures 1C and 4A), which lost their stratification, corroborating the importance of IGF-1 for retinal organoid development. The highest number of photoreceptors was observed in the IGF-1 treated group and the lowest number was observed in the no IGF-1 group (Figure 4B). Mature photoreceptors are observed in retinal organoids from day 150.[26,27] In accordance with this, only a few rods with typical morphology were present in the IGF-1 and NBI-31722 + IGF-1 groups (Figure 4A[e,e′,q,q′]); however, the low numbers due to early differentiation stage precluded any firm statistical conclusions. Similarly Cone opsins (OPN1SW and OPN1LW/MW) were present only in IGF-1 group (Figure 4A[f,f′,g,g′]).

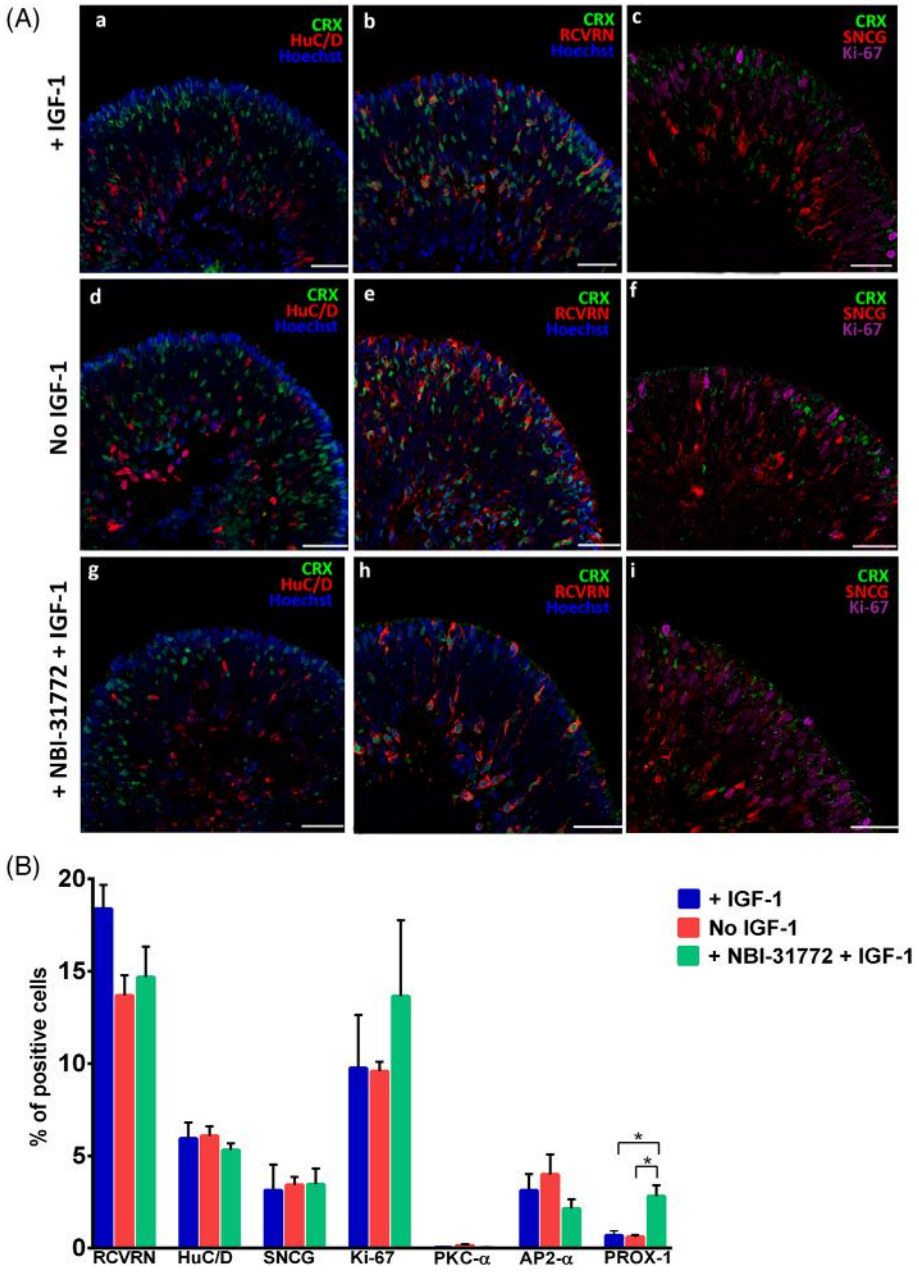

FIGURE 3 The impact of IGFBP inhibition in the middle stage of retinal organoid differentiation: day 90. A, Immunohistochemical analysis of human retinal organoids at day 90 of differentiation, in three different culture conditions: with IGF-1 (a-c), without IGF-1 (d-f), or with IGF-1 and NBI-31772 (g-i). Antibodies used against HuC/D (red), RCVRN (red), SNCG (red), and Ki-67 (purple). Hoechst nuclear stain (blue) and endogenous GFP (green) also shown. B, Immunohistochemistry quantification analysis. Data are shown as mean ± SEM (n = 5). Scale bars = 50 μm. CRX, cone rod homeobox; GFP, green fluorescent protein; IGF-1, insulin-like growth factor 1; IGFBP, insulin growth factor binding protein; RCVRN, Recoverin

Together these data suggest an important role for IGF-1 in photoreceptor development. Photoreceptor cells started to form ribbon synapsis demonstrated by the immunoreactivity of Bassoon and Ribeye, respectively (Figure 4A[c,d]). Putative synaptic connections were not observed in the control group (Figure 4A[j,k]), strengthening the necessity of IGF-1 for correct organoid development, synaptogenesis, and lamination. HuC/D^{+} cells were found at the basal site of the organoids treated with IGF-1 and/or inhibitor (Figure 4A[a,m]), being more numerous in the control group as showed by the quantification analysis (Figure 4B). In contrast, there were less AP2-α^{+} cells in this group compared to the other conditions (Figure S5C,G,K), suggesting that the large amount of HuC/D^{+} cells represented mainly Retinal Ganglion Cells. This was corroborated by the significant increase in the fraction of SNCG^{+} (Figure S5A,E,I) in the no IGF-1 group. A similar increase in Retinal Ganglion Cells number was also observed at day 37 but not day 90. We speculate that this changeable effect may be due to the specific IGFBPs being used to mediate IGF-1 functions at different stages of development. As we have indicated above, IGFBP3, IGFBP7, and IGFBP5 are likely to mediate IGF-1 functions in the early, mid, and late stages of differentiation, respectively. This differential binding of IGFBPs to IGF-1 may channel its activities through IGF-1R shown in our previous work [7] to be expressed in the ONL or IGF-2R, which is expressed in the RGCs, inner and outer plexiform layers of the adult human retina. This coupled with different patterns of IGFBP expression in retina (eg, IGFBP-7 is only expressed in the RGC layer of human adult retina) may explain the changeable effect on RGCs in the control group (no IGF-1) at the three differentiation time points examined. Horizontal cells (Figure S5C,G,K) were found throughout the organoids and just few PKC-α^{+} cells, if not

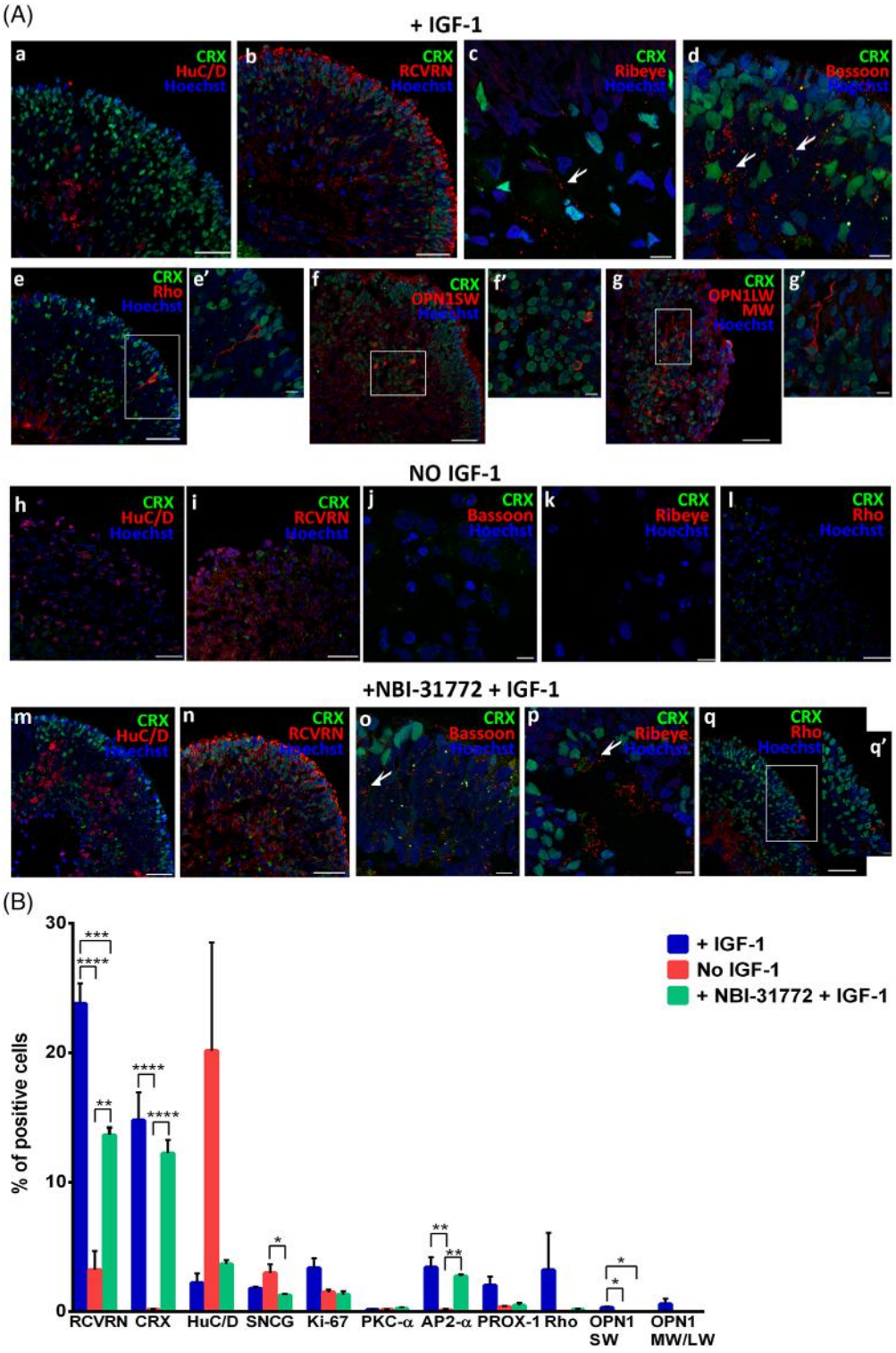

FIGURE 4 The impact of IGFBP inhibition in the later stages of retinal organoid differentiation: day 120. A, Immunohistochemical analysis of human retinal organoids at day 120 of differentiation in three different culture conditions: with IGF-1 (a-g′), without IGF-1 (k-l), or with IGF-1 and NBI-31772 (m-q′). Antibodies used against HuC/D (red), RCVRN (red), Bassoon and Ribeye (red, white arrows), and mature photoreceptors: Rhodopsin (red) (e, e′, q, q′), OPN1SW (red) (f, f′), and OPN1LW MW (red) (g, g′). Hoechst nuclear stain (blue) and endogenous GFP (green) also shown. B, Immunohistochemistry quantification analysis. Data are shown as mean ± SEM (n = 5). Scale bars = 50 μm (a, b, e, f, g, h, i, l, m, n, and q) and 10 μm (c, d, e′, f′, g′, j, k, o, p, and q′). CRX, cone rod homeobox; GFP, green fluorescent protein; IGF-1, insulin-like growth factor 1; IGFBP, insulin growth factor binding protein; RCVRN, Recoverin and Rho, Rhodopsin

absent (control group) were present spanning the neural retina of the organoid (Figure S5B,F,J). IGF-1 and NBI-31772 + IGF-1 treated organoids showed long and abundant Müller glia cells projecting from the inner apical side to the outer edge of the organoids (Figure S5D,L). Although the organoids grown without IGF-1 contained Müller glia cells, they were much shorter and situated mainly at the outer edge of the organoid and not dispersed throughout the structure (Figure S5H).

In summary, our data show an important role for IGFBPs and IGF-1 in various stages of retinal organoids generation and maturation. The effects are specific to each stage of development and most likely mediated by different member of the IGFBP family. It is interesting to note that another widely used differentiation method, which involves embryoid body (EB) formation, followed by plating in 2D culture conditions, dislodging and culturing the organoids under 3D

conditions does not use IGF-1 supplementation in the media, but employs instead BMP-4 at the very early stages of differentiation, which raises interesting questions on the necessity of IGF-1 for generation of retinal organoids from pluripotent stem cells.[28-30] We cannot exclude that organoids generated with such method are not producing IGF-1 endogenously to sufficient levels. Neither can we comment on downstream pathways or target genes being activated by IGF-1 or BMP-4 unless both methods are studied in parallel in the same lab and with the same cell lines.

4 | CONCLUSION

The purpose of this project was to study the role of IGFBPs in retinal development. Aligning with the literature,[7] this project confirmed the positive effects of IGF-1 on retinal organoid development and maturation, as IGF-1 treated organoids showed the most efficient retinal organoid generation, retinal lamination as well as the highest number of photoreceptors. The photoreceptor development was affected in the NBI-31722 + IGF-1 treated group and this was associated at the early stages of differentiation with the lack of retinal lamination and a skew toward the interneuronal cell fate, which supports the hypothesis that IGFBPs enhance the IGF-1 function during early retinal development. This conclusion was corroborated by the no IGF-1 control group, which showed the worst photoreceptor development, lack of retinal lamination at the early stages of organoid development, and a skew toward the development of interneuronal fates.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

D.Z.: study design, performed research, data collection and analysis, figure preparation, manuscript writing; M.M.M.: performed research, data analysis, figure preparation and contributed to the manuscript writing; S.M., B.D.: performed research, data collection and analysis; R.B., J.A.-A.: data analysis; M.L.: study design, performed research, data collection and analysis, manuscript writing and fund raising. All authors approved the final version of manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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